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T cell immunity and HCV reinfection after cure of chronic hepatitis C with an interferon-free antiviral regimen

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Abstract

Background and Rationale—Memory CD8+ T cells generated by spontaneous resolution of HCV infection rapidly control secondary infections and reduce the risk of virus persistence. Here, CD8+ T cell immunity and the response to reinfection was assessed in a chimpanzee cured of an earlier chronic infection with an interferon-free antiviral regimen.

Results—CD8+ T cells expanded from liver immediately before and two years after cure of chronic infection with two direct acting antivirals (DAA) targeted epitopes in the E2, NS5a, and NS5b proteins. A second infection to assess CD8+ T cell responsiveness resulted in rapid suppression of HCV replication by week 2, but viremia rebounded 3 weeks later and the infection persisted. The E2, NS5a and NS5b proteins remained dominant CD8+ T cell targets after re-infection. Resurgent HCV replication was temporally associated with mutational escape of NS5a and NS5b class I epitopes that had also mutated during the first chronic infection. Two epitopes in E2 remained intact throughout both persistent infections. Intrahepatic CD8+ T cells targeting intact and escape-prone epitopes differed in expression of phenotypic markers of functional exhaustion two years after successful DAA therapy, and in the capacity to expand in liver upon reinfection.

Conclusions—The intrahepatic HCV-specific CD8+ T cell repertoire established during chronic infection was narrowly focused but very stable after cure with DAA. Existing intrahepatic CD8+ T cells targeting dominant epitopes of the challenge virus failed to prevent persistence. Vaccination

after DAA cure may be necessary to broaden the T cell response and reduce the risk of a second persistent infection.

Spontaneous resolution of acute primary hepatitis C virus (HCV) infection in chimpanzees and humans generates long-lived T cell memory(1, 2). These memory CD4⁺ and CD8⁺ T cells appear to be important for rapid control of secondary HCV infections(2) and a 4–5 fold reduction in the risk of a persistent outcome when compared with primary infections in HCV naïve human subjects(3, 4). Chronic HCV infection results in functional exhaustion of CD8⁺ T cells and mutational escape of some class I epitopes encoded by the virus(1, 2). CD8⁺ T cell exhaustion is characterized by low expression of CD127, the IL-7 α receptor important for self-renewal of memory populations, and high expression of co-inhibitory receptors like PD-1, CTLA-4, 2B4, and TIM-3 in blood(5, 6) and liver(7). This phenotype is tempered to some degree by mutational escape of class I HCV epitopes early in the course of infection(8–10). Because exhaustion is caused in part by constant antigenic stimulation of CD8⁺ T cells(11, 12), cure of chronic infection has the potential for at least partial restoration of the response. The capacity of CD8⁺ T cells to recover after successful antiviral therapy and respond to a secondary infection has not been widely studied. Circulating CD8⁺ T cells from patients cured with pegylated type I interferon (pegIFN) and ribavirin failed to produce antiviral cytokines or proliferate after *ex vivo* stimulation with HCV antigens(13, 14). Control of HCV replication in a cell co-culture model was also impaired, suggesting that damage caused by chronic infection is permanent even after antigen production is terminated by therapy(13–15). Much less is known about the ability of virus-specific CD8⁺ T cells to control HCV replication upon reinfection, a more stringent test of functionality. Some humans successfully treated with pegIFN and ribavirin do spontaneously resolve secondary infections(16, 17). Expansion of one HCV-specific CD8⁺ T cell population was described in the blood of a human subject who developed a secondary resolving infection several months after cure of chronic hepatitis C with pegIFN and ribavirin(10). This observation is consistent with the potential for effective CD8⁺ T cell immunity to secondary infection despite profound impairment of the response during the earlier chronic infection.

New small molecules inhibitors of non-structural HCV proteins like NS3, NS5a and NS5b may cure most chronic infections without the need for type I IFN(18–20), a cytokine that is sometimes necessary for CD8⁺ T cell differentiation(21–23) but under some circumstances also limits effector function or survival(24–29) and contributes to virus persistence in murine models(30, 31). With oral dosing, a shorter duration of therapy, and reduced toxicity of direct acting antivirals (DAA) it is predicted that treatment will become more common. The perceived potential for HCV reinfection is one factor that might be considered in DAA treatment decisions, especially for people who acquired the infection through injection drug use and have an ongoing risk of re-exposure to the virus. Indeed, reinfection has already been documented in a subject after successful treatment with 2 DAA (daclatasvir and sofosbuvir) in the context of a clinical trial(32). Understanding the factors that determine if exhausted T cells recover after antiviral cure and respond to reinfection is therefore of practical importance. It is not yet known if the intrahepatic CD8⁺ T cell repertoire is stable in the years following cure or whether CD8⁺ T cells targeting intact or escaped epitopes expand upon reinfection. Whether the pace of CD8⁺ T cell immunity is accelerated or

delayed as in primary HCV infection, and the capacity of the virus to escape the response by mutation, has also not been studied. Here we addressed these questions in a chimpanzee that was cured of chronic hepatitis C after a 28 day course of treatment with small molecule inhibitors of the HCV NS5a and NS5b proteins.

Materials and Methods

Infection and antiviral treatment of chronic HCV infection

Chimpanzee (*Pan troglodytes*) CH5835 was maintained under standard conditions for humane care at the Michale E. Keeling Center for Comparative Medicine and Research, M. D. Anderson Cancer Center. The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Michale E. Keeling Center. Chimpanzee 5835 was homozygous for the Patr (*Pan troglodytes*) A*0901 and B*0101 class I alleles as determined by genotyping(33). Persistent infection was established after challenge with the HCV J4/91 genotype 1b virus (R. Purcell, unpublished). Seven years later NS5a(34) and NS5b(35) inhibitors synthesized from published structures were administered orally at 60 mg and 2 mg/Kg, respectively, once per day for 28 days. Approximately two years (658 days) after treatment, chimpanzee 5835 was re-challenged intravenously with the identical stock of J4/91 HCV virus used to initiate the first infection.

Intrahepatic immune responses

Liver tissue obtained via transcutaneous biopsy was homogenized and CD8+ T cells positively isolated with paramagnetic beads were co-cultured with irradiated (5000R) autologous PBMC pulsed with peptides (18 amino acids overlapping by 11 residues) spanning the entire HCV J4/91 polyprotein (Genbank AF054250)(36). After 2–3 weeks of culture, cells were expanded with anti-CD3 antibodies and irradiated human PBMC. Three to 4 weeks later, cells were tested for HCV specificity by IFN- γ ELISpot(36).

Interferon- γ ELISpot Assay

CD8+ T cells (5×10^4) were co-cultured with irradiated (20,000R) B-LCL (5×10^3) pulsed with HCV peptides to present antigen in ELISpot plates pre-coated with anti-IFN- γ antibodies. Plates were incubated for 36 hours and developed for enumeration of spot forming cells (SFC)(36).

Tetramer analysis

Mononuclear cells were isolated from liver tissue collected by needle biopsy and incubated immediately (i.e. without *ex vivo* culture) with E2₆₈₀ (allophycocyanin fluorophore) and NS5a₁₉₉₂ (phycoerythrin fluorophore) labeled tetramers for 30 minutes at 4°C. After co-staining with antibodies to CD8, CD3, CD127, and PD-1 for 20 minutes at 4°C, cells were fixed and analyzed by flow cytometry. Live and dead cells were discriminated with an amine-reactive viability dye. Cells expressing CD4, CD14, CD16, and CD19 were gated out of the analysis.

Virus Sequencing

RNA extracted from chimpanzee serum (100 µl) was reverse transcribed using random hexamers and reverse transcriptase. cDNA was amplified with nested sets of PCR primers that flanked the Patr class I epitopes. PCR primers are described in Table 1. Nested-PCR products containing HCV inserts were cloned and sequenced by the Laboratory for Genomics & Bioinformatics (University of Oklahoma Health Sciences Center).

HCV mRNA and transfection

HCV mRNA was amplified and transfected into target cells to assess CD8+ T cell recognition of the wild type and I2430V substituted NS5b₂₄₂₃ epitope as previously described(36). A fragment spanning nucleotides 6481 to 9361 and containing the NS5a and NS5b genes of HCV J4/91 virus was amplified by PCR using forward primer 5'-atactcgagaccatgtccggtcgtggctaagggatgtttgg-3' and reverse primer 5'-atatctagattatcatcggttggggagcaggtaatgcctac-3'. The resulting DNA fragment was cloned into the pGem-11z vector and the sequence was verified as described above. Site-directed mutagenesis was used to introduce the nucleotide substitution leading to an I2430V coding change. As a control, the gene encoding enhanced green fluorescent protein (EGFP) was also cloned into the pGem-11z vector. Plasmids were then linearized and used for the generation of capped and poly(A) tailed mRNA. Autologous B-LCL were electroporated with 5 µg of viral mRNA and cultured for 24 before mixing with a CD8+ T cell line at a 1:1 ratio in an intracellular cytokine staining assay as described(36). The CD8+ T cell lines used to test recognition of wild-type and mutant peptides were derived from liver 14 days before cure of the first chronic infection.

Results

Chimpanzee 5835 was persistently infected with the HCV-J4/91 (genotype 1b) strain of HCV for 7 years. Viremia was stable at approximately 5×10^4 IU of HCV RNA/ml of serum but dropped sharply 3 days after the start of antiviral therapy. HCV RNA remained below the limit of detection (12 IU/ml of serum) during 28 days of treatment and was not detected through 24 months of follow-up (Figure 1A).

We first sought to define the breadth and stability of the intrahepatic HCV-specific CD8+ T cell repertoire after cure of infection. CD8+ T cells were isolated from liver 14 days before and 20 months after termination of chronic infection and then expanded in culture. Recognition of HCV antigens was then assessed by IFN- γ ELISpot assay. The analysis revealed that the CD8+ T cell repertoire established during chronic infection was remarkably stable after cure because the E2, NS5a and NS5b proteins were dominant targets at both time points (Figure 2A). Mapping studies revealed that the response was narrowly directed against 2 Patr class I restricted epitopes in E2 (designated E2₆₃₀ and E2₆₈₀) and 1 epitope each in NS5a (NS5a₁₉₉₂) and NS5b (NS5b₂₄₂₃) (Table 1 and Figure 2A). At day 644 after cure we observed robust *ex vivo* expansion of CD8+ T cells targeting the pooled NS5a and NS5b peptides and the individual NS5a₁₉₉₂ and NS5b₂₄₂₃ epitopes (Figure 2B). The E2-specific response was comprised predominantly of E2₆₈₀-and not E2₆₃₀-specific CD8+ T cells (Figure 2B).

To assess the responsiveness of these intrahepatic HCV-specific CD8⁺ T cells, chimpanzee 5835 was rechallenged with the J4/91 virus approximately 2 years (658 days) after cure of the chronic infection. HCV RNA was present in serum at day 7 but was undetectable at days 14 and 21 (Figure 1B). Viremia rebounded at day 28 and persistent infection ensued at a low but relatively stable setpoint ($\sim 5 \times 10^3$ IU/ml serum) (Figure 1B). Intrahepatic CD8⁺ T cells still recognized the NS5a and NS5b proteins, and the associated NS5a₁₉₉₂ and NS5b₂₄₂₃ epitopes, 85 days after secondary infection (Figure 2C). The E2-specific responses detected before re-challenge were lost by day 85 (Figure 2C). Except for weak activity against the core/E1 peptide pool, the virus-specific CD8⁺ T cell response did not broaden to other proteins including NS3 and NS4 that are often dominant targets of cellular immunity in HCV infection (Figure 2C).

We next sought to determine if the rebound in virus replication at day 28 was caused by mutational escape of the dominant class I epitopes in E2, NS5a, and NS5b. Sequencing of the virus revealed that the E2₆₃₀ and E2₆₈₀ epitopes were intact just before cure of the first chronic infection and did not mutate during the second infection (Figure 3A). Mutations in the NS5a₁₉₉₂ and NS5b₂₄₂₃ epitopes were observed during the first chronic infection (Figure 3A). As expected, both epitopes were intact at day 7 after reinfection with the wild-type J4/91 virus, just before replication was transiently controlled (Figure 3A). Amino acid substitutions appeared in both epitopes when the virus re-emerged at day 28 post-infection. The NS5b₂₄₂₃ epitope acquired the same mutation (I2430V) present during the first infection (Figure 3A) and provided effective escape from CD8⁺ T cell recognition (Figure 3B). The amino acid substitutions in the NS5a₁₉₉₂ epitope during the first chronic infection (Q1996R) and at day 28 of the second infection (T1993I) provided an equivalent measure of immune escape (Figure 3C). The doubly substituted epitope (T1993I/Q1996R) that eventually emerged at day 140 was recognized less efficiently than either singly substituted epitope (Figure 3C).

To better understand why CD8⁺ T cells targeting the intact E2 epitopes failed to prevent persistence, Patr class I tetramers were used to track expansion and expression of the PD-1 and CD127 receptors. Tetramers were constructed for each of the four dominant T cell populations (E2₆₃₀, E2₆₈₀, NS5a₁₉₉₂, and NS5b₂₄₂₃). With the exception of T cells targeting epitope NS5a₁₉₉₂, frequencies in blood were below the limit of detection at all time points before and after the second infection (supporting Figure 1). The liver needle biopsies were of sufficient size to simultaneously compare the frequency and phenotype of CD8⁺ T cell populations targeting the intact E2₆₈₀ and escape-prone NS5a₁₉₉₂ epitopes. On study day 0 the E2₆₈₀-specific CD8⁺ T cells were present in liver at a frequency of 0.26% (Figure 4 A and B), but were below the threshold for reliable quantification in blood (supporting Figure 1). This is consistent with sequestration of HCV-specific CD8⁺ T cells in the chronically infected liver. CD8⁺ T cells targeting the intact E2₆₈₀ epitope did not expand in liver (Figure 4A and B) or blood (supporting Figure 1) after re-infection. Through 6 months of follow-up, frequencies in liver were nearly identical to those measured just before re-infection, suggesting a defect in responsiveness (Figure 4B). Just before reinfection the NS5a₁₉₉₂-specific CD8⁺ T cells were present in liver at a frequency of 0.39% (Figure 4A) and in blood at 0.07% (supporting Figure 1). It is notable that blood frequencies were not substantially different than those measured 2 years earlier just before and during DAA

therapy (supporting Figure 2). Massive expansion of the NS5a₁₉₉₂-specific population was observed in liver (Figure 4A and B) and blood (supporting Figure 1) by day 14, although the peak frequency in the blood was substantially lower than in liver. Partial contraction of the intrahepatic response was observed by day 28 when virus containing the T1993I escape mutation appeared in serum. Frequencies in liver then remained stable through day 56 but by day 189 had declined sharply from the peak (Figure 4B).

Immediately before reinfection most of the intrahepatic NS5a₁₉₉₂-specific CD8⁺ T cells in liver expressed PD-1, but at low mean fluorescence intensity (MFI) (Figure 5 A and C). PD-1 MFI increased sharply in liver and blood by day 14 as expected for an activated cell population. PD-1 intensity on liver-resident CD8⁺ T cells then declined but remained above pre-infection baseline values as the second persistent infection was established (Figure 5A and C). Expression of PD-1 on circulating CD8⁺ T cells peaked at day 14 and then dropped even though the infection persisted (supporting Figure 3). By day 189 after the second infection, PD-1 on circulating CD8⁺ T cells declined to levels observed during the first chronic infection (supporting Figure 2). CD127 was expressed on approximately 60% of intrahepatic NS5a₁₉₉₂-specific T cells before reinfection, but was rapidly lost after virus challenge and did not recover (Figure 5A and C, left panel) despite emergence of an NS5a₁₉₉₂ epitope escape variant at day 28 (Figure 3). Downregulation of CD127 was more transient on circulating NS5a₁₉₉₂-specific CD8⁺ T cells when compared with those in liver. Expression declined at day 14 but gradually recovered to levels observed before re-infection (supporting Figure 3) and also just before and during treatment of the first chronic infection (supporting Figure 2).

The intrahepatic E2₆₈₀-specific CD8⁺ T cells had an exhausted phenotype two years after cure of chronic infection. Most lacked expression of CD127 and PD-1 was elevated when compared with the responsive NS5a₁₉₉₂-specific population (Figure 5B and C, right panel). This phenotype did not change substantially after reinfection. PD-1 levels remained high on E2₆₈₀-specific CD8⁺ T cells and expression of CD127 was below 20 percent through 6 months of follow-up (Figure 5B and C, right panel), consistent with exhaustion and an apparent absence of activation.

Discussion

Chimpanzee 5835 was part of a larger study to combine DAA treatment and vaccination to restore cellular immunity and prevent rebound of virus replication in chronic hepatitis C. A sustained virological response in this animal after 28 days of DAA treatment provided a unique opportunity to characterize HCV-specific CD8⁺ T cells in liver before and after cure. The repertoire of HCV-specific CD8⁺ T cells present in liver during chronic infection was remarkably stable for at least two years after DAA-mediated cure. It is likely that the NS5a and NS5b-specific CD8⁺ T cells were maintained in liver after cure by cytokine-driven homeostatic proliferation based on the pattern of CD127 expression. How E2-specific CD8⁺ T cells that lack CD127 were maintained after termination of viremia is not certain. Constant stimulation by viral antigens is thought to maintain these populations during chronic infection(12). No HCV genomes were detected in plasma during 2 years of follow-up and so intermittent low-level virus replication sufficient to maintain the E2-specific CD8⁺ T cells

seems unlikely, at least in this case(37). We can't exclude the possibility that HCV antigens are cleared slowly from the liver after virus replication is terminated and provide enough stimulation to maintain CD8+ T cells that are apparently exhausted.

A second infection of the animal resulted in HCV persistence after a brief period of control. CD8+ T cells targeting the E2, NS5a, and NS5b proteins remained dominant when the breadth of the response was reassessed at day 85 after the second infection. The only evidence for broadening of the response was activity against core/E1 that was too weak to characterize further. It is possible that this response was a remnant of broader T cell activity against other epitopes that occurred before day 85. Alternatively, it is possible that CD8+ T cells specific for other epitopes in immunodominant proteins like NS3 and NS4 may have been permanently deleted or lost by attrition over 7 years of chronic infection and thus unable to respond to a second infection. Re-infection with a heterologous HCV strain or genotype could conceivably elicit a broader response against novel epitopes not previously targeted by CD8+ T cells.

The virus that emerged at day 28 after a brief 2–3 week period of control had acquired escape mutations in the dominant NS5a and NS5b class I epitopes. It is noteworthy that these escape mutations developed at a time of very weak replication because HCV genomes were below the threshold for detection (12 GE/ml serum) on days 14 and 21. The very close temporal relationship between the rebound in virus replication and escape of these dominant epitopes indicates that the mutations are most likely a cause and not an effect of HCV persistence in the setting of secondary infection. The breadth of the intrahepatic CD8+ T cell repertoire against HCV, and the proportion of class I epitopes that escape or remain intact, is highly variable in the chronically infected liver of humans and chimpanzees(6, 38, 39). It is conceivable that a broader repertoire of HCV-specific CD8+ T cells targeting escape-prone epitopes could promote resolution, especially if an accelerated memory response outpaced acquisition of mutations in multiple class I epitopes. However, a response that is narrowly focused on a small number of escaped and intact HCV class I epitopes, as observed in his animal, is not uncommon in humans(38, 39). Under this circumstance secondary infections might be expected to persist unless the response is broadened.

HCV established a second persistent infection even though CD8+ T cells targeting at least 3 dominant epitopes were present in liver at the time of virus challenge. Our results suggest that failure to contain HCV replication was caused either by (i) mutational escape from CD8+ T cells that had a memory phenotype (CD127^{high}PD-1^{low}) and expanded massively after reinfection, or (ii) poor expansion of CD8+ T cells with an exhausted (CD127^{low}PD-1^{high}) phenotype that targeted intact epitopes. A memory response by circulating CD127^{high}PD-1^{low} CD8+ T cells was also described during a secondary resolving infection in a human subject(10), but the breadth of the response contributing to control and sequence differences between viruses that established the first and second infections was not known. Epitope escape early in the course of persistent infection leads to higher CD127 and lower PD-1 expression(8–10), and better antiviral activity by CD8+ T cells co-cultured with HCV-infected hepatoma cells(15). Our study suggests that patterns of CD127 and PD-1 expression, and mutational escape in class I epitopes, may also predict

recovery of CD8+ T cells after antiviral cure and the capacity to respond to secondary infection.

This study has limitations. As noted above, chronic hepatitis C was unexpectedly cured in a single animal after a very short course of therapy with two DAA. While this event provided a unique opportunity to study changes in the intrahepatic T cell repertoire, it is almost certainly not representative of all secondary infections in individuals cured of chronic hepatitis C. Infection outcome may depend on variables such as the breadth of the CD8+ T cell repertoire in the persistently infected liver and how many epitopes carry escape mutations. Limitations of liver sampling restricted our analysis to one escaped and one intact epitope. The biopsy was too small to simultaneously assess CD8+ T cell effector function or test for presence of occult HCV genomes in liver after cure. It is important to emphasize that these studies can now be conducted only in humans and will be challenging to design. In this study most CD8+ T cells visualized in liver were not present in blood even after reinfection. CD8+ T cells are also largely sequestered to liver in human subjects(38, 39) and patterns of co-inhibitory receptor expression can differ from those in blood(7). Also, the sequence of HCV strains that established primary human infections are often not known except in the case of medical accidents. It is therefore difficult to unambiguously identify escape mutations that occur during primary infection and determine if they are conserved in the HCV strain that establishes secondary infection after DAA cure. Our study of chimpanzee 5835 may, despite its limitations, provide an important framework for interpretation of human studies where longitudinal sampling of liver through cure and reinfection is not possible.

This study provides, to the best of our knowledge, the first description of the intrahepatic CD8+ T cell repertoire through two distinct chronic infections in the same individual. It indicates that the intrahepatic repertoire is stable after DAA cure, but may not change substantially upon reinfection with closely related HCV subtypes. The potential for recognition of new epitopes, particularly upon reinfection with distinct HCV genotypes that co-circulate in some communities, remains to be determined. Mutational escape of class I epitopes also appeared to be an important contributor to persistence even though a memory CD8+ T cell response developed within days of exposure to virus. The analysis also suggests that at least some CD8+ T cells targeting intact epitopes do not recover after cure and are unable to prevent persistence of a secondary infection. For those who are successfully treated with new DAA regimens but remain at risk for exposure to the virus, vaccination after cure to broaden selective CD8+ T cell memory may be necessary to provide the same protection afforded by spontaneous resolution of acute hepatitis C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

B-LCL	B lymphoblastoid cell lines
DAA	direct acting antiviral
HCV	hepatitis C virus
IFN	interferon
Patr	Pan troglodytes
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death 1
MFI	mean fluorescence intensity
SFC	spot forming cells

References

1. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J Clin Invest.* 2009; 119:1745–1754. [PubMed: 19587449]
2. Walker CM. Adaptive immunity to the hepatitis C virus. *Adv Virus Res.* 2010; 78:43–86. [PubMed: 21040831]
3. Cox AL, Thomas DL. Hepatitis C virus vaccines among people who inject drugs. *Clin Infect Dis.* 2013; 57 (Suppl 2):S46–50. [PubMed: 23884065]
4. Grebely J, Prins M, Hellard M, Cox AL, Osburn WO, Lauer G, Page K, et al. Hepatitis C virus clearance, reinfection, and persistence, with insights from studies of injecting drug users: towards a vaccine. *Lancet Infect Dis.* 2012; 12:408–414. [PubMed: 22541630]
5. Lauer GM. Immune responses to hepatitis C virus (HCV) infection and the prospects for an effective HCV vaccine or immunotherapies. *J Infect Dis.* 2013; 207 (Suppl 1):S7–S12. [PubMed: 23390305]
6. Klenerman P, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. *Gut.* 2012; 61:1226–1234. [PubMed: 21873736]
7. Kroy DC, Ciuffreda D, Cooperrider JH, Tomlinson M, Hauck GD, Aneja J, Berger C, et al. Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors. *Gastroenterology.* 2014; 146:550–561. [PubMed: 24148617]
8. Rutebemberwa A, Ray SC, Astemborski J, Levine J, Liu L, Dowd KA, Clute S, et al. High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *J Immunol.* 2008; 181:8215–8225. [PubMed: 19050238]
9. Bengsch B, Seigel B, Ruhl M, Timm J, Kuntz M, Blum HE, Pircher H, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog.* 2010; 6:e1000947. [PubMed: 20548953]
10. Kasproiewicz V, Kang YH, Lucas M, Schulze zur Wiesch J, Kuntzen T, Fleming V, Nolan BE, et al. Hepatitis C virus (HCV) sequence variation induces an HCV-specific T-cell phenotype analogous to spontaneous resolution. *J Virol.* 2010; 84:1656–1663. [PubMed: 19906915]
11. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A.* 2004; 101:16004–16009. [PubMed: 15505208]
12. Wherry EJ. T cell exhaustion. *Nat Immunol.* 2011; 12:492–499. [PubMed: 21739672]

13. Missale G, Pilli M, Zerbini A, Penna A, Ravanetti L, Barili V, Orlandini A, et al. Lack of full CD8 functional restoration after antiviral treatment for acute and chronic hepatitis C virus infection. *Gut*. 2012; 61:1076–1084. [PubMed: 22337949]
14. Abdel-Hakeem MS, Bedard N, Badr G, Ostrowski M, Sekaly RP, Bruneau J, Willems B, et al. Comparison of immune restoration in early versus late alpha interferon therapy against hepatitis C virus. *J Virol*. 2010; 84:10429–10435. [PubMed: 20668076]
15. Seigel B, Bengsch B, Lohmann V, Bartenschlager R, Blum HE, Thimme R. Factors that determine the antiviral efficacy of HCV-specific CD8(+) T cells ex vivo. *Gastroenterology*. 2013; 144:426–436. [PubMed: 23142136]
16. Grady BP, Schinkel J, Thomas XV, Dalgard O. Hepatitis C virus reinfection following treatment among people who use drugs. *Clin Infect Dis*. 2013; 57 (Suppl 2):S105–110. [PubMed: 23884057]
17. Grebely J, Pham ST, Matthews GV, Petoumenos K, Bull RA, Yeung B, Rawlinson W, et al. Hepatitis C virus reinfection and superinfection among treated and untreated participants with recent infection. *Hepatology*. 2012; 55:1058–1069. [PubMed: 22031335]
18. Poordad F, Lawitz E, Kowdley KV, Cohen DE, Podsadecki T, Siggelkow S, Heckaman M, et al. Exploratory study of oral combination antiviral therapy for hepatitis C. *N Engl J Med*. 2013; 368:45–53. [PubMed: 23281975]
19. Zeuzem S, Soriano V, Asselah T, Bronowicki JP, Lohse AW, Mullhaupt B, Schuchmann M, et al. Faldaprevir and deleobuvir for HCV genotype 1 infection. *N Engl J Med*. 2013; 369:630–639. [PubMed: 23944300]
20. Drenth JP. HCV treatment--no more room for interferonologists? *N Engl J Med*. 2013; 368:1931–1932. [PubMed: 23607592]
21. Le Bon A, Durand V, Kamphuis E, Thompson C, Bulfone-Paus S, Rossmann C, Kalinke U, et al. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *J Immunol*. 2006; 176:4682–4689. [PubMed: 16585561]
22. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol*. 2005; 174:4465–4469. [PubMed: 15814665]
23. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *J Exp Med*. 2005; 202:637–650. [PubMed: 16129706]
24. Gil MP, Salomon R, Louten J, Biron CA. Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo. *Blood*. 2006; 107:987–993. [PubMed: 16210337]
25. Marshall HD, Urban SL, Welsh RM. Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. *J Virol*. 2011; 85:5929–5939. [PubMed: 21471240]
26. McNally JM, Zarozinski CC, Lin MY, Brehm MA, Chen HD, Welsh RM. Attrition of bystander CD8 T cells during virus-induced T-cell and interferon responses. *J Virol*. 2001; 75:5965–5976. [PubMed: 11390598]
27. Welsh RM, Bahl K, Marshall HD, Urban SL. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog*. 2012; 8:e1002352. [PubMed: 22241987]
28. Fraietta JA, Mueller YM, Yang G, Boesteanu AC, Gracias DT, Do DH, Hope JL, et al. Type I Interferon Upregulates Bak and Contributes to T Cell Loss during Human Immunodeficiency Virus (HIV) Infection. *PLoS Pathog*. 2013; 9:e1003658. [PubMed: 24130482]
29. Gracias DT, Stelekati E, Hope JL, Boesteanu AC, Doering TA, Norton J, Mueller YM, et al. The microRNA miR-155 controls CD8(+) T cell responses by regulating interferon signaling. *Nat Immunol*. 2013; 14:593–602. [PubMed: 23603793]
30. Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, Aronow BJ, et al. Blockade of chronic type I interferon signaling to control persistent LCMV infection. *Science*. 2013; 340:202–207. [PubMed: 23580528]
31. Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, Schreiber RD, et al. Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science*. 2013; 340:207–211. [PubMed: 23580529]

32. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, Lawitz E, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. *N Engl J Med.* 2014; 370:211–221. [PubMed: 24428467]
33. Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, et al. Analysis of a successful immune response against hepatitis C virus. *Immunity.* 1999; 10:439–449. [PubMed: 10229187]
34. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, Serrano-Wu MH, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature.* 2010; 465:96–100. [PubMed: 20410884]
35. Carroll SS, Ludmerer S, Handt L, Koeplinger K, Zhang NR, Graham D, Davies ME, et al. Robust antiviral efficacy upon administration of a nucleoside analog to hepatitis C virus-infected chimpanzees. *Antimicrob Agents Chemother.* 2009; 53:926–934. [PubMed: 19075052]
36. Callendret B, Bukh J, Eccleston HB, Heksch R, Hasselschwert DL, Purcell RH, Hughes AL, et al. Transmission of clonal hepatitis C virus genomes reveals the dominant but transitory role of CD8(+) T cells in early viral evolution. *J Virol.* 2011; 85:11833–11845. [PubMed: 21900166]
37. Veerapu NS, Raghuraman S, Liang TJ, Heller T, Rehmann B. Sporadic reappearance of minute amounts of hepatitis C virus RNA after successful therapy stimulates cellular immune responses. *Gastroenterology.* 2011; 140:676–685. e671. [PubMed: 21040725]
38. Koziel MJ, Dudley D, Afdhal N, Grakoui A, Rice CM, Choo QL, Houghton M, et al. HLA class I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. *Journal of Clinical Investigation.* 1995; 96:2311–2321. [PubMed: 7593618]
39. Lauer GM, Barnes E, Lucas M, Timm J, Ouchi K, Kim AY, Day CL, et al. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology.* 2004; 127:924–936. [PubMed: 15362047]

Figure 1. Changes in HCV viremia after DAA treatment and HCV J4/91 reinfection

(A) After 7 years of chronic infection with the HCV J4/91 virus, chimpanzee 5835 was treated daily for 28 days the NS5a and NS5b inhibitors as depicted by the gray shaded area. Serum RNA levels were measured at three time points just before initiation of treatment and at multiple time points through 658 days of follow-up. (B) Rechallenge with HCV J4/91 was undertaken at study day 0, which was 23.5 months (658 days) after the initiation of DAA therapy. HCV RNA levels in blood were determined by the Roche COBAS Ampliprep/COBAS Taqman HCV test and data are presented as the number of international units (IU) per mL of serum.

Figure 2. Repertoire of intrahepatic HCV-specific CD8+ T cells

Left Panels. The frequency of intrahepatic CD8+ T cells recognizing HCV proteins at (A) day 14 before cure of the first infection, (B) day 567 after DAA cure (also day 91 before reinfection) and (C) day 85 after rechallenge with HCV. The response was measured against pooled peptides representing HCV proteins shown on the X-axis. Right Panels. At each time point the IFN- γ ELISpot assay was also used to detect the presence of intrahepatic CD8+ T cells directed against 4 dominant class I peptide epitopes (E2₆₃₀, E2₆₈₀, NS5a₁₉₉₂, and NS5a₂₄₂₃) mapped from positive pools. Data are presented as the number of IFN- γ spot forming cells (SFC) per million cultured CD8+ T cells.

Figure 3. Analysis of mutational escape in dominant HCV epitopes

(A) HCV RNA isolated from serum after 7 years of chronic infection (day 0 of DAA treatment), and days 7, 28, and 140 after re-exposure to the J4/91 virus was sequenced to identify mutations in the indicated class I epitope. (B). Recognition of autologous BLCL expressing the wild-type NS5b gene and the same gene encoding the I2430V amino acid substitution by CD8⁺ T cells specific for the NS5b₂₄₂₃ epitope. The CD8⁺ T cell lines used in these assays were generated from liver 14 days before cure of the first chronic infection. The percentage of CD8⁺ T cells positive for intracellular IFN- γ production by flow cytometry is shown. (C). Recognition of BLCL pulsed with the indicated peptide representing the NS5a₁₉₉₂ wild-type epitope and the T1993I, Q1996R, and doubly substituted variants. Data are presented as spot forming cells (SFC) per million cells in an IFN- γ ELISpot assay.

Figure 4. Expansion of HCV-specific CD8+ T cells in liver after HCV reinfection

(A) The frequency of CD8+ T cells targeting the NS5a₁₉₉₂ (top row) or E2₆₈₀ (bottom row) was determined at the indicated day after reinfection by direct visualization from liver with tetramers. Numbers in each box indicate the frequency of CD8+ T cells targeting each epitope as a percentage of total liver CD8+ T cells analyzed. (B) The kinetic relationship between virus replication and the frequency of NS5a₁₉₉₂ and E2₆₈₀-specific CD8+ T cells.

Figure 5. Phenotype of intrahepatic HCV-specific CD8⁺ T cells

CD8⁺ T cells were co-stained with the NS5a₁₉₉₂ (A) or E2₆₈₀ (B) tetramer and antibodies to CD127 or PD-1. The percentage of cells in each quadrant at the indicated time point is shown. Day 0 is the day of reinfection, 658 days after DAA curative therapy was initiated. (B) Percentage of CD127 expression (left Y axis) and mean fluorescence intensity (MFI) of PD-1 staining (right Y axis) is displayed for NS5a₁₉₉₂ (left panel) and E2₆₈₀ (right panel) - specific CD8⁺ T cells.

Table 1

Patr class I restricted epitopes.

Epitope	J4/91 sequence	Patr class I	PCR primers ^I
E2 ₆₃₀	aa630- RMYVGGVEHRL	A*0901	FP-J4 nt2064 5'-GGTAACCGCACCTTGATCTG-3' RP-J4 nt2453 5'-ACCGTACAGGTATTGCACGTC-3'
E2 ₆₈₀	aa680- TTLPALSTGLI	B*0901	FP-J4 nt2064 5'-GGTAACCGCACCTTGATCTG-3' RP-J4 nt24531 5'-ACCGTACAGGTATTGCACGTC-3'
NS5a ₁₉₉₂	aa1992- KTWLQSKLL	B*0901	FP-J4 nt6217 5' GGCTTCACCAGTGGATTAATGA 3' RP-J4 nt6429 5' GGCAGGTGGTTTGCATGAT 3'
NS5a ₂₄₂₃	aa2423- YTWTGALI	B*0901	FP-J4 nt7471 5'-ACAAAGGGTCCGACGTTGA-3' RP-J4 nt7724 5'-GGATGTTGTGGCGTAGACCAT-3'

^I Forward (FP) and reverse (RP) primers used for PCR-mediated amplification of HCV J4/91 sequences containing the indicated class I restricted epitope. The J4/91 sequence position of the first nucleotide (nt) in the primer is indicated.