Early Interferon Therapy for Hepatitis C Virus Infection Rescues Polyfunctional, Long-Lived CD8+ Memory T Cells†

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The majority of acute hepatitis C virus (HCV) infections progress to chronicity and progressive liver damage. Alpha interferon (IFN-α) antiviral therapy achieves the highest rate of success when IFN-α is administered early during the acute phase, but the underlying mechanisms are unknown. We used a panel of major histocompatibility complex class I tetramers to monitor the phenotypic and functional signatures of HCV-specific T cells during acute HCV infection with different infection outcomes and during early IFN therapy. We demonstrate that spontaneous resolution correlates with the early development of polyfunctional (IFN-γ- and IL-2-producing and CD107α+) virus-specific CD8+ T cells. These polyfunctional T cells are distinguished by the expression of CD127 and Bcl-2 and represent a transitional memory T-cell subset that exhibits the phenotypic and functional signatures of both central and effector memory T cells. In contrast, HCV-specific CD8+ T cells in acute infections evolving to chronically expressed low levels of CD127 and Bcl-2, exhibited diminished proliferation and cytokine production, and eventually disappeared from the periphery. Early therapeutic intervention with pegylated IFN-α rescued polyfunctional memory T cells expressing high levels of CD127 and Bcl-2. These cells were detectable for up to 1 year following discontinuation of therapy. Our results suggest that the polyfunctionality of HCV-specific T cells can be predictive of the outcome of acute HCV infection and that early therapeutic intervention can reconstitute the pool of long-lived polyfunctional memory T cells.

Hepatitis C virus (HCV) infection resolves spontaneously during the acute phase in a minority of infected individuals while the majority develop persistent viremia and chronic hepatitis over a period of years or decades (28). Understanding the immune response leading to spontaneous resolution during acute HCV infection has been hampered by the asymptomatic nature of the disease. Studies in the chimpanzee model and high-risk populations like intravenous drug users (IDUs) or healthcare workers following HCV exposure have demonstrated the absolute requirement for the CD8+ and CD4+ HCV-specific T-cell responses to prevent viral persistence (reviewed in reference 12 and 50). A cellular immune response is induced in most infected individuals, resulting in spontaneous resolution or transient control of viremia. However, this response is not sustained in individuals who develop viral persistence. The inefficiency of this initial immune response and viral recurrence in individuals who progress to chronic infection are likely due to the loss of CD4+ T-cell help (16, 20, 56, 57) and the rapid emergence of viral escape mutants in targeted CD8+ cytotoxic T lymphocyte epitopes (9). Individuals who spontaneously resolve HCV infection develop long-lived memory T-cell responses (54) that can be protective upon reexposure (51). In contrast, cellular immune responses are mostly undetectable in the blood of persistently infected individuals and are mostly localized in the liver (19, 27). Visualization of HCV-specific T cells ex vivo using major histocompatibility complex class I (MHC-I) tetramers has demonstrated that HCV-specific CD8+ T cells are severely impaired in function in chronically infected individuals, as demonstrated by the lack of proliferation (60) and cytokine production (22) in response to specific antigen stimulation. At present it is difficult to establish if such aberrant responses are the cause or the effect of persistent viremia and T-cell exhaustion.

Memory T cells in humans are classified based on the expression of the chemokine receptor CCR7 and CD45RA into three subsets: central memory (Tcm), defined as CCR7+ CD45RA− with a high proliferative capacity and lower gamma interferon (IFN-γ) production; effector memory (Tem), defined as CCR7− CD45RA− with lower proliferative capacity and higher IFN-γ production; and terminally differentiated effectors, defined as CCR7− CD45RA+ and having the lowest proliferative capacity and highest IFN-γ production (46, 47). Additional markers have been used to identify different subsets or the maturation status of memory T cells like the costimulatory molecules CD27 and CD28, interluekin-7 (IL-7) receptor alpha (CD127) as a marker of cells destined to become memory T cells (29), and programmed death receptor-1 (PD-1) as a marker for T-cell exhaustion in several viral infec-
tions (14). Recent studies have associated spontaneous resolution of acute HCV infection with upregulation of CD127 (17, 58) and downregulation of PD-1 (18, 39, 42, 59). In contrast, other studies have demonstrated that CD127+ HCV-specific CD8+ T cells are detectable in persistently infected individuals (4, 42) and that PD-1 levels do not correlate with the outcome of acute HCV infection (8, 35), suggesting that PD-1 may not be a marker for T-cell exhaustion but, rather, activation. In addition, virus-specific memory T cells in chronic HCV infection were shown to be arrested at an early maturation stage as CD27+ CD28- cells (3). However, the contribution of the different memory T-cell markers to defining functionally competent HCV-specific T-cell populations associated with viral clearance and the development of such subsets during clearly defined acute HCV infection remain elusive.

There is no vaccine for HCV, and the current therapy, a combination of pegylated IFN-α (PEG-IFN-α) and ribavirin, is effective in only approximately 50% of chronically infected individuals (15). Nevertheless, response to therapy is enhanced if it is started early during the acute phase (32, 34, 62), suggesting progressive damage to the host defense system with prolonged infection. The role of the immune response in determining the outcome of HCV therapy has been controversial, with some studies demonstrating an enhanced immune response in individuals who respond to therapy (31, 33) and others showing no correlation and even a decline in the immune response following therapeutic elimination of the virus (37, 43). Most importantly, a comprehensive analysis of the functional and phenotypic signatures of HCV-specific T cells when therapy is introduced early during the acute phase is lacking.

In this study, we used a panel of five MHC-I tetramers and nine-color multiparametric flow cytometry to monitor longitudinally the phenotypic and functional changes in HCV-specific T cells in a unique cohort of IDUs at high risk of HCV infection before and during acute HCV infections that progressed to spontaneous resolution or viral persistence. In addition, we followed a subset of these individuals during early IFN therapy. We demonstrate that acute resolving HCV infections are characterized by the early development of polyfunctional CD127hi Bcl-2hi CD8+ T cells consistent with a transitional TEM+ cell profile. Most importantly, early therapeutic intervention with PEG-IFN-α rescued long-lived polyfunctional memory T cells expressing high levels of CD127 and Bcl-2 (CD127hi Bcl-2hi) cells.

MATERIALS AND METHODS

Study subjects and clinical follow-up. HCV acutely infected subjects were recruited among high-risk IDUs participating in the St. Luc cohort study or methadone treatment program or presenting to the hepatology clinic at St. Luc Hospital of the Centre Hospitalier de l’Université de Montréal. This study was approved by the institutional ethics committee (SLS05.014 and SL05.025) and conducted according to the Declaration of Helsinki. All participants signed informed consent upon enrolment. Acute HCV infection was defined as either (i) detection of positive HCV RNA in the absence of HCV antibodies at recruitment, followed by seroconversion (patients R1, C2, T1, T4); or (ii) a positive HCV antibody test following a previous negative test in the presence of positive HCV RNA, with time intervals ranging between 63 and 126 days (patient R2, 126 days; patient T2, 63 days; patient T3, 90 days). In addition, patient CI presented to the hepatology clinic with symptomatic acute HCV infection including elevated serum alanine aminotransferase levels and HCV RNA and antibody-positive tests following a high-risk exposure. All patients tested negative for human immunodeficiency virus (HIV) and hepatitis B virus. Duration of infection was defined as the time (in weeks) from the first positive HCV RNA test. Spontaneous viral resolution or persistent infection was defined as the absence or presence of HCV RNA at 12 weeks postenrollement. In accordance with the clinical guidelines at the time, IFN therapy was offered to participants if they tested HCV RNA positive at 12 weeks after HCV detection (61). Patients who accepted treatment received 12 to 16 weeks of PEG-IFN-α2a (Pegasys®/Roche Diagnostics, Welwyn Garden City, Hertfordshire, United Kingdom) (180 μg/week) and no ribavirin. HLA typing was performed by the core facility of the Fonds de la Recherche en Santé du Québec (FRSQ) AIDS and Infectious Disease Network, Montreal, QC, Canada, using standard sequence-specific primer-PCR high-resolution techniques as previously described (63).

HCV RNA testing and quantification. Qualitative HCV RNA tests were performed using an automated COBAS AmpliPrep/COBAS Amplipcr HCV test, version 2.0 (sensitivity, 50 IU/ml) (Roche Molecular Systems, Inc., Branchburg, NJ). HCV genotyping was done using standard sequencing for the NS5B region, and HCV RNA was quantified by the COBAS Amplicor HCV Monitor test, version 2.0 (sensitivity, 600 IU/ml) (Roche Molecular Systems). Both tests were performed by the Laboratoire de Santé Publique du Québec (Ste Anne-de-Bellevue, QC, Canada) as part of the clinical follow-up of patients. Additional HCV RNA quantification was performed using an in-house quantitative real-time PCR assay adapted from T. Takeuchi et al. (55). Briefly, total viral RNA was extracted from 200 μl of plasma collected in EDTA using a QiAmp Mini Elute Spin Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol, and eluted in 25 μl. Quantitative PCR was performed in duplicate using 10 μl of extracted RNA in a 25 μl reaction volume of one-step reverse transcription-PCR using a Quantitect Probe RT-PCR kit (Qiagen). The following primers were used: forward primer, 5'-CGGGAGAGCCATAGTGG-3' (clone J1); reverse primer, 5'-AGTACCACAAGGCCTTTT-3'; probe, 5'-6-FAM-CTGGGGAACGGTTGTAAC-IBFQ-3', where FAM is 6-carboxyfluorescein and IBFQ is Iowa Black fluorescence quencher). Real-time PCR was performed using a Rotor Gene 3000 Instrument (Corbett Research, Sydney, Australia) as follows: reverse transcription for 30 min at 50°C and PCR initial activation step for 15 min at 95°C; followed by 50 cycles of two-step PCR (15 s at 95°C and 60 s at 58°C). HCV plasma RNA concentration was calculated using the standard curve generated from patient plasma of known HCV RNA content.

Peptides and peptide-HLA class I tetramers. Overlapping peptides corresponding to the HCV H77 genotype 1a polypeptide reference sequence used in the enzyme-linked immunospot screening assays were obtained from the Biodefense and Emerging Infections Research Resources Repository, Manassas, VA. All other peptides were synthesized by Eastern Quebec Proteomics Core Facility, Quebec, QC, Canada. MHC-I tetramers were synthesized by the National Immunomonitoring Laboratories, Montreal, QC, Canada and are as follows: HLA-A1-restricted HCV NS3 peptide consisting of residues 1436 to 1444 (ATDALMTGY) (HLA-A1/NS3-1436), HLA-A2-restricted HCV NS3 peptide consisting of residues 1073 to 1081 (CINGWCWTV) (HLA-A2/NS3-1073), HLA-A2-restricted HCV NS3 peptide consisting of residues 1346 to 1354 (GLGTELNR) (HLA-A2/NS3-1346), HLA-B7-restricted HCV core peptide consisting of residues 41 to 49 (GPRLLVRAT) (HLA-B7/core-41), and HLA-B8-restricted HCV NS3 peptide consisting of residues 1395 to 1403 (HSDKKCDCE) (HLA-B8/NS3-1395). The control tetramers were the following: HLA-A2-restricted cytomegalovirus (CMV) pp65 peptide consisting of residues 495 to 503 (NLVPVMATV), HLA-B7-restricted CMV pp65 peptide consisting of residues 1014 to 1023 (TPRTGGGAM), and HLA-A2-restricted influenza A virus (Flu) peptide consisting of residues 1007 to 1015 (GILGFVFTL).

Flow cytometry antibodies and reagents. Directly conjugated antibodies against the following surface molecules were used: CCR7-phycocerythrin (PE)-Cy7 (clone 3D12), CD3-allophycocyanin (APC) (clone UCHT1), CD3-Pacific Blue (clone SP34-2), CD4-Pacific Blue (clone RPA-T4), CD8-Alexa Fluor 700 or CD8-Pacific Blue (clone RPA-T8), CD28-peridinin chlorophyll protein-Cy5.5 (clone 1L293), CD127-Alexa Fluor 647 (clone HIL-7R-M21), and PD-1-APC (clone MH4A) (all from BD Biosciences, San Jose, CA); CD27-APC-Alexa Fluor 750 (clone 0323) (eBioscience, San Diego, CA); CD3-energy-coupled dye (PE-Texas Red; clone UCHT1) and CD45RA-energy-coupled dye (clone 2H4) (both from Beckman Coulter, Marseille, France); and CD127-Alexa Fluor 488 (clone HCD17) (Biologend, San Diego, CA). The following intracellular antibodies were used: IL-2-PE (clone MQ1-17H12), IFN-γ-Alexa 700 (clone B72), Bcl-2-fluorescein isothiocyanate (clone 100), and CD107a-PE-Cy5 (clone H4A3), all from BD Biosciences. Live cells were identified using an Aqua Live/Dead fixable dead cell Stain Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol. “Fluorescence minus one” control stains were used to determine background.

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levels of staining. Multiparameter flow cytometry was performed using a standard BD LSR II instrument equipped with blue (485 nm), red (633 nm), and violet (405 nm) lasers (BD Biosciences), to systematically perform nine-color staining using FACS Diva software (BD Biosciences). Compensation was performed with single fluorochromes and BD CompBeads (BD Biosciences). Data files were analyzed using FlowJo software, version 5.6.3 for Mac (Tree Star, Inc., Ashland, OR). Polyclonal data were exported using Boolean gates in FlowJo and further analyzed using PESTLE software (version 1.5.4) and SPICE software (version 4.1.6) obtained from M. Roederer, National Institutes of Health, Bethesda, MD. Polyclonal T cells were defined as viable CD3+ CD6+ cells that produce IFN-γ and IL-2 and express CD107a simultaneously (IFN-γ-IL-2-CD107a+). A cutoff of 0.02% of specific IFN-γ production was considered for any polyclonality analysis.

Multiparametric phenotypic characterization of HCV-specific T cells. All flow cytometry assays were performed on cryopreserved samples. For phenotype analysis, 2 × 10⁶ peripheral blood mononuclear cells (PBMCs) were stained with freshly prepared tetramer-PE for 30 min at room temperature and washed in fluorescence-activated cell sorting (FACS) buffer (1× phosphate-buffered saline [PBS], 1% fetal bovine serum [FBS], 0.02% NaN₃) to remove any non-specific intracellular fluorescence on the cell surface.

Intracellular cytokine staining (ICS) and CD107a degranulation assay. A total of 2 × 10⁶ PBMCs were incubated with anti-CD107a and either dimethyl sulfoxide (0.1%) as a negative control or HCV peptide (5 to 30 μg/ml) at 37°C in R-10 medium (RPMI medium [Invitrogen, Carlsbad, CA] supplemented with 10% FBS). Following 1 h of stimulation, 10 μg/ml of brefeldin A (Sigma-Aldrich) and 6 μg/ml of monensin sodium salt (Sigma-Aldrich) were added, and cells were incubated for a total of 16 h. Cells were washed with FACS buffer, stained for intracellular IFN-γ and IL-2, and fixed in FACS Fix buffer. For intracellular CD107a detection, PBMCs were first stained with tetramers and surface markers, lysed with a BD Lysing and Permeabilizing Kit (BD Biosciences) according to the manufacturer’s instructions, stained with anti-CD1 and anti-CD8 antibodies, and then washed twice and fixed in FACS Fix buffer.

Intracellular cytokine staining (ICS) and CD107a degranulation assay. A total of 2 × 10⁶ PBMCs were incubated with anti-CD107a and either dimethyl sulfoxide (0.1%) as a negative control or HCV peptide (1 μg/ml) at 37°C in R-10 medium (RPMI medium [Invitrogen, Carlsbad, CA] supplemented with 10% FBS). Following 1 h of stimulation, 10 μg/ml of brefeldin A (Sigma-Aldrich) and 6 μg/ml of monensin sodium salt (Sigma-Aldrich) were added, and cells were incubated for a total of 16 h. Cells were washed with FACS buffer, stained for viability and cell surface antigens, and permeablized using BD Cytofix/Cytoperm (BD Biosciences) and then stained with anti-CD127 and anti-CD107a, and fixed in FACS Fix buffer. For intracellular CD107a detection, PBMCs were first stained with tetramers and surface markers, lysed with a BD Lysing and Permeabilizing Kit (BD Biosciences) according to the manufacturer’s instructions, stained with anti-CD1 and anti-CD8 antibodies, and then washed twice and fixed in FACS Fix buffer.

Identification of acute HCV infection and longitudinal phenotypic analysis of HCV-specific CD8+ T cells. Eight subjects acutely infected with HCV were identified among IDUs, the group at highest risk of new HCV infections, as described in Materials and Methods. Patients’ demographics and characteristics are listed in Table 1. HLA typing and the tetramers used are listed in Table 2. The overall breadth of the immune response was monitored at the earliest time point by an IFN-γ enzyme-linked immunospot assay using a panel of overlapping peptides corresponding to the HCV H77 genotype 1a polyprotein reference sequence. As documented in the literature, the immune response was broader in the patients who resolved spontaneously (data not shown). A panel of five different HLA class I tetramers corresponding to predefined dominant HCV responses was then used together with a comprehensive nine-color panel of phenotypic markers for T-cell maturation (CD27, and CD28), T-cell memory (CCR7, CD45RA, and CD127), and T-cell exhaustion (PD-1). Control tetramers containing CMV or Flu peptides were used in most patients (Table 2). Representative phenotyping results, using HLA-A2/NS3-1073 tetramer from patient R1 with acute resolving HCV and patient T4 with chronic evolution are presented in Fig. 1A and B, respectively. HCV tetramer-positive cells were not de-

### Table 1. Characteristics and demographics of acute HCV-infected patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender*</th>
<th>Age (yr)</th>
<th>Route of infection</th>
<th>HIV infection status</th>
<th>Genotype</th>
<th>Highest viral load (IU/ml of plasma)</th>
<th>Acute infection outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>M</td>
<td>45</td>
<td>IDU</td>
<td>Negative</td>
<td>1</td>
<td>&lt;10⁰⁶</td>
<td>Spontaneously resolved</td>
</tr>
<tr>
<td>R2</td>
<td>M</td>
<td>39</td>
<td>IDU</td>
<td>Negative</td>
<td>ND⁺</td>
<td>&lt;10⁰⁶</td>
<td>Spontaneously resolved</td>
</tr>
<tr>
<td>C1</td>
<td>M</td>
<td>23</td>
<td>IDU</td>
<td>Negative</td>
<td>1a</td>
<td>1,030,000</td>
<td>Chronic evolution</td>
</tr>
<tr>
<td>C2</td>
<td>M</td>
<td>24</td>
<td>IDU</td>
<td>Negative</td>
<td>3a</td>
<td>1,400,000</td>
<td>Chronic evolution</td>
</tr>
<tr>
<td>T1</td>
<td>F</td>
<td>29</td>
<td>IDU</td>
<td>Negative</td>
<td>1</td>
<td>1,170</td>
<td>Resolved after therapy</td>
</tr>
<tr>
<td>T2</td>
<td>M</td>
<td>32</td>
<td>IDU</td>
<td>Negative</td>
<td>1b</td>
<td>9,300</td>
<td>Resolved after therapy</td>
</tr>
<tr>
<td>T3</td>
<td>M</td>
<td>30</td>
<td>IDU</td>
<td>Negative</td>
<td>1a</td>
<td>11,000,000</td>
<td>Resolved after therapy</td>
</tr>
<tr>
<td>T4</td>
<td>M</td>
<td>35</td>
<td>IDU</td>
<td>Negative</td>
<td>1a</td>
<td>30,200,000</td>
<td>Therapy relapse</td>
</tr>
</tbody>
</table>

* M, male; F, female.

⁺ Determined by COBAS Amplicor HCV Monitor test, version 2.0 (sensitivity, 600 IU/ml).

⁺⁺⁺ Determined by real-time quantitative PCR assay (sensitivity, 1,000 IU/ml).

⁺⁺ ND, not determined.
tectable in patient R1 at 9 weeks prior to his first positive RNA test or in patient T4 at 2 weeks postdetection of viremia (PDV). Although HLA-A2/NS3-1073 tetramer frequency was very high in patient R1 compared to patient T4 (4.5% versus 0.18%), there was no difference in the phenotype of HCV-specific cells in both patients at the early stages of infection as cells were primarily CD27⁺ CD28⁻ CCR7⁻ CD45RA⁻, expressing low levels of CD127 (CD127lo) and PD-1 (PD-1⁺). Nevertheless, as patient R1 eliminated the virus and despite a decline in the frequency of tetramer-positive cells, CD127 expression was gradually upregulated. Cells detectable at a late follow-up time point at 86 weeks PDV were 94% positive for CD127 (Fig. 1A). In patient T4, as viremia persisted, the tetramer-positive cells lost CD127 and upregulated PD-1 expression (Fig. 1B). Similar results were obtained with other patients studied; HCV tetramer-positive cells detected in all patients early were primarily CD27⁺ CD28⁻ CCR7⁻ CD45RA⁻ CD127⁻ PD-1⁻ (data not shown) and did not change as infection progressed to chronicity or spontaneous resolution, with the exception of CD127 and PD-1. Reexamining the phenotype of HCV tetramer-positive CD8⁺ T cells at the latest follow-up time point prior to commencement of therapy demonstrated that CD127 was upregulated on HCV-specific CD8⁺ T cells in patients R1 and R2, who spontaneously resolved acute HCV infection (Fig. 1C), and slightly upregulated or unchanged in patients T1 and T2, who exhibited low viral loads of 1,170 and 9,300 IU/ml plasma, respectively (Table 1). Similar to previous reports, PD-1 expression did not necessarily correlate with infection outcome (8, 35). Expression was generally low during the early phase of infection in all patients but then was upregulated on HCV-positive cells in patients T3 and T4 (Fig. 1C), who also exhibited the highest viral loads, around 10⁷ IU/ml (Table 1).

Spontaneously resolved HCV infection is associated with the early development of polyfunctional T cells. Given that we could not establish an early phenotypic marker for T cells associated with spontaneously resolved HCV infection except CD127, we hypothesized that CD127 expression delineates a unique subset of long-lived memory T cells that have antiviral effector functions, similar to earlier observations in murine and human viral infections (29, 30). To evaluate longevity of the CD127⁺ cells, we monitored the longitudinal coexpression of the antiapoptotic molecule Bcl-2 and CD127 in tetramer-positive cells in patients R1 and R2. As illustrated in Fig. 2A, HLA-A2/NS3-1073 tetramer-positive cells in patient R1 and HLA-B8/NS3-1395 tetramer-positive cells in patient R2 expressed low levels of CD127 and Bcl-2 at the first detection of viremia although it was partially controlled (<600 IU/ml). As virus was completely eliminated, tetramer-positive cells gradually upregulated CD127 and Bcl-2, with distinct enrichment in the CD127⁺ Bcl-2⁺ tetramer population that represented 81% and 69% of tetramer-positive cells at the latest follow-up time point in patients R1 and R2, respectively (Fig. 2A). Furthermore, upregulation of CD127 and Bcl-2 was associated with increased proliferative capacity, as observed in a CFSE dilution assay (Fig. 2B). The proliferation of HLA-A2/NS3-1073 tetramer-positive cells increased from 44% at the first detection of viremia to nearly 97% by week 86 PDV despite a fourfold decline in tetramer frequency from 4.5% to 0.95% (Fig. 2A), consistent with a memory T-cell profile (24–26).

Given that polyfunctionality has been associated with control of several viral infections, high IFN-γ MFIs, and a TC1 cell phenotype (24–26), we defined the polyfunctionality of IFN-γ-producing cells to estimate the proportion of cells producing both IFN-γ and IL-2; IFN-γ and CD107a; or IFN-γ, IL-2, and CD107a simultaneously. Polyfunctional T cells (IFN-γ⁺ IL-2⁺ CD107a⁺) were detected at the earliest time point studied despite active viral replication. The proportion of polyfunctional T cells (Fig. 2D) increased over time, in particular, in the long-term memory phase representing 15% and 26% of all IFN-γ-producing cells in patients R1 and R2, respectively. Most importantly, the majority of HCV-specific cells exhibited at least two functions (Fig. 2D).

**Acute HCV with chronic evolution is associated with diminished function and rapid loss of HCV-specific T cells.**

**TABLE 2. Patient HLA types and tetramers used**

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA allele(s)</th>
<th>HCV tetramer</th>
<th>Control tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>A0201, A3101</td>
<td>B4403, B5101</td>
<td>HLA-A2/NS3-1073</td>
</tr>
<tr>
<td>R2</td>
<td>A0101, A2402</td>
<td>B0801, B3906</td>
<td>HLA-B8/NS3-1395</td>
</tr>
<tr>
<td>C1</td>
<td>A0201, A6802</td>
<td>B4402</td>
<td>HLA-A2/NS3-1073</td>
</tr>
<tr>
<td>C2</td>
<td>A0101, A0201</td>
<td>B0702, B4402</td>
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<td>A0101, A0201</td>
<td>B2703, B4402</td>
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</tr>
<tr>
<td>T2</td>
<td>A0301, A2601</td>
<td>B0702, B4002</td>
<td>HLA-A2/NS3-1073</td>
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<tr>
<td>T3</td>
<td>A0201, A0205</td>
<td>B4901, B5101</td>
<td>HLA-A2/NS3-1073</td>
</tr>
<tr>
<td>T4</td>
<td>A0201, A6802</td>
<td>B1801, B4002</td>
<td>HLA-A2/NS3-1073</td>
</tr>
</tbody>
</table>
hypothesized that HCV acutely infected individuals who develop persistent infection fail to develop or sustain polyfunctional virus-specific T cells. We thus monitored the phenotype and function of HCV-specific T cells longitudinally in patient C1 by using two different HCV tetramers, HLA-A2/NS3-1073 and HLA-A2/NS3-1406 (Fig. 3A). Although the two populations of tetramer-positive cells were detectable at a considerable frequency (0.1 to 0.4%) up to 18 weeks PDV in patient C1, the tetramer-positive cells remained CD127lo Bcl-2lo (Fig. 3A, middle panel). Furthermore, CD8\(^+\) T cells interacting with both tetramers became undetectable in the peripheral blood by week 41 PDV (Fig. 3A). Longitudinal analysis of the functionality of the cells demonstrated weak proliferative capacity against the NS3-1073 peptide during the early time points of the infection and diminished proliferation over time (Fig. 3A, middle panel). Furthermore, ICS analysis in response to stimulation with a mixture of the NS3-1073 and NS3-1406 peptides demonstrated very low or undetectable cytokine production or CD107a degranulation. Polyfunctionality could not be assessed due to the very low level of IFN-\(\gamma\) production.

FIG. 1. Longitudinal phenotyping of virus-specific T cells during acute HCV infection. Representative longitudinal tetramer staining and phenotyping data from patient R1 with acute resolving HCV infection prior to and after detection of viremia (A) and patient T4 with chronic evolution (B). Tetramer frequency is represented as percentage of CD3\(^+\) CD8\(^+\) T cells. Black represents HLA-A2/HCV-1073 tetramer-positive cells, and red represents total CD3\(^+\) CD8\(^+\) T cells in the same donor. Percentages of tetramer-positive cells in each quadrant are shown. (C) Summary of longitudinal phenotypic changes in CD127 and PD-1 expression on HCV-tetramer-positive cells during acute HCV at the earliest and latest follow-up time points prior to commencement of therapy. Each symbol represents one HCV tetramer as indicated in the legend according to the following key: A2-1073, HLA-A2/NS3-1073; B8-1395, HLA-B8/NS3-1395; A1-1436, HLA-A1/NS3-1436; B7-41, HLA-B7/core-41; A2-1406, HLA-A2/NS3-1406. Patient numbers are included. Wks, weeks.
were observed in patient C2 using HLA-B7/core-41 tetramer, with a disappearance of tetramer-positive cells by week 17 PDV, low level expression of CD127 and Bcl-2 on tetramer-positive cells, and diminished proliferation and cytokine production (Fig. 3B).

CD127 distinguishes a unique polyfunctional, memory T-cell population. Given the observed correlation between upregulation of CD127 and increased proliferation and effector functions, in particular, in spontaneously resolved HCV infection, we sought to confirm that CD127 distinguishes a unique
T-cell subset destined to become polyfunctional long-lived memory T cells. We sorted CD3$^+$ CD127$^{hi}$ or CD3$^+$ CD127$^{neg}$ T cells from the PBMCs of patient R2 at week 17 PDV. Postsorting phenotypic characterization using the HLA-B8/NS3-1395 tetramer demonstrated that, indeed, CD127 tetramer-positive cells were all Bcl-2$, consistent with long-lived memory T-cell profiles (Fig. 4A). Furthermore, CD127$^{hi}$ tetramer-positive cells proliferated extensively in response to peptide stimulation, demonstrating proliferation of 99% of tetramer-positive cells (Fig. 4B) versus 65% of tetramer-positive cells in the presorting sample (Fig. 2B). Proliferation was not performed on CD127$^{neg}$ cells due to the limited number of recovered cells. Finally, ICS and polyfunctionality analysis demonstrated that the CD127$^{hi}$ population was polyfunctional in response to stimulation with the cognate peptide with production of IFN-$\gamma$ and IL-2 and degranulation, while CD127$^{neg}$ cells produced very few cytokines (Fig. 4C), despite a higher frequency of HLA-B8 tetramer-positive cells (1.58% in the CD127$^{neg}$ versus 0.25% in CD127$^{hi}$ populations) (Fig. 4A). Similar results were observed in patient R1 using sorted cells from week 57 PDV (see Fig. S2 in the supplemental material). These results confirm that CD127 marks a distinct population of polyfunctional HCV-specific T cells in these individuals with spontaneously resolved infections.

Early therapeutic intervention rescues polyfunctional long-lived memory T cells. Patients T1, T2, and T3 remained persistently infected for up to 15 to 22 weeks PDV and exhibited predominantly a CD127$^{lo}$ phenotype of HCV tetramer-specific T cells from the early onset of viremia (Fig. 1C). In accordance with published data and clinical guidelines at the time showing that most acute resolving cases of HCV infection resolve within the first 12 weeks of infection and recommending IFN therapy for patients who remain HCV RNA positive after 12 weeks (61), the patients received IFN monotherapy with no ribavirin for 12 to 16 weeks. The duration of treatment was based on the reports of Kamal et al. demonstrating that treatment administered for 8 to 12 weeks was effective to achieve sustained virologic response (SVR) in genotypes other than genotype 1 and among patients who present a rapid virologic response, i.e., HCV RNA negative at week 4, regardless of the genotype (32, 34).

We hypothesized that early IFN therapy will induce upregulation of Bcl-2 and may rescue HCV-specific T cells from deletion, as observed in patients C1 and C2 who declined therapy. We thus monitored the phenotype and function of the HCV tetramer-specific cells in these subjects prior to, during, and up to 1 year following discontinuation of antiviral therapy. Patient T1, who exhibited a low plasma viral load at the start of therapy (1,170 IU/ml), responded to two HCV tetramers, HLA-A1/NS3-1436 and HLA-A2/NS3-1073. This patient received IFN therapy between 15 and 27 weeks PDV (12 weeks total). Prior to commencement of therapy, HLA-A1/NS3-1436

FIG. 3. Chronically evolving acute HCV infection is associated with low-level expression of CD127 and Bcl-2 and effector functions. Representative data from patient C1 using HLA-A2/NS3-1073 (A2-1073) and HLA-A2/NS3-1406 (A2-1406) tetramers (A) and patient C2 using HLA-B7/core-41 (B7/core-41) tetramer (B) are shown. Top panel depicts tetramer frequency relative to HCV RNA viral load. Middle panel represents longitudinal expression of CD127 and intracellular Bcl-2 in HLA-A2/NS3-1073 tetramer positive (Tet$^+$) cells in patient C1 (A) and HLA-B7/core-41 tetramer-positive cells in patient C2 (B); proliferation of HCV-specific T cells in response to NS3-1073 peptide in patient C1 or core-41 peptide in patient C2 is depicted as a bar graph representing the percentage of proliferating (CFSElo) tetramer-positive (Tet$^+$) cells. The bottom panel represents longitudinal cytokine production and CD107a degranulation following stimulation with a mixture of NS3-1073 and NS3-1406 peptides for patient C1 (A) or core-41 peptide for patient C2 (B), represented as percent specific expression in viable CD3$^+$ CD8$^+$ T cells.
tetramer-positive cells were primarily CD127<sup>lo</sup> Bcl-2<sup>lo</sup> (Fig. 5A). Within 2 weeks of starting therapy, a rapid shift was observed in the phenotype of HLA-A1/NS3-1436 tetramer, CD127, and Bcl-2. Black represents HLA-A1/NS3-1436 tetramer-positive cells, and red represents total CD3<sup>+</sup> CD8<sup>+</sup> T cells; overall tetramer frequency is represented in the upper right quadrant, and percentages of tetramer-positive cells in each quadrant are shown. (B) CFSE proliferation of CD127<sup>hi</sup> cells gated on CD3<sup>+</sup> CD8<sup>+</sup> T cells. The percentage of proliferating (CFSE<sup>lo</sup>) tetramer-positive cells is represented in the upper left quadrant; the percentage of total cells in each quadrant is represented in the bottom left quadrant. Postsorting, CD127<sup>neg</sup> cells were too few to perform this assay. ND, not detected. (C) Cytokine production and CD107a degranulation of CD127<sup>neg</sup> and CD127<sup>hi</sup> cells stimulated with NS3-1395 peptide. Pie chart represents polyfunctional analysis for CD127<sup>hi</sup> cells, gated on viable CD3<sup>+</sup> CD8<sup>+</sup> IFN-γ<sup>-</sup> cells. Red represents three functions (IFN-γ, IL-2<sup>+</sup>, and CD107a<sup>+</sup>), blue represents two functions (IFN-γ<sup>-</sup> and CD107a<sup>-</sup>), green represents two functions (IFN-γ<sup>-</sup> and IL-2<sup>-</sup>), and yellow represents one function (IFN-γ<sup>-</sup>); numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ-producing cells in the CD127<sup>neg</sup> population was too low to perform the polyfunctional analysis.

FIG. 4. CD127 expression delineates a unique polyfunctional T-cell subset. CD3<sup>+</sup> T cells from PBMCs of patient R2 at week 17 PDV were sorted into CD127<sup>neg</sup> and CD127<sup>hi</sup> cells and tested for their proliferative capacity and cytokine production. (A) Pre- and postsorting purity of cells stained with HLA-B8/NS3-1395 tetramer, CD127, and Bcl-2. Black represents HLA-B8/NS3-1395 tetramer-positive cells, and red represents total CD3<sup>+</sup> CD8<sup>+</sup> T cells; overall tetramer frequency is represented in the upper right quadrant, and percentages of tetramer-positive cells in each quadrant are shown. (B) CFSE proliferation of CD127<sup>hi</sup> cells gated on CD3<sup>+</sup> CD8<sup>+</sup> T cells. The percentage of proliferating (CFSE<sup>lo</sup>) tetramer-positive cells is represented in the upper left quadrant; the percentage of total cells in each quadrant is represented in the bottom left quadrant. Postsorting, CD127<sup>neg</sup> cells were too few to perform this assay. ND, not detected. (C) Cytokine production and CD107a degranulation of CD127<sup>neg</sup> and CD127<sup>hi</sup> cells stimulated with NS3-1395 peptide. Pie chart represents polyfunctional analysis for CD127<sup>hi</sup> cells, gated on viable CD3<sup>+</sup> CD8<sup>+</sup> IFN-γ<sup>-</sup> cells. Red represents three functions (IFN-γ, IL-2<sup>+</sup>, and CD107a<sup>+</sup>), blue represents two functions (IFN-γ<sup>-</sup> and CD107a<sup>-</sup>), green represents two functions (IFN-γ<sup>-</sup> and IL-2<sup>-</sup>), and yellow represents one function (IFN-γ<sup>-</sup>); numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ-producing cells in the CD127<sup>neg</sup> population was too low to perform the polyfunctional analysis.

tetramer-positive cells were primarily CD127<sup>lo</sup> Bcl-2<sup>lo</sup> (Fig. 5A). Within 2 weeks of starting therapy, a rapid shift was observed in the phenotype of HLA-A1/NS3-1436 tetramer-positive T cells, with upregulation of CD127 and Bcl-2 upon virus elimination (Fig. 5A). This patient achieved SVR, defined as an absence of HCV RNA up to 24 weeks after the discontinuation of therapy. A CD127<sup>+</sup> Bcl-2<sup>+</sup> tetramer population was selected for during therapy and remained detectable up to 58 weeks after discontinuation of therapy (84 weeks PDV) (Fig. 5A). Similar results were observed in this patient using the HLA-A2/NS3-1073 tetramer (data not shown), in patient T2 using HLA-B7/core-41 tetramer (Fig. 5B), and in patient T3, who exhibited very high viral load (11 × 10<sup>6</sup> IU/ml) prior to therapy but nevertheless responded to treatment and achieved SVR (Fig. 5C). Furthermore, although the patients exhibited different levels of PD-1 expression on HCV-specific T cells, PD-1 expression was downregulated in all patients upon viral clearance (data not shown). The overall expression
of CD127, Bcl-2, and PD-1 was not affected in total CD8 T cells in any of the patients studied during IFN therapy (data not shown). Furthermore, control staining using CMV or Flu tetramers demonstrated that there was no change in phenotype of these T-cell populations before, during, and after IFN therapy (see Fig. S3 in the supplemental material), thus confirming that the observed phenotypic changes are unique to HCV-specific T cells and are related to viral clearance rather than immune modulation by IFN-α.

In addition, we monitored the function of HCV-specific T cells using CFSE proliferation assays and ICS. All patients demonstrated a good proliferative response at the early stages of infection, which increased following initiation of therapy (Fig. 5, bottom panels), suggesting that proliferation might not be predictive of the outcome of acute HCV. In contrast, very weak or no IFN-γ, IL-2, or CD107a expression was detected against the various epitopes tested prior to therapy, and cells were primarily monofunctional (IFN-γ producers) (Fig. 6). A marked increase in the IFN-γ MFI, restoration in all functions, and generation of polyfunctional T cells were observed in all patients following the initiation of therapy and coincident with virus elimination (Fig. 6). Notably, although HCV-specific T cells declined in frequency over time, they remained detectable and polyfunctional up to 1 year following discontinuation of therapy in all patients studied (Fig. 5 and 6). Polyfunctional cells constituted 11 to 21% of specific T cells at the latest follow-up, suggesting a long-lived memory T-cell response.

Transient restoration of HCV-specific immune responses during therapy followed by loss upon viral recurrence. We monitored longitudinally the phenotype and function of patient T4, who experienced recurrence in viremia following the discontinuation of IFN therapy. This patient responded to the HLA-A2/NS3-1073 tetramer and exhibited a very high viral load (Table 1) (Fig. 7A). During the first 17 weeks PDV, the HLA-A2/NS3-1073-specific cells were mostly CD127lo Bcl-2lo (Fig. 7A). This patient underwent IFN therapy for 16 weeks

FIG. 5. Early treatment with PEG-IFN-α rescues CD127+ Bcl-2+ HCV-specific T cells. Longitudinal phenotypic characterization and proliferative response of HCV tetramer-positive T cells in patient T1 using HLA-A1/NS3-1436 tetramer (A1-1436 Tet) and treated between weeks 15 to 27 (A), patient T2 using HLA-B7/core-41 tetramer (B7-Core 41 Tet) and treated between weeks 32 and 54 (B), and patient T3 using HLA-A2/NS3-1073 tetramer (A2-1073 Tet) and treated between weeks 32 and 48 (C). The shaded area represents the period of PEG-IFN-α therapy. The top panel depicts the respective tetramer(s) frequency relative to HCV RNA viral load. The bottom panel represents the expression of CD127 and Bcl-2 on tetramer-positive cells and proliferation of HCV-specific T cells in the presence of their specific peptides depicted as bar graphs representing the percentage of proliferating (CFSElo) tetramer-positive cells. Tet+, tetramer positive.

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between weeks 21 to 36 PDV, with recurrence of viremia at 8 weeks after the discontinuation of therapy (week 44 PDV). Given the nature of the cohort studied, it is difficult to determine whether this recurrence in viremia is a true relapse or a new infection. We sequenced a stretch of 50 amino acids spanning the NS3-1073 epitope in patient T4 prior to therapy and upon recurrence of viremia on week 44 PDV and observed the same viral RNA sequence in 13/13 and 14/14 molecular clones, respectively (data not shown), suggesting that this is a recurrence of viremia and not a new infection. Interestingly, patient T4 rapidly developed CD127^-Bcl-2^- HCV tetramer-positive cells and demonstrated enhanced pro-

![Image of diagrams showing longitudinal analysis of cytokine production, IFN-MFI, and CD107a degranulation following specific peptide stimulation and polyfunctionality in patients T1, T2, and T3.](http://jvi.asm.org/)

FIG. 6. Early treatment with PEG-IFN-α rescues polyfunctional HCV-specific T cells. Longitudinal analysis of cytokine production, IFN-γ MFI, and CD107a degranulation following specific peptide stimulation and polyfunctionality in patient T1 (A and B), patient T2 (C and D), and patient T3 (E and F). The shaded areas represent the PEG-IFN-α administration period between weeks 15 to 27 in patient T1, weeks 32 to 54 in patient T2, and weeks 32 to 48 in patient T3. For polyfunctionality, red represents three functions (IFN-γ*, IL-2*, and CD107a*), blue represents two functions (IFN-γ* and CD107a*), green represents two functions (IFN-γ* and IL-2*), and yellow represents one function (IFN-γ*); numbers on the pie chart represent the percentage of each population. Polyfunctional analysis was not performed before therapy when the frequency of IFN-γ-producing cells was mostly lower than the cutoff threshold (0.02%) for polyfunctionality analysis. A1-1436, HLA-A1/NS3-1436; B7-core 41, HLA-B7/core-41; A2-1073, HLA-A2/NS3-1073; Tet, tetramer.
liferative capacity at week 28 PDV (7 weeks after the beginning of therapy) (Fig. 7A). The cells also became polyfunctional as they acquired the capacity to produce IFN-γ, IL-2, and CD107a (Fig. 7C). However, the proportion of polyfunctional T cells declined by week 36 PDV. NS3-1073-specific T cells remained detectable by tetramer 3 weeks later at week 39 PDV and exhibited 65% proliferation (Fig. 7A) but were primarily IFN-γ monofunctional at 88% (Fig. 7C) and had a lower

FIG. 7. Transient restoration of CD127 and Bcl-2 expression and limited polyfunctionality in HCV-specific T cells during therapy, followed by loss upon viral recurrence in patient T4. Longitudinal phenotypic characterization and proliferative response of HLA-A2/NS3-1073 (A2-1073) tetramer-positive T cells in patient T4 are shown. (A) The top panel represents the A2/1073 tetramer frequency relative to HCV RNA viral load. The bottom panel represents the expression of CD127 and Bcl-2 on tetramer-positive cells; CFSE proliferation of HCV-specific T cells in the presence of NS3-1073 peptide is depicted as bar graphs representing the percentage of proliferating (CFSElo) tetramer-positive cells. (B) Longitudinal analysis of cytokine production, IFN-γ MFI, and CD107a degranulation following NS3-1073 peptide stimulation. The shaded areas represent the period of administration of PEG-IFN-α therapy between weeks 21 to 36. (C) Polyfunctionality of HCV peptide-specific T cells in each patient. Red represents three functions (IFN-γ+, IL-2+, and CD107a+), blue represents two functions (IFN-γ+ and CD107a+), green represents two functions (IFN-γ+ and IL-2+), and yellow represents one function (IFN-γ-); numbers on the pie chart represent the percentage of each population. The frequency of IFN-γ-producing cells at some points prior to therapy was lower than the cutoff threshold (0.02%) for the polyfunctionality analysis. Tet, tetramer.
IFN-γ MFI. Furthermore, as this patient relapsed at week 44 PDV, tetramer-positive cells and cytokine production were undetectable in peripheral blood (Fig. 7A and B). Another follow-up time point at week 93 PDV yielded similar results (data not shown). To confirm that the NS3-1073-specific cells were not present at a low frequency undetectable by tetramers, we attempted to generate a T-cell line from the peripheral blood of this patient using repeated peptide stimulation at week 93 PDV. These attempts were unsuccessful, suggesting that the NS3-1073-specific cells were either completely eliminated from peripheral blood or localized to the liver or were extremely defective in their proliferative capacity.

Phenotypic and functional changes observed are not due to changes in targeted epitopes. To confirm that the phenotypic changes observed over time are not due to the emergence of escape mutations in this region, we sequenced the HCV epitope region targeted in the main tetramers used in this study. At least two time points were sequenced for each patient, early upon recruitment and late prior to starting IFN therapy. The following epitopes were sequenced: HLA-A2/NS3-1073, HLA-A2/NS3-1406, and HLA-A1/NS3-1436. As illustrated in Table 3 the dominant viral sequence in all epitopes did not change in any of the patients tested. A mismatch occurred between the autologous virus sequence and the peptide sequence used in the tetramer and ICS assay in patients T1 and T3. In patient T1, a Y→F change was detected in the HLA-A1-restricted NS3-1436 epitope; this change was previously shown not to influence T-cell function (36). Similarly, an I→V mutation was observed in the HLA-A2/NS3-1073 autologous virus sequence of patient T3, which is a natural variant of this epitope, previously described (36). It is unlikely that such a mismatch is responsible for the changes in phenotype and function of HCV-specific T cells during therapy.

Table 3. Sequencing of HCV epitopes studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time point</th>
<th>HLA-A2/NS3-1073</th>
<th>HLA-A2/NS3-1406</th>
<th>HLA-A1/NS3-1436</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sequence</td>
<td>No. of positive clones/no. of clones tested</td>
<td>Sequence</td>
</tr>
<tr>
<td>C2</td>
<td>Early</td>
<td>CINGVCWTV</td>
<td>13/13</td>
<td>KLVALGINAV</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>----</td>
<td>8/9</td>
<td>----</td>
</tr>
<tr>
<td>T1</td>
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<td>----</td>
<td>8/8</td>
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<td></td>
<td>Late</td>
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<td>8/8</td>
<td>----</td>
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<tr>
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<td>-V---------</td>
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<tr>
<td></td>
<td>Late</td>
<td>-V---------</td>
<td>10/10</td>
<td>----</td>
</tr>
<tr>
<td>T4</td>
<td>Early</td>
<td>----</td>
<td>13/13</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>----</td>
<td>10/10</td>
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* Dashes indicate identity with the reference sequence.

**DISCUSSION**

We have performed a comprehensive multiparametric phenotypic and functional characterization of the HCV-specific immune response during clearly defined acute infection in a unique cohort of IDUs with spontaneous resolution and chronic evolution and during early IFN therapy. We demonstrate that spontaneous resolution is associated with early emergence of polyfunctional CD8+ T cells. These polyfunctional T cells were defined by the expression of CD127 and Bcl-2. Despite a measurable immune response detected at the early time points, HCV-specific T cells failed to upregulate CD127 and Bcl-2 expression in individuals who developed persistent viremia, and these cells eventually disappeared from peripheral blood. Notably, early therapeutic intervention reconstituted a long-lived polyfunctional memory T cell response.

In agreement with previous reports, we demonstrate that PD-1 expression does not correlate with the outcome of acute HCV infection (8, 35), except at the highest viral loads. We also demonstrate that spontaneous resolution correlates with upregulation of CD127 on HCV-specific cells and intracellular content of the antiapoptotic molecule Bcl-2, a common characteristic of long-lived memory T cells (30). We further demonstrate that CD127 expression distinguishes a unique subset of HCV-specific memory T cells bearing the phenotypic signature of TEm cells (CCR7−CD45RA−) and yet bearing the functional signatures of both TCM (rapid proliferation and high...
IL-2 production) and T_{EM} (high IFN-γ production and cytotoxic potential). These cells are polyfunctional in nature, which is defined as having the capacity to produce more than one cytokine in addition to cytotoxic and proliferative capacities (26, 48). Such polyfunctional T cells were recently correlated with control of HIV infection (1, 6), response to HIV vaccines (24, 41), and antiretroviral therapy (44). We establish that these polyfunctional memory T cells in the context of HCV are CD27^+ CD28^+ CCR7^− CD45RA^− CD127^+ Bcl-2^+. A similar memory T-cell subset at a transitional or early maturation stage (CD27^+ CD28^+ CD127^− ) with a short replicative history and strong telomerase activity was recently described (45). We propose that this polyfunctional T-cell subset represents transitional effector memory T cells that are long-lived but also constitute the first responders upon antigen encounter in the periphery. Although this T-cell subset is distinguished primarily by CD127 expression and such a phenotype was reported in other viral infections like hepatitis B virus (7), it is unlikely that CD127 alone can be used as a marker of such polyfunctional memory T cells or to predict the outcome of HCV infection or therapy. Such simplistic models of defining maturation of virus-specific T cells and their functional capacity are no longer sufficient, but comprehensive systematic phenotypic, functional, and even genomic profiling will be essential. Studies using a larger cohort of patients are needed to accurately define the phenotypic and functional signatures of these polyfunctional T cells and should be included in monitoring the efficacy of any protective or therapeutic vaccines for HCV, as recently suggested for HIV vaccine trials (48).

Several factors may contribute to the induction of polyfunctional T cells and establishment of T-cell memory (30). The most important factor is unarguably CD4^+ T-cell help. Indeed, CD4^+ T cells play an essential role in establishment and maintenance of CD8^+ memory T cells in various models of murine and human infections (49, 52, 53). In particular, acute HCV infections that progress to chronicity have been linked to the inability to sustain a CD4^+ helper T-cell response (16, 56, 57). Depletion studies in the chimpanzee model have demonstrated that loss of CD4^+ T cells results in loss of function, a decline in HCV-specific CD8^+ T cells, and enhanced emergence of escape mutations in targeted cytotoxic T lymphocyte epitopes (20). The role of CD4^+ T-cell help and the mechanisms of cross talk between CD4^+ and CD8^+ T cells during the memory phase that probably occur via interaction with dendritic cells still remain to be elucidated (10, 11).

Longevity is one of the unique characteristics of memory T cells. HCV-specific memory T cells were detected in spontaneously resolved infections for up to 20 years in the absence of any detectable viremia (54). Similarly, in the present study, we demonstrate that although HCV-specific memory T cells declined over time, they remained detectable at a reasonable frequency for up to 1 year following spontaneous resolution or SVRs to IFN therapy. All patients were tested at multiple time points by the most sensitive PCR assays (sensitivity, 50 IU/ml plasma) and were consistently negative, suggesting that HCV-specific T-cell memory is antigen independent and that establishment of a long-lived memory response is due to successful viral elimination and prevention of persistent T-cell activation and exhaustion. Nevertheless, it is still possible that very low-level residual virus or intermittent viral replication (40) contributes to long-term maintenance of virus-specific memory T-cell populations.

PEG-IFN-α therapy for HCV is most effective when initiated early during the acute phase (32, 34, 62), but the potential contribution of adaptive cellular immunity in this enhanced response is controversial, with several reports demonstrating a decline in the immune response during therapy (31, 33, 37, 43). In the present study, we do not observe any significant drop in the frequency of HCV-specific T cells during therapy. Although we do observe a gradual decline over time, HCV-specific T cells remained detectable for up to 1.5 years of follow-up. Such differences between the various studies might be due to the therapeutic regimen or the nature of the dominant immune response generated in each patient and to how accurately it matches the autologous virus sequence.

We demonstrate a major change in the immune response following therapy while the circulating viral population remains unchanged, thereby excluding a potential influence for viral escape on the immune response to the epitopes studied. However, we did not perform a detailed analysis of viral escape in these patients, and it remains an important mechanism for HCV immune evasion. It is still possible that viral escape did occur in other regions and may have contributed to viral persistence. Furthermore, the increased polyfunctionality of virus-specific T cells may induce a new selection pressure and trigger mutational escape. A more comprehensive analysis of polyfunctionality in relation to full-length viral sequencing is essential to elucidate this point.

We demonstrate that early therapeutic intervention can rescue a polyfunctional immune response with the same phenotypic and functional characteristics as memory T cells induced following spontaneous resolution. However, it remains unclear if such a reconstituted response is the cause or the effect of enhanced virus clearance during early therapy. We favor the hypothesis that reconstitution of a polyfunctional immune response is a consequence of virus elimination and prevention of continued T-cell exhaustion, similar to acute resolving HCV and other viral infections (30). It is likely that there is a limited window of time when IFN therapy can rescue such a polyfunctional response before HCV-specific T cells become severely exhausted and eliminated from circulation. In agreement with this hypothesis, our results demonstrated that HCV-specific cells eventually became undetectable in patients C1 and C2, who declined HCV therapy. Similarly, several reports in the literature have demonstrated that HCV-specific T cells are mostly undetectable in the peripheral blood of chronically infected individuals and mostly localized in the liver or targeting epitopes that underwent escape mutations, thus preventing exhaustion of the specific T cells.

It is tempting to speculate that the development of a polyfunctional T-cell response, when treatment is initiated early, will prevent relapse upon discontinuation of therapy. The inability to rescue such a polyfunctional response when treatment is initiated later during the chronic phase may increase the possibility of virus recurrence and relapse upon discontinuation of therapy. Indeed, our results demonstrating that patient T4, who exhibited viral recurrence, developed primarily a monofunctional immune response favor this hypothesis. Furthermore, large-scale studies demonstrated that the end of treatment response to PEG-IFN-α without ribavirin is approx-
imately 56% when treatment is initiated late in the chronic phase (15) and 83% when treatment is initiated during the acute phase (62). However, the SVR rate is much lower in individuals treated during the chronic phase, reaching only 29% (15) versus 71% when treatment is started during the acute phase (62).

The reconstitution of polyfunctional HCV-specific T cells after early IFN therapy argues in favor of early administration of IFN therapy. Our results demonstrating that the recovered HCV-specific T cells bear the same phenotype and functional signatures as memory T cells generated upon spontaneous resolution suggest that this immune response may be protective upon reexposure and further underscore the importance of the early administration of therapy. Studies in the chimpanzee have demonstrated that prior resolution of one HCV infection can induce protection from chronic infection upon re-exposure (51). Although such a protective response is difficult to evaluate in humans, two reports have demonstrated that high-risk IDUs who have already resolved one HCV infection are less likely to be reinfected than individuals who are HCV naive, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23). Alcoholics, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23). Alcoholics, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23).

Naïve, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23). Alcoholics, despite repeated high-risk exposures (21, 38). IDUs currently represent the main reservoir of HCV (2, 23).


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53. Sun, J. C., M. A. Williams, and M. J. Bevan. 2004. CD8+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat. Immunol. 5:927–933.


