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The Frequency of CD127$^+$ Hepatitis C Virus (HCV)-Specific T Cells but Not the Expression of Exhaustion Markers Predicts the Outcome of Acute HCV Infection

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T cells are exhausted and overexpress inhibitory molecules in chronic hepatitis C virus (HCV) infection. It is unclear whether this is the cause or consequence of HCV persistence. By studying serial blood and liver samples of chimpanzees during acute infection, we demonstrate that the early expression of the memory precursor marker CD127 on HCV-specific T cells, but not the expression of inhibitory molecules on those T cells or their ligands in the liver, predicts the outcome of acute infection.

Acute hepatitis C virus (HCV) infection is spontaneously cleared by a minority (20 to 30%) of infected patients, and clearance typically occurs in the context of vigorous HCV-specific T-cell responses (1–5). In contrast, HCV-specific T cells are impaired in chronic HCV infection, as evidenced by decreased proliferation, cytokine production, and cytolytic activity (6, 7). This impaired phenotype has been attributed to a variety of inhibitory mechanisms, which include increased levels of regulatory T cells (Tregs) and inhibitory cytokines (8–12) as well as T-cell exhaustion due to upregulation of inhibitory molecules such as programmed cell death 1 (PD-1) (13–19), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (16, 17), and T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) (14, 15). *In vitro* blockade of these inhibitory receptors reverses functional exhaustion and restores proliferation and effector function of chronic phase CD8$^+$ T cells (13, 15–19).

To date it is not clear whether the observed T-cell exhaustion is the cause or consequence of chronic HCV infection. High levels of PD-1 on HCV-specific T cells and PD-1 mRNA in the liver were reported in acutely infected patients (20) and chimpanzees (21) that later progressed to chronic hepatitis. In contrast, other studies demonstrated high PD-1 levels on HCV-specific T cells in acute HCV infection irrespective of the infection outcome (11, 22). These contradictory results may be due to heterogeneous study cohorts because the exact time point of infection was not known and the genotypes and sequences of the infecting virus differed among patients.

Using serial blood and liver biopsy samples from chimpanzees that had been infected with HCV genotype 1a in a previous study (23), we investigated whether the outcome of HCV infection can be predicted by the phenotype of HCV-specific CD8$^+$ T cells at the earliest testable time point, i.e., when these T cells first appear in the blood. Chimpanzees Ch6461 and Ch6455 cleared HCV, whereas chimpanzees Ch6475, Ch6411, and Ch6412 developed persistent infection (Fig. 1A and B). Of note, Ch6412 temporarily controlled HCV at undetectable serum titers from week 19 to 23 prior to developing persistent viremia.

As previously described (24), CD8β mRNA levels increased in all chimpanzees during the acute phase of hepatitis, indicative of T-cell recruitment to the liver (Fig. 1C). To determine the intrahepatic mRNA level of T-cell surface markers, their ligands, and cytokines that are known to inhibit HCV-specific T cells, we extracted total RNA from serial liver biopsy specimens using the RNeasy minikit (Qiagen, Valencia, CA) and reverse transcribed it with the first-strand cDNA synthesis kit (Marligen Biosciences, Ijamsville, MD). mRNA levels of genes of interest were determined in duplicate with TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The amount of specific mRNA was normalized to mean levels of β-actin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and β7 mRNA as endogenous references and is presented as fold increase over preinfection mRNA levels.

As shown in Fig. 1D, intrahepatic mRNA levels of PD-L1 and galectin-9, which are known to inhibit CD8 T cells, increased within 2 weeks of acute HCV infection in parallel with viremia. Thus, these ligands were upregulated prior to upregulation of mRNA levels of the PD-L1-receptor PD-1 and the galectin-9 receptor Tim-3, which are typically expressed on liver-infiltrating lymphocytes (Fig. 1E). In addition, mRNA levels of the lymphocyte inhibitory receptors CTLA-4 and lymphocyte activation gene 3 (LAG-3) increased (Fig. 1E). Importantly, expression levels of intrahepatic mRNA of these markers did not interfere with HCV clearance in the two chimpanzees with self-limited infection. Likewise, mRNA levels of immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor β (TGF-β) and the Treg marker Foxp3 did not differ between the two groups of chimpanzees (Fig. 1F). These data indicate that acute phase levels of

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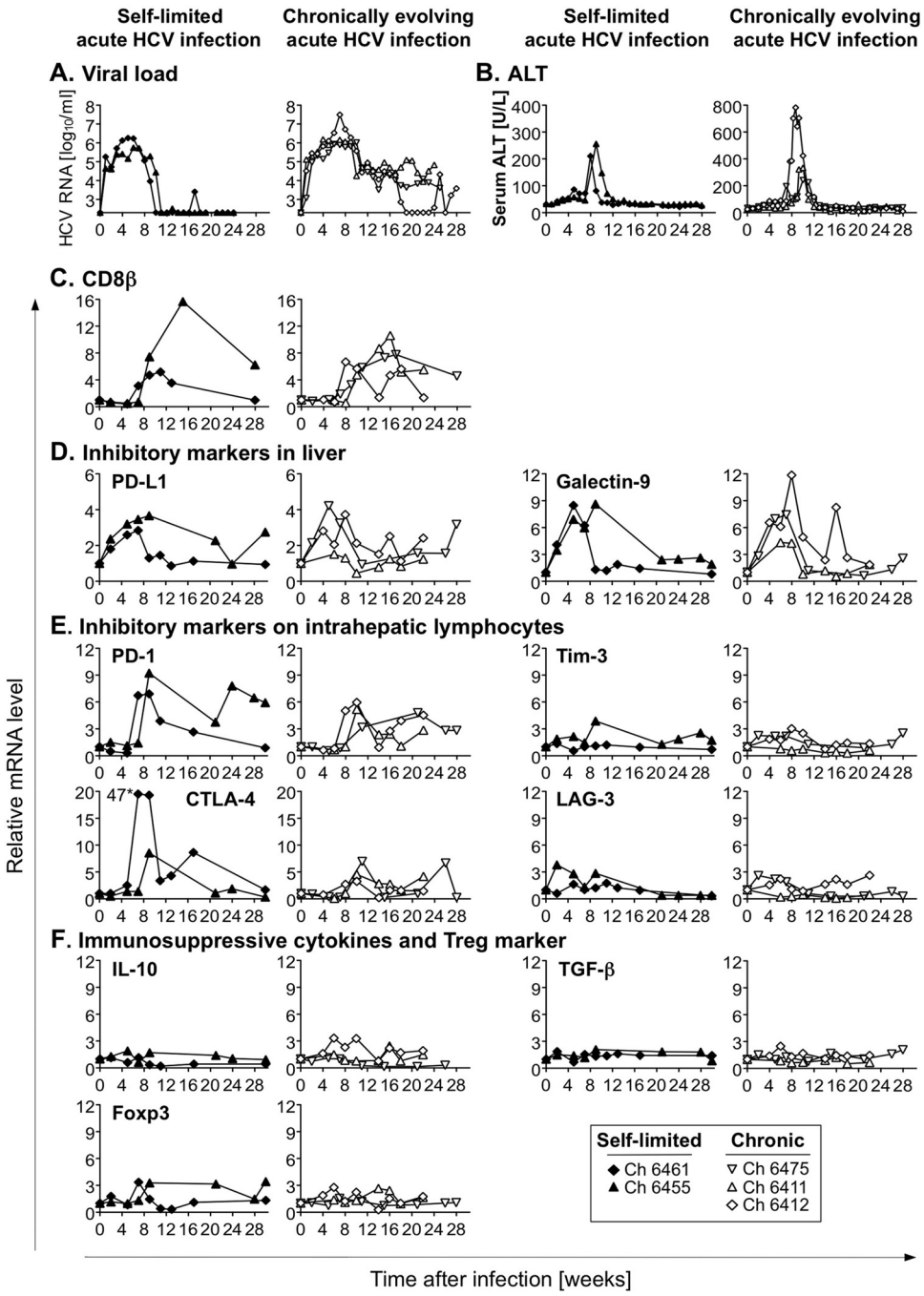

FIG 1 Intrahepatic mRNA expression levels of T-cell-inhibitory molecules during acute HCV infection. (A to C) Five chimpanzees were intravenously challenged with serum containing 100 chimpanzee 50% infectious doses (CID_{50}) of HCV genotype 1a in a protocol approved by the Public Health Service Interagency Model Committee (National Institutes of Health) and the Animal Care and Use Committee (Center for Biologics Evaluation and Research) at an Association for Assessment and Accreditation of Animal Care-accredited facility (23). Serum HCV RNA titers (A), alanine aminotransferase (ALT) levels (B), and intrahepatic CD8β mRNA levels (C) have previously been reported (23, 24) and are shown for reference purposes. (D to F) Serial liver biopsy specimens were analyzed for mRNA levels of PD-L1 and galectin-9 (D), of PD-1, Tim-3, CTLA-4, and LAG-3 (E), and of IL-10, TGF-β, and Foxp3 (F). Intrahepatic mRNA levels were normalized to mean levels of β-actin, GAPDH, and β7 mRNA as endogenous references and were expressed as fold increases over preinfection levels. The CTLA-4 mRNA value next to the asterisk specifies an off-scale value.

inhibitory molecules, which are typically increased in the chronic phase of HCV infection, do not differ among chimpanzees with differential outcomes of infection.

To characterize the quality and magnitude of the HCV-specific T-cell response, we used (i) *Pan troglodytes* class I tetramers (NIAID Tetramer Facility of the NIH AIDS Research and Reference Reagent Program) to analyze by flow cytometry HCV-specific T-cell responses against epitopes that we had previously

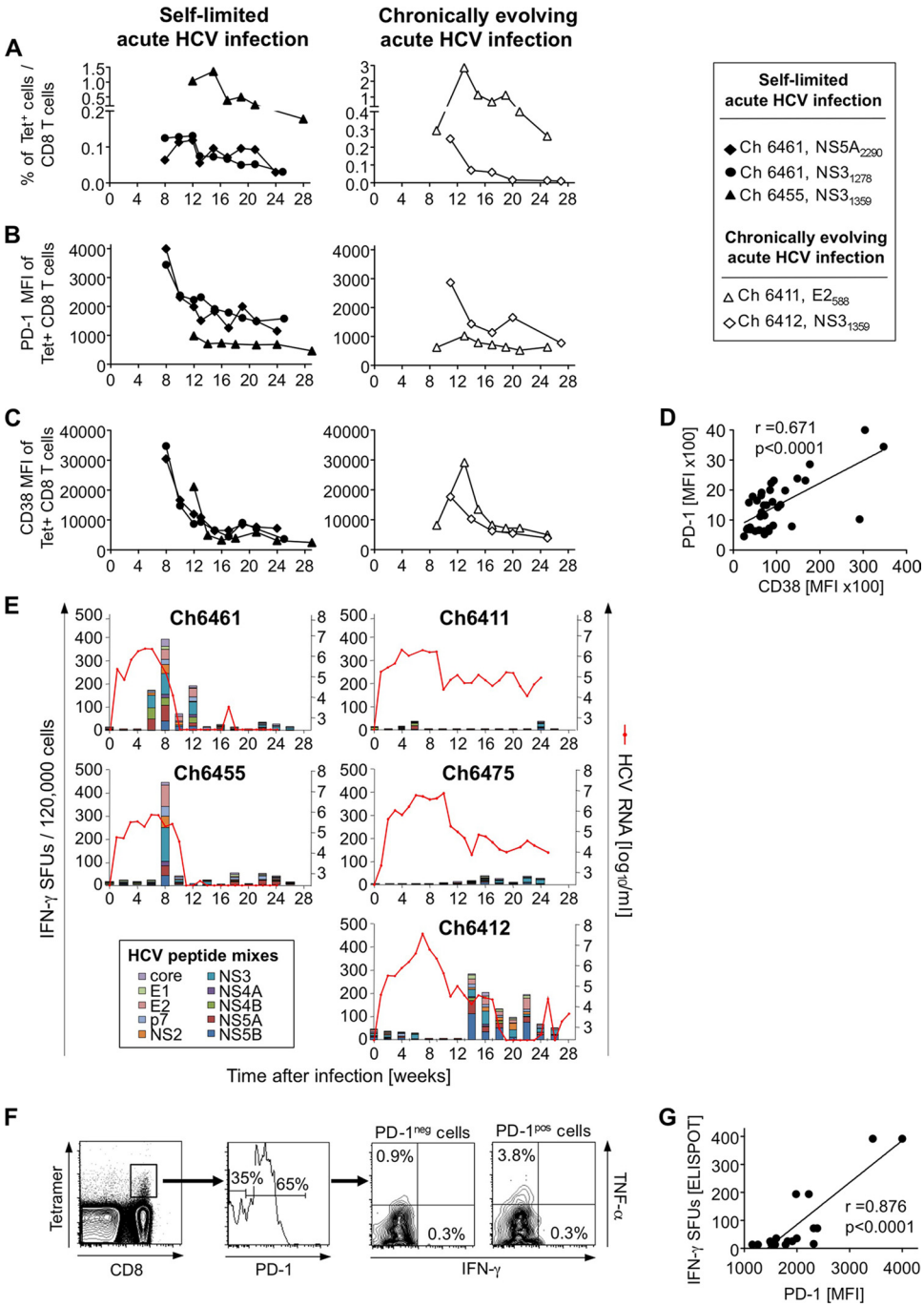

FIG 2 Characterization of HCV-specific T-cell responses in acute HCV infection. (A) The frequency of HCV-specific T cells in the blood was evaluated with *P. troglodytes* class I tetramers. (B to D) The levels of PD-1 (B) and CD38 (C) expression (MFIs) and the correlation between the CD38 MFI and the PD-1 MFI (D) were determined on tetramer$^+$ CD8$^+$ T cells during the first 6 months of HCV infection using previously described techniques (25). *r*, correlation coefficient, linear regression analysis. (E) *Ex vivo* IFN-γ ELISpot assays were performed in duplicate by stimulating PBMCs with 18 mixes of overlapping HCV peptides (600 peptides total) spanning the entire HCV polyprotein sequence using a previously described technique (35). HCV RNA titers were previously reported (23, 24) and are shown as red lines for reference purposes. (F) PD-1$^+$ and PD-1$^-$ subsets of NS3$_{1278}$-stimulated tetramer$^+$ CD8$^+$ T cells (Ch6461, week 9 after infection) were assessed for IFN-γ and TNF-α secretion using a previously described technique (25). (G) The overall T-cell IFN-γ response against overlapping peptides (IFN-γ ELISpot) was correlated with the PD-1 MFIs of tetramer$^+$ CD8$^+$ T cells at time points throughout the course of infection (Ch6461) in a linear regression analysis. SFU, spot-forming unit.

mapped in these chimpanzees (24) and (ii) a set of 600 overlapping pentadecamer HCV peptides that matched the sequence of the infection virus (Mimotopes, Clayton, Australia) to analyze the total T-cell response in gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays. Due to the limited number of lymphocytes that can be isolated from liver biopsy specimens, these assays were performed with peripheral blood mononuclear cells (PBMCs). As shown in Fig. 2A, the frequency of

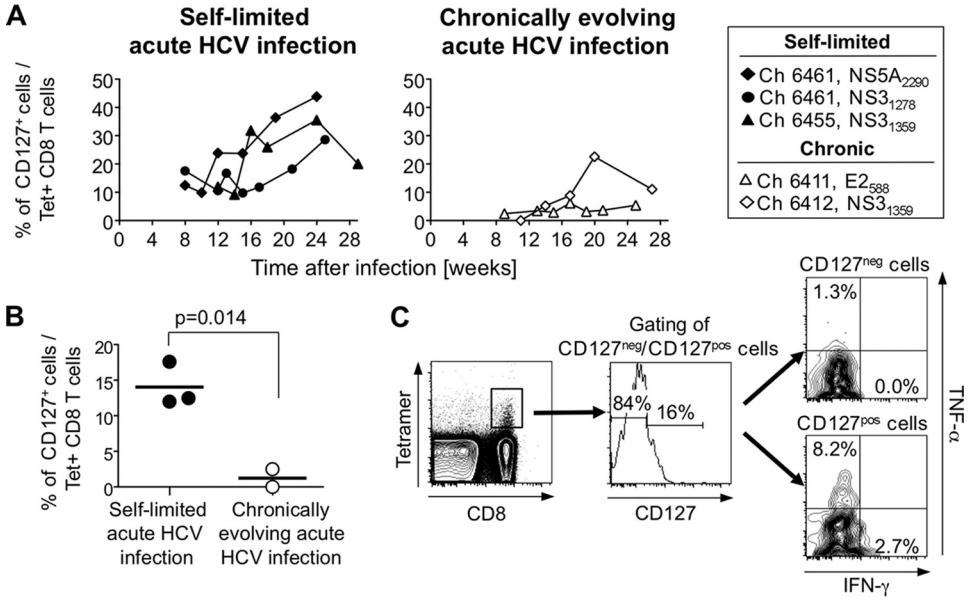

FIG 3 Differential CD127 expression on HCV-specific T cells in acute HCV infection. (A) The frequency of CD127$^+$ cells within the tetramer$^+$ CD8$^+$ T-cell population was determined during the first 6 months of HCV infection using previously described techniques (25). (B) The percentages of CD127$^+$ cells within the tetramer$^+$ CD8$^+$ T-cell population were compared between chimpanzees with self-limited and chronically evolving courses at the time point at which tetramer$^+$ T cells were first detected in the blood (unpaired Student *t* test). (C) CD127$^+$ and CD127$^-$ subsets of NS3$_{1278}$-stimulated tetramer$^+$ CD8$^+$ T cells (Ch6461, week 9 after infection) were assessed for IFN-γ and TNF-α secretion using a previously described technique (25).

tetramer$^+$ CD8$^+$ T cells in the blood was highest in the early acute phase of hepatitis C (weeks 8 to 14) and did not predict the outcome of acute HCV infection. Consistent with intrahepatic PD-1 mRNA levels, the mean fluorescence intensity (MFI) of PD-1 on tetramer$^+$ T cells in the blood peaked at the two earliest time points with detectable tetramer$^+$ T cells and decreased thereafter in both groups of chimpanzees (Fig. 2B). The MFI for CD38, a T-cell activation marker, tended to be higher on tetramer$^+$ cells of chimpanzees with self-limited hepatitis C than on those of chimpanzees with chronically evolving hepatitis C at the first study time point (Fig. 2C).

Overall, there was a strong correlation between PD-1 and CD38 MFIs ($r = 0.671$, $P < 0.0001$) (Fig. 2D). Furthermore, the time points with the highest PD-1 and CD38 expression on tetramer$^+$ CD8 T cells in self-limited acute HCV infection corresponded to the time points with strongest HCV-specific T-cell responses in the ELISpot assay (Fig. 2B and E). To confirm the functionality of tetramer$^+$ CD8$^+$ T cells in the context of their PD-1 status at the single-cell level, we assessed IFN-γ and tumor necrosis factor alpha (TNF-α) production of tetramer-stained peptide-stimulated PBMCs by flow cytometry at an early time point after infection using a previously described technique (25). As shown in Fig. 2F for Ch6461 at week 9 after infection, a greater percentage of PD-1$^+$ than PD-1$^-$ T cells produced TNF-α. This was supported by the close correlation between PD-1 expression levels on tetramer$^+$ T cells and the overall frequency of HCV-specific IFN-γ-producing T cells in this chimpanzee ($r = 0.876$, $P < 0.0001$) (Fig. 2G). Based on these findings we propose that PD-1 is an activation rather than exhaustion marker in acute HCV infection and that upregulation of PD-1 on HCV-specific T cells during the acute phase of infection does not interfere with HCV clearance. Indeed, as shown in other scenarios PD-1 expression can be transient on T-cell receptor (TCR)- and/or cytokine-stimulated T cells (26, 27). This may differ from chronic HCV infection, where PD-1 marks exhausted T cells with impaired function (13–19).

Next, we studied HCV-specific CD8$^+$ T cells for the expression of CD127, a marker of memory precursor cells (28). While the overall frequency of CD127$^+$ tetramer$^+$ T cells was lower in HCV-infected chimpanzees than in previous studies on HCV-infected patients (29), differential CD127 expression was observed during the early phase of acute infection when virus was still detectable even in animals that subsequently cleared the infection. Indeed, at the time point when tetramer$^+$ cells were first detectable in the blood, the frequency of CD127$^+$ cells within the tetramer$^+$ population was significantly higher in chimpanzees with self-limited acute infection than in chimpanzees with chronically evolving infection ($P = 0.014$, Student's *t* test) (Fig. 3B).

Because CD127 expression on T cells has been found to increase upon mutational escape of the targeted epitopes (9, 30), we sequenced HCV in all chimpanzees. A 5.2-kb HCV product was amplified by reverse transcription-PCR (RT-PCR) to sequence the E2 epitope with primer 5′-AYGTTCYGRTGRAGRTGGAT-3′ and the NS3 epitopes with primer 5′-ARCCRGTCATGAGRGCA TC-3′ (31). A 0.8-kb HCV product was amplified to sequence the NS5A epitope based on a previous report (32) but using RT-PCR primer 5′-TTACGACCCCCCTTCTC-3′, first-round PCR primers 5′-ACACTCGCTGCCACTGTGG-3′ and 5′-TYGACCATGA CCCGTCGC-3′, second-round PCR primers 5′-AGGAACATGT GGAGTGGG-3′ and 5′-GATTCRGTGAGGACCACC-3′, and sequencing primer 5′-GATTCRGTGAGGACCACC-3′. PCR conditions were 94°C (2 min) followed by 35 cycles of 94°C (20 s), 55°C (30 s), and 68°C (1 min 20 s), with a final extension at 70°C (10 min). The results from at least 17 molecular clones per sample confirmed that the observed differential CD127 expression in the early acute phase of HCV infection was not due to sequence vari-

TABLE 1 HCV epitope sequence during early acute and during chronic HCV infections

Chimpanzee (infection outcome)	Epitope location[a]	Wk after infection[b]	Amino acid sequence[c]	No. of clones with indicated sequence/no. of clones sequenced
Ch6461 (self limited)	$NS3_{1278–1285}$	0	GVDPN**IRTGVRTI**TTGSP	
		8*	-----**--------**-----	17/17
	$NS5A_{2290–2298}$	0	LPVWA**RPDYNPPLV**ETWKK	
		8*	-----**---------**-----	22/24
			-----**Q--------**-----	1/24
			-----**-----L---**-----	1/24
Ch6455 (self limited)	$NS3_{1359–1367}$	0	SVTVS**HPNIEEVAL**STTGE	
		8*	-----**---------**-----	16/18
			--I--**---------**-----	1/18
			-----**---------**----R	1/18
Ch6411 (chronic)	$E2_{588–596}$	0	TDCFR**KHPEATYSR**CGSGP	
		9*	-----**---------**-----	22/23
			-----**--L------**-----	1/23
		26	-----**---------**-----	7/21
			-----**----T----**-----	11/21
			-----**-----L---**-----	1/21
			-----**-----S---**-----	1/21
			-----**T---T----**-----	1/21
Ch6412 (chronic)	$NS3_{1359–1367}$	0	SVTVS**HPNIEEVAL**STTGE	
		11*	-----**---------**-----	24/24
		26	----P**---------**-----	23/23

[a] $NS3_{1278}$ and $NS5A_{2290}$ are Patr-B*0301/02 restricted, $NS3_{1359}$ is Patr-B*1301 restricted, and $E2_{588}$ is Patr-A*0401 restricted.
[b] The HCV sequence that is indicated for the week 0 time point is the sequence of the HCV inoculum. *, first time point at which tetramer$^+$ CD8 T cells were detected in the blood.
[c] The epitope sequence is in boldface and underlined. Hyphens indicate amino acids identical to those at inoculation.

ations in T-cell epitopes (Table 1). After the acute phase, the percentage of CD127$^+$ cells gradually increased to 28 to 44% of the tetramer$^+$ T-cell population in those chimpanzees that cleared HCV. The increase in CD127$^+$ HCV-specific T-cell responses occurred while the bulk IFN-γ ELISpot response declined (Fig. 3A and 2E), which suggests an expansion of a small population of antigen-independent HCV-specific T cells, as observed during antiviral therapy. Expression of CD127, the IL-7 receptor, may enable these cells to respond effectively to minute amounts of IL-7 and to proliferate as antigen-independent memory precursors. This is consistent with the finding that CD127$^+$ tetramer$^+$ T cells mounted better TNF-α and IFN-γ responses upon stimulation with their cognate peptide than their CD127$^-$ counterparts (Fig. 3C) and that the higher frequency of CD127$^+$ tetramer$^+$ T cells in chimpanzees with self-limited hepatitis C in the early phase of acute infection corresponded to the early and strong induction of the total HCV-specific T-cell response observed in the IFN-γ ELISpot assay (Fig. 3A and 2E).

In contrast to results for chimpanzees with self-limited acute infection, the percentage of CD127$^+$ HCV-specific T cells remained low in those chimpanzees with chronically evolving hepatitis (Fig. 3A). This is consistent with reports on decreased CD127 levels on HCV-specific T cells of patients who were studied late in the course of HCV infection (5, 9, 30, 33, 34). Solely Ch6412, which experienced transient viral control at weeks 19 to 23 after infection, displayed a transient increase in the CD127$^+$ T-cell frequency (Fig. 3A). Reemergence of HCV to high titers at week 24 in this chimpanzee coincided with the failure to maintain an increasing frequency of CD127$^+$ T cells. Again, this fluctuation in the size of the CD127$^+$ HCV-specific T-cell population was not due to HCV sequence variations in the corresponding T-cell epitopes (Table 1).

Thus, the frequency of CD127$^+$ cells in the HCV-specific CD8$^+$ T-cell population in the early phase of acute hepatitis, when all chimpanzees were still viremic, rather than the expression of inhibitory molecules on HCV-specific T cells or the intrahepatic levels of their ligands or inhibitory cytokines correlated with the subsequent infection outcome.

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