Characterizing CD8+ T cells in Hepatitis C Virus Reinfection

by
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ABSTRACT

Hepatitis C virus (HCV) infects millions of people worldwide with the majority of infections becoming chronic. Chronic infection can result in severe morbidity and mortality. Although recently developed therapies are increasingly more effective at clearing virus, many hurdles prevent universal access to treatment. Therefore, a vaccine to prevent progression to chronic disease is an appropriate public health solution. Interestingly, about 25% of initial HCV infections are spontaneously resolved without treatment. However, the immune correlates of protection are not fully elucidated. Individuals who clear the initial infection can become reinfected, but upon reinfection tend to have attenuated viral kinetics, suggesting a role for the adaptive immune response.

Clearance of a reinfection models a successful immune response upon re-exposure. In this analysis, we examine the protective role of CD8 T cells during HCV reinfections using a prospective cohort study of individuals at high-risk of acquiring HCV in Baltimore, Maryland. The cellular immune response during reinfection was characterized by assessing breadth, specificity, and magnitude of anti-viral cytokines produced upon recognition of HCV epitopes. We then phenotyped HCV-specific CD8 T cells to examine the dynamics of activation, exhaustion, maturation, and memory of the immune response during reinfection.

The results suggest an association between reinfection events and enhanced magnitude and breadth of T cell responses. Additionally, the HCV-specific CD8 T cells are not
dampened upon reinfection but are capable of becoming activated and vary in T cell subsets upon reinfection.
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Finally, I would like to thank my family and friends who have provided immense moral support throughout my thesis even though you had little understanding of what I was saying.
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INTRODUCTION

Public health implications

HCV epidemiology

Hepatitis C virus (HCV) infects 170 million people worldwide, with an increase in global prevalence of anti-HCV antibodies from roughly 122 million to 185 million persons between 1990 and 2005 (Hanafiah 2013). In the United States, an estimated 3.6 million or 1.3% of the general population have anti-HCV antibodies, though only 2.7 million or 1.0% of the population have chronic HCV infection (Denniston 2014). However, evaluation of the burden of disease due to HCV is conservative because acute illness is generally clinically mild thus typically unrecognized and infrequently diagnosed, and high at-risk populations such as homeless and incarcerated persons are not included in most estimates (Westbrook 2014, Denniston 2014). Rates of HCV seroprevalence are dramatically higher in correctional facilities compared to the general population, with seroprevalence estimates ranging from 27-30% of male and 38-49% of female inmates compared to 1.3% of the general United States population (Baillargeon 2003, Macalino 2004). Therefore, expanded prevalence studies that include underrepresented high-risk groups not contained in previous studies such as the homeless, incarcerated people, veterans, health care workers, and those on long-term dialysis estimated that approximately 2.0% of the United States population have HCV infection (Chak 2011). Prevalence of chronic HCV infection in the United States is greatest in those born between 1945 and 1965, representing 81% of chronic HCV infection (Denniston 2014). However, incidence of HCV infection increased in every age group in 2012 compared to 2010 and 2011, with the greatest increase in the 20-29 year age group reporting 0.75 to
1.73 cases per 100,000 people (CDC 2014). Rising incidence indicative of an emerging epidemic is present in people aged 24 or younger, of white race, residing in suburban areas, and who used oral prescription-type opioids prior to using heroin (Kleven 2012, Pollini 2013, Valdiserri 2014). While injection drug use is the most common risk factor for acquiring HCV, because transmission occurs primarily through percutaneous exposure to blood, other methods of acquiring HCV include a history of receiving a blood product transfusion prior to 1992, receiving body tattoos in a non-professional parlor, and, though infrequently, in men who have sex with men (Williams 2011, Tohme 2010, Tohme 2012). The prevalence of HCV antibodies is between 75% and 90% among people who inject drugs (Spach 2014).

**Mortality and morbidity associated with HCV**

Substantial morbidity and mortality from HCV occur due to chronic infection. In Western countries, chronic HCV infection is the leading cause of end-stage liver disease, hepatocellular carcinoma, and liver related death, and is the leading indication for liver transplant (Westbrook 2014). An estimated 27% of cirrhosis and 25% of hepatocellular carcinoma is attributable to HCV infection globally (Perz 2006). Moreover, HCV infection accounted for 366,000 deaths in 2002 worldwide, with an estimated 211,000 from cirrhosis and 155,000 from liver cancer (Perz 2006). In the United States, deaths due to HCV surpassed deaths due to HIV in 2007 when 15,106 HCV-related deaths were reported but only 12,734 HIV-related deaths were reported (Ly 2012).
Treatments and prevention

Although there is no current vaccine to prevent HCV infection, efficacy, as gauged by viral clearance and tolerability of medications, has improved with recent therapeutic developments. The initial regimen of interferon alfa monotherapy had a viral clearance rate of less than 10%, but this has shifted to more than 70% with the current therapies (Liang 2013). Interferon alfa induces interferon-stimulated genes that inhibit HCV replication by inducing antiviral functions, but therapies have evolved to be interferon-free, to improve tolerability, and to decrease duration of therapy (Liang 2013, Kohli 2014). Although direct-acting antivirals target HCV replication and processing at various steps in the life cycle, there are drawbacks to such targeted action, such as genotype-specific efficacy. For example, boceprevir and telaprevir, NS3/NS4A serine protease inhibitors that prevent proteolytic processing of the HCV polyprotein, have narrow coverage beyond genotype 1 infection (Liang 2013, Kohli 2014). Additionally, selection of viral variants by direct-acting antivirals confers resistance and thus combination therapy is required to prevent viral resistance (Pawlotsky 2011).

Even though current therapies are highly effective at clearing HCV infection, there are many barriers to effective clearance. First, acute infection presents with mild or no symptoms and thus is typically unrecognized, so knowledge of infection and need to seek care is minimal and the infection remains undiagnosed (Westbrook 2014). Second, the population most at risk for HCV infection are people who inject drugs (PWID), for whom obstacles to receiving effective HCV care include poverty, homelessness, addiction, mental health disorders, social marginalization and instability, and comorbidities such as
HIV (Williams 2011, Edlin 2005). The complexity of treatment for HIV and HCV coinfection often requires a specialist. An economic barrier is the cost of treatment ranging from $8,000 for only pegylated-interferon and ribavirin therapy to $41,700 for triple therapy, although newly approved simeprevir and sofosbuvir are $66,000 and $84,000 (Chan 2013, Fair Pricing Coalition 2013).

Finally, an immunologically interesting and unsettling point of discussion is that recovery does not confer protection from reinfection (Callendret 2014). In other words, continued exposure to HCV will negate the benefits of treatment. Therefore, methods beyond treatment to prevent progression to chronic infection that induces the morbidity and mortality caused by HCV are necessary. One approach to examining disease progression prevention is by studying the immune response of those able to spontaneously clear infection. Approximately 25% of those infected with HCV are able to clear the virus (Osburn 2010). Furthermore, rapid clearance of HCV reinfection indicates that a successful memory immune response was generated upon challenge, which is ideal for examining correlates of protection in a vaccine candidate. Studying the difference between an effective immune response able to clear the initial infection and subsequent reinfections to those unsuccessful at clearing infection will aid in determining correlates of protection.
**HCV pathogenesis**

**Virus characteristics**

First discovered in 1989 and formerly known as non-A, non-B hepatitis agent, hepatitis C virus is an enveloped, positive-stranded RNA virus from the genus hepacivirus and in the family *flaviviridae* containing approximately 9.6 kilobases (Choo 1989). After entry into hepatocytes, the virus uncoats and the genome is translated into a single polypeptide of roughly 3000 amino acids and is cleaved into three structural proteins at the amino-terminal region: core, E1, and E2; and seven nonstructural proteins at the carboxy-terminal region: p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Lindenbach 2013a, Bartenschlager 2004). The surface glycoproteins E1 and E2 mediate binding and entry into hepatocytes while the nonstructural proteins manage HCV replication via RNA synthesis, dampening host defense mechanisms, and assembling the virus (Lindenbach 2013a, Mazumdar 2011, Lindenbach 2013b).

**HCV genome variability**

A major challenge in the study of HCV is the variability of the virus genome. Genetic variability of HCV occurs both between and within genotypes. Because the virally encoded RNA polymerase lacks proofreading function, HCV has a high rate of evolution and thus diversity. HCV strains are classified based on phylogenetic analysis of sequence similarity into seven genotypes with more closely related groups within each genotype further classified into subtypes (Simmonds 1994). Sequences between genotypes differ at 30-35% of nucleotides while sequences within genotypes differ at 10-20% of their nucleotides (Messina 2015, Smith 2014). In one study examining about 60% of the
world's countries, or 90% of the world's population, the most prevalent genotype was genotype 1 accounting for 46.2% of cases, followed by genotype 3 accounting for 30.1% of cases globally (Messina 2015).

Additional HCV variability occurs within an infected individual, termed quasispecies. Using detailed virus phylogenetic characterization tools such as next-generation sequencing or pyrosequencing as opposed to Sanger sequencing, multiple variants of HCV can be seen in early HCV infection although a dominant variant is present in most samples (Abdelrahman 2015, Beerenwinkel 2011). Furthermore, during acute HCV infection, variants within an individual range from 1 to 7.8% based on phylogenetic analysis (Herring 2005). The diversity of viruses within an individual poses major challenges to vaccine development and drug therapies.

**Immune Response**

**Innate immune response to HCV**

Although most viral pathogens induce a successful immune response able to clear infection, HCV has developed mechanisms to evade the immune response and persist. Central to host defense against viral pathogens is recognition of the invading pathogen as nonself by the innate immune system. Pathogen-associated molecular patterns, or PAMPs, are microbial components such as glycoproteins, lipopolysaccharides, proteoglycans, and nucleic acid motifs that are conserved through various microorganisms capable of recognition by the innate immune system by pattern recognition receptors, or PRRs (Akira 2006). Pathogen recognition leads to signaling of
proteins that activate antiviral programs. HCV is detected by innate sensing mechanisms such as retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) genes such as RIG-I and melanoma differentiation-associated protein 5 (MDA-5) that bind to the polyuridine-rich motif of the 3’ non-translated region and its replication intermediate in the HCV genome, endosomal sensor toll-like receptor 3 (TLR3) detecting double-stranded RNA (dsRNA), toll-like receptor 7 (TLR7) that senses single-stranded RNA oligonucleotides, and protein kinase R (PKR) that binds to dsRNA (Saito 2008, Horner 2013, Jensen 2012, Chattergoon 2014). Recognition of HCV through most sensors activates kinases that phosphorylate signaling molecules to induce downstream transcription of antiviral and immunomodulatory genes, type I and type III interferons, and other proinflammatory cytokines that influence protein synthesis, growth regulation, and apoptosis (Horner 2013, Jensen 2012). The interferons induced by innate sensing mechanisms inhibit viral replication in infected cells and signal to uninfected neighboring cells to enter an antiviral state (Li 2013). An important role of innate immune sensing is coordinating the adaptive immune response. Through antigen presentation, co-stimulatory molecules, and cytokines, the innate immune system promotes the initiation of the highly-specific adaptive immune response.

Adaptive immune response: humoral immunity

Although the mechanism for delay in antibody production is not known, anti-HCV antibodies are not detected until 7-10 weeks after exposure to HCV, while antiviral immune responses are often generated 5-7 days after exposure to other viruses (Netski 2005). The efficacy of neutralizing antibodies in viral clearance is not well understood.
Evidence of neutralizing antibody activity has been demonstrated by clearing established HCV infection in liver chimeric mice and protecting genetically humanized mice from HCV infection (de Jong 2014, Law 2008). In humans, there is evidence of association between rapid induction of neutralizing antibodies during the acute phase of infection and viral clearance (Pestka 2007). However, infected humans developing antibodies that neutralize distantly related HCV genotypes fail to control HCV replication with evidence of selection for escape variants (von Hahn 2007). Moreover, viral clearance can occur independent of antibody contribution, and cross-neutralizing antibody was not protective against heterologous virus challenge (Cooper 1999, Post 2004, Barth 2011). Further studies suggest an association between clearance of reinfection and generation of cross-reactive neutralizing antibodies, but this was not true for all subjects (Osburn 2010). Although the role of neutralizing antibodies remains unclear, this evidence indicates that other methods of adaptive immunity contribute more to HCV viral clearance.

**Adaptive immune response: cellular immunity**

Cellular immune responses are thought to play a vital role in clearance of HCV. More significantly, virus-specific CD8 T cells are the primary adaptive immune effector cells facilitating HCV clearance. Briefly, CD8 T cells recognize 8-10 amino acid long peptides bound to major histocompatibility complex (MHC) class I molecules on antigen presenting cells or infected target cells. Virus-specific CD8 T cells have effector function through cytolytic properties, such as production of perforin and granzymes, Fas-FasL interaction, or production of antiviral cytokines such as IFN-γ upon recognition of antigen presented via human leukocyte antigen (HLA). In acute infection, HCV-specific
CD8 T cells begin to appear 6-8 weeks after infection (Cox 2005, Thimme 2002). This delay in cellular response is due to delaying of priming of CD8 T cells rather than recruitment of HCV-specific T cells (Shin 2011). Delays of cellular immune responses compared to other viral infections are seen in both chronic and cleared HCV infections (Cox 2005).

Spontaneous resolution of infection is associated with robust HCV-specific CD8 T cell responses. Successful responses are associated with increased breadth and magnitude of epitopes recognized, and enhanced ability to produce cytokines upon epitope recognition (Lauer 2004, Abdel-Hakeem 2014, Osburn 2010, Ciuffreda 2008). The ability of CD8 T cells to produce several effector cytokines and molecules is associated with HCV clearance (Badr 2008).

During early infection, CD4 T cells target epitopes broadly regardless of outcome (zur Wiesch 2012). However, there is an inverse correlation between the number of detected responses of CD4 T cells and time to onset of symptoms in infections that become chronic (zur Wiesch 2012). No such correlation is apparent for spontaneously resolved infections (zur Wiesch 2012). Spontaneous resolution is correlated with vigorous CD4 T cell proliferative responses (zur Wiesch 2012). Importantly, the loss of broad CD4 T cell response is not causative of progression to chronic HCV but rather a result of other mechanisms that induce a contraction of responses (zur Wiesch 2012). In CD4 T cell depletion studies, HCV RNA levels were lower in reinfection after CD4 T cell depletion when compared to levels in primary infection where CD4 T cells were intact, indicating
that CD4 T cells are not the primary antiviral effector cells in HCV clearance (Grakoui 2003).

**Viral escape of immune system**

Although an immune response occurs upon HCV infection, it is ineffective and leads to chronic infection in approximately 75% of adults exposed (Thomas 105, CDC 2014). Pressure from both cellular and humoral immunity during chronic infection leads to selection of escape variants and continued viremia (von Hahn 2007). The high replication rate and lack of proofreading activity in the RNA-dependent-RNA polymerase leads to multiple variants that can escape the adaptive immune response.

**Genetic associations with infection outcome**

Genetic factors have been implicated in the protective function in many diseases. For example, subjects with CCR5 receptor deletion display resistance to HIV infection (Steinberger 2000). In HCV, some genetic associations with infection outcome have been defined as well. Spontaneous resolution of infection was associated with a single nucleotide polymorphism (rs12979860) 3 kilobases upstream of the gene encoding the type III interferon (IFN)-λ3, also known as IL28B, and the C/C genotype (Thomas 2009). Several class I and class II HLA alleles are associated with HCV viral clearance. HLA class I alleles with the most reproducibly positive association with spontaneous clearance are *HLA-B*27, *HLA-B*57, and *HLA-Cw*01 (Schmidt 2011). Additional studies have noted positive associations with viral clearance and MHC class I alleles *HLA-A*11:01, *HLA-A*03, and *HLA-Cw*01:02 and MHC class II alleles *HLA-DRB1*03 and *HLA-
DQB1*03:01 (Mina 2015, Thio 2001). The association of HLA molecules with outcome further supports the importance of the adaptive immune response to reinfection clearance.

Current knowledge of chronic versus acute versus reinfected clearers

Importance of reinfections

Understanding the immune correlates of protection is the first step to developing an effective vaccine. Both chimpanzee and human studies noted that HCV reinfection resolved by the immune response is associated with more rapid viral clearance, decreased HCV RNA serum titer, and decreased liver disease compared to primary infection (Nascimbeni 2003, Osburn 2010). These observations are indicative of an adaptive immune component involved in immunity to HCV reinfection. Although clearance of the initial infection and never becoming reinfected is indicative of a protective immune response, the ability to clear reinfection guarantees not only continued exposure, but a successful response upon rechallenge. The goal of prevention of progression to chronic infection is achieved in reinfections that are cleared.
EXAMINING BREADTH AND SPECIFICITY OF T CELL EPITOPES DURING REINFECTION

Introduction

Current studies of HCV reinfection

The most efficient and effective way to study the immune response during reinfections is by prospective studies of populations at high-risk for acquiring HCV. Because only 20-30% of initial infections are cleared, these studies need to be large to ensure that reinfections are observed (Osburn 2010). Therefore, few studies have characterized the human immune response during HCV reinfection. The Montreal Acute Hepatitis C Injection Drug User Cohort Study characterized the cellular immune response during HCV reinfection by examining the magnitude and breadth of the T cell response, proliferative capacity upon HCV-peptide stimulation, cytokines produced by HCV-specific T cells, and phenotyping HCV-specific T cells (Abdel-Hakeem 2014). Additionally, a prospective study of intravenous drug users in Baltimore characterized reinfection by examining infection kinetics, breadth and magnitude of T cell response, and appearance of neutralizing antibodies (Osburn 2010). Significant findings indicated that spontaneous resolution of reinfections is associated with an increase in breadth and magnitude of the HCV-specific T cells (Abdel-Hakeem 2014, Osburn 2010). The proliferative capacity of HCV-specific CD4 and CD8 T cells in subjects who clear reinfection is greater than in those who become chronically infected upon reinfection (Abdel-Hakeem 2014). Similar to those who resolve acute infection, spontaneous resolution of reinfection is associated with enhanced cytokine production by HCV-
specific T cells compared to reinfections that become chronic (Abdel-Hakeem 2014). Interestingly, clearance of reinfection was more suggestive of an association between the level of individual cytokine production rather than the polyfunctionality of the HCV-specific CD4 T cells, but in CD8 T cells clearance was associated with both the level of cytokine production and polyfunctionality (Abdel-Hakeem 2014).

Although the Montreal prospective cohort provides valuable insight into reinfection T cell dynamics, the subject history is incomplete. Information on genotypes for many of the initial infecting HCV subtype were not provided because subjects entered the study with anti-HCV antibodies already present, indicating a previous infection. Obtaining viral sequence information during acute infection reduces the possibility that a reinfection is not merely a spike in HCV RNA during the initial infection since variable levels of viremia, including brief periods below the level of detection of standard assays has been documented (Abdelrahman 2015, Cox 2005b). In addition, without identification of the infection in the initial acute phase of infection, it is not possible to know how many preceding infections the patient has experienced. Therefore, it is integral to ensure a strict definition of reinfection as in our BBAASH cohort.
Methods

Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort

As a prospective study of HCV, subjects at high risk for infection were recruited to enter the Baltimore Before and After Acute Study of Hepatitis (BBAASH) in Baltimore, Maryland. Participants were 18-65 year old active injection drug users with no detectable anti-HCV antibodies or HCV virus in peripheral blood. Drug and injection practice counseling in addition to referral to medical treatment, needle exchange, and other services were provided for study subjects. Written informed consent was obtained from each study subject, and the study protocol was approved by the Institutional Review Board of the Johns Hopkins School of Medicine. Blood was donated by study subjects at one month intervals for anti-HCV antibody and HCV RNA viral load testing on either serum or plasma. Upon HCV RNA detection, units of blood were drawn every three months to obtain larger amounts of peripheral blood mononuclear cells (PBMCs) for cellular analysis. A reinfection was defined as phylogenetic difference of the Core-E1 region by 5%, and at least 60 days of aviremia determined by either viral load COBAS Taqman reverse transcription PCR quantitative assay and/or qualitative Core-E1 genotype assay between infections. The subject characteristics are summarized in Tables 1-3 below.
<table>
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<th>Patient code</th>
<th>Sex</th>
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<th>HLA class II allele</th>
<th>^IL28b alleles</th>
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Notes: All study subjects are Caucasian. 1. *IL28b* SNP: rs12979860; 2. Reinfection defined as a new infection following at least 60 days of aviremia determined by quantitative viral load RT-PCR and qualitative Core-E1 detection with greater than 5% phylogenetic difference in HCV Core-E1 nucleotides; 3. N/A signifies genetic information not available. Arrows (→) indicate new infection while dashes (/) indicate a change in dominant genotype during an infection.
Table 2. Summary of reinfected subjects

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<th>Female (n=3)</th>
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<td>86%</td>
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<td>60%</td>
<td>67%</td>
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Note: Genetic information unavailable for one subject

Table 3. Individual chronic study subject characteristics for FACS analysis

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<th>HLA class II allele</th>
<th>1IL28b alleles</th>
<th>Genotype of infections</th>
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<tr>
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Limitations to study design

Although the prospective BBAASH monthly study enables in-depth opportunities to examine HCV infection dynamics, inherent limitations restrict complete comprehensive analysis of the immune system. These limitations can be grouped into the technical aspects, subject characteristics, and virus kinetics.

The first, technical limitations, refers to the assays used to qualitatively and quantitatively assess the virus, and the method to determine recognized HCV epitopes. The limit of detection for genotyping is 100 International Units (IU)/mL, while the limit of detection for quantitative determination of viral load is 50 IU/mL. This means that virus could be circulating but is below limit of detection of the assay. Additionally, the ELISpot assay that qualitatively and quantitatively defines recognized T cell epitopes uses genotype 1a virus peptides as antigens, and recognition of epitopes restricted to other genotypes may be less well detected in this assay. Although there are other genotypes of HCV in circulation, 1a is the most common genotype, representing roughly 75% of total genotypes prevalent in the US (Messina 2015).

The second constraint that limits the analysis of the immune system is subject characteristics. In order to perform cellular analysis, large blood donations, called units, are required to have enough PBMCs. Units can be drawn no more frequently than every 8 weeks. If subjects are ineligible for a large blood draw, for example having poor veins or anemia, then a unit cannot be obtained and cellular analysis cannot be performed at that time point.
The characteristics of HCV infection in conjunction with the cohort study format limit reinfection analysis. HCV reinfection is associated with attenuated virus kinetics such as shorter duration of viremia and decreased magnitude of circulating virus (Osburn 2010). Subjects in the BBAASH cohort donate blood monthly, but the decreased duration of viremia upon reinfection permits a reinfection to be cleared between the monthly draws and thus not be detected. Another characteristic of HCV infection that limits comprehensive analysis is a control comparison. Chronically infected individuals tend to lose T cell responses over time, and thus time-matching the course of infection of a chronic infection to a reinfection time point starting from the initial infection is not ideal. Therefore, in the following analyses, each reinfection is treated as a ‘new infection’ and compared to both the acute infection of the same subject and the acute infection of a chronically infected subject. However, HCV-specific T cell responses in acute infection are not apparent until 6-8 weeks after infection (Cox 2005a, Thimme 2002). In our study, we compare the T cell response in reinfection to the earliest time point that HCV-specific responses were detected in the primary infection. The strict definition of reinfection as 60 days of aviremia followed by infection with a new HCV virus with at least 5% difference in Core-E1 sequence ensures analysis of reinfection rather than recurrence of viremia from the initial infection.

**Determination of HCV viral levels**

A quantitative value of HCV viral level was determined from either serum or plasma stored at -80° Celsius from monthly visits using a quantitative reverse-transcriptase
polymerase chain reaction (RT-PCR) assay with the Abbott RealTime HCV RNA assay. The sensitivity for this assay is 50 IU/mL.

**HCV genotyping**

HCV genotype was determined by performing phylogenetic analysis of HCV Core-E1 sequence obtained from either patient serum or plasma stored at -80°C. Viral RNA was extracted using QIAGEN's QIAamp® MinElute® Virus Spin Kit according to the manufacturer's instructions. The positive control was plasma from subjects with known sequence and viral load while the negative control was Millipore water in place of plasma. RNA was extracted from 600 µl (cleared subjects) and 200 µl (acutely infected) of plasma or serum. An RT-PCR reaction was performed first, and the products were converted to cDNA in a second round. Samples were run on a 1% agarose/tris-borate EDTA gel with ethidium bromide for approximately 30 minutes at 100-130 volts. Bands were detected using a transilluminator. If positive, the PCR product was purified using a Qiagen kit following manufacturer's instructions. DNA was sequenced at the Core Sequencing facility at Johns Hopkins. Sequences were trimmed to equal length and aligned with BioEdit using ClustalX.

**Enzyme-Linked ImmunoSpot (ELISpot) assay**

Using a multichannel pipet, an ELISpot plate was coated with recombinant mouse anti-human IFN-γ antibody (MAbTech clone#1-D1K). The ELISpot plate was incubated at 4°C Celsius overnight. Cells were taken immediately from -160°C Celsius liquid nitrogen storage to 37°C Celsius water bath. Using drip method, 50% FBS in RPMI (R50) was
added to cells, washed twice, and resuspended to final resting concentration of 3 million cells per milliliter in tissue culture flask overnight in CO₂ incubator at 37° Celsius. After overnight incubation, mouse anti-human IFN-γ antibody was discarded from ELISpot plates and each plate was washed four times with sterile 1X PBS. To block the plate, 10% FBS in RPMI (R10) was added to each well and incubated for two hours at 37° Celsius. PBMCs that had rested overnight were resuspended to a final concentration of 4 million cells per milliliter in R10. After blocking the ELISpot plate for two hours, R10 was discarded. HCV peptide diluted to 20 µg/ml in R10 was added to each well. Using a multichannel pipet, 200,000 cells were added to each well. In other words, 200,000 PBMCs at 2 million cells per milliliter were being stimulated by 10 µg/ml of peptide in a total volume of 100 µl of R10. PBMCs with peptide were incubated for 15-20 hours at 37° Celsius. After incubation, the plate contents were discarded and blotted dry. The plate was washed 7 times with wash buffer (0.05% Tween20 in 1X PBS). Using a multichannel pipet, biotinylated mouse anti-human IFN-γ monoclonal antibody (MAbTech clone 7-B6-1) detection antibody was added to each well and incubated for 3 hours at room temperature. After incubation, the plate contents were discarded and the plate was washed 4 times. Alkaline phosphatase-conjugated anti-biotin monoclonal antibody (aBio-ALKPHOS, Vector Laboratories) was added to each well of the plate and incubated for 2 hours at room temperature. After incubation, the contents of the plate were discarded and washed 4 times with wash buffer. Plates were developed with filtered BCIP/NBT solution for 10 minutes at room temperature. After developing, contents of plate were discarded and plate was washed 4 times with UltraPure water to stop
development. The plate gasket was removed to allow the plate to dry overnight. Spot-forming cells were quantified at the JHU Immunology core.

**Results**

**Greater magnitude, but not breadth, is associated with HCV reinfection.**

The breadth and specificity of T cell responses was examined using ELISpot. Previous studies have indicated that clearance of reinfection is associated with increase in breadth of HCV-specific T cell response and higher magnitude of interferon gamma production (Abdel-Hakeem 2014). At each visit where large numbers of PBMCs were available, cells were tested on the same plate in an interferon gamma ELISpot assay with individual peptides that previous ELISpot screens indicated were recognized by that subject's T cells. The peptides tested included both optimal epitopes (peptides that were previously determined to be of optimal length for recognition) and longer peptides from overlapping peptide pools that span the whole HCV polyprotein. The magnitude of the response is indicated by the total number of SFCs while the breadth of the response is defined as the number of different epitopes recognized. For each reinfection, the magnitude and breadth of the anti-HCV cellular immune response was obtained using ELISpot (Figure 1A-H). A summary of the viral RNA levels and HCV T cell epitopes recognized and magnitude of the response is presented in Figures 2A-B. If multiple units were tested during a reinfection, the response with the greatest magnitude was used for analysis.
The total magnitude of the anti-HCV interferon gamma response for the first reinfection is greater than the magnitude of response during the primary infection for every subject tested. This increase is seen in both reinfections that become chronic and reinfections that are cleared. There is a significantly greater interferon gamma response to HCV peptides in the first reinfection than the primary infection (Figure 3A). However, subsequent reinfections are not uniformly associated with continued increases in the magnitude of the T cell response. While the magnitude of the T cell response remains greater than in primary infection, some second and third reinfections are associated with declining magnitude of the T cell response compared to the magnitude at the first reinfection.

In contrast, the breadth of the T cell response is not significantly greater with reinfection than in primary infection. While new T cell epitopes are almost uniformly targeted with each new infection, there is also loss of T cell epitope recognition such that the number of total epitopes recognized during each reinfection is not always greater than the number of epitopes recognized during the initial infection. There is no statistically significant difference between the number of total epitopes recognized during the first reinfection than during the primary infection (Figure 3B). These results suggest that an increase in magnitude, but not an increase in breadth, is associated with reinfection.

We compared the total number of epitopes recognized during the last available reinfection time point to the number of epitopes recognized during the initial infection. There was a statistically significant increase in the number of new epitopes recognized upon the last reinfection to the initial infection for all subjects (p=0.019 paired t-test; p=0.016 Wilcoxon Signed rank test). However, there was no statistically significant
difference in the number of new epitopes recognized upon first reinfection compared to initial infection (p=0.156 Wilcoxon Signed Rank Test; p=0.125 Paired t-test).

**Cleared reinfections and persistent reinfections have no statistically significant difference in the breadth or magnitude of anti-HCV T cell interferon gamma secretion.**

In contrast to primary infection, most reinfections are cleared. However, roughly 17% persist with resultant chronic infection (Osburn 2010). In this analysis, three subjects were unable to clear a reinfection. Although two subjects became persistently infected during their first reinfections, the third developed persistent infection following the second reinfection. In order to control for a persistent reinfection occurring on the second reinfection, one subject was randomly chosen using a random number generator from the three subjects who cleared second reinfections and the second reinfection from that subject was chosen for analysis. The first reinfection was used as a comparator for the two subjects with persistence upon the first reinfection.

A comparison of the breadth and magnitude of the fold change of cleared versus persistent reinfections revealed no statistically significant differences. The magnitude of interferon gamma produced during reinfection compared to the initial infection is presented as the fold change in Figure 4A. However, there was no difference in the magnitude of anti-HCV response between those unable to clear the reinfection and those who clear reinfections. This suggests that the magnitude does not predict outcome of
reinfection. However, the small number of subjects limits the power to detect statistical significance in this study.

Similar findings were seen with breadth of response. The ratio of the number of total epitopes recognized during the reinfection to the number of epitopes recognized in the first infection was compared between cleared reinfections and persistent reinfections. There was no difference between cleared reinfections and persistent reinfections in the total number of epitopes recognized in reinfection versus initial infection (Figure 4B). Further analysis investigated whether there was a difference in the number of new responses that developed upon reinfection between cleared reinfections and persistent reinfections. There was no statistically significant difference between cleared reinfections and persistent reinfections in the development of new responses upon reinfection compared to initial infection (Figure 4C). These results indicate that the breadth of anti-HCV epitopes recognized upon reinfection does not correlate with outcome of reinfection.

Although an increase in breadth of response from initial infection to first reinfection for all subjects, (including those who clear and those who develop persistence upon reinfection) was not significant, we wanted to examine if there were statistically significant differences in subjects who clear all reinfections. No statistically significant association was found in the total number of epitopes recognized from initial infection to first reinfection in subjects who clear all reinfections (p=0.250 Wilcoxon Signed Rank Test, Paired t-test failed normality). A similar analysis was performed looking at the number of total epitopes recognized upon reinfection compared to initial infection in
subjects who become chronically reinfected. Similarly, there was no statistically significant change in the total number of epitopes recognized comparing initial infection to persistent reinfection in subjects who do not clear reinfection (p=0.478 Paired t-test).

An indication of a robust immune response is the ability to develop new responses. T cells may respond to epitopes during reinfection that were not recognized during the initial infection, suggesting an ongoing response to new HCV exposures. We analyzed this development in various ways. First, as all subjects are reinfected at least once, we compared the number of new epitopes recognized during first reinfection in those who clear infection to those who develop persistent reinfection, but this was not significantly different between the two groups (p=0.250 Mann-Whitney Rank Sum Test; t-test failed normality). As previously described, the subject who became persistently infected upon second reinfection was randomly matched to a second reinfection of a subject who cleared. However, some subjects become reinfected multiple times and recognize new epitopes during every reinfection. To take into account multiple reinfections, we took the average of all the new epitopes gained during every reinfection and compared this average between subjects who clear all reinfections and subjects with persistence upon reinfection. However, there was no statistically significant difference in the average number of new epitopes in all reinfections between subjects who do and do not clear reinfection (p=0.250 Mann-Whitney Rank Sum Test because t-test failed normality).

A hallmark of chronic HCV infection is the loss of interferon gamma secretion in response to HCV peptides over time. To examine if subjects who are unable to clear reinfection have similar loss, we compared the number of epitopes not recognized during
the first reinfection that were recognized during the initial infection between subjects who clear the reinfection and subjects who do not clear the reinfection. For the subject who became chronic upon second reinfection, the epitopes recognized during the second reinfection was compared to the initial infection. This was randomly matched to another subject and the same analysis performed. However, there was no statistically significant difference in the number of epitopes lost in repeated clearers and in reinfections that become chronic (p=0.482, t-test).

No immunodominance in epitopes recognized by reinfected subjects.

To examine whether reinfection is associated with recognition of a particular epitope or region of the HCV polypeptide, all of the HCV epitopes recognized by each reinfected subject were mapped to the HCV polyprotein. Epitopes are recognized throughout with no apparent immunodominance (Figure 5). Further analysis was performed to determine if persistent reinfection is associated with responses to a common epitope. There was no apparent immunodominance in reinfections that become persistent. The red dots in Figure 5 indicate epitopes recognized by subjects at the reinfection that becomes persistent. These results suggest no difference in the region of the HCV polyprotein targeted by T cells from subjects who clear reinfections compared to those who do not clear reinfection.
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Figure 1A. Reinfected Clear Subject 57

Figure 1A-H. Plot of viral load and epitope response. Each slice of the pie chart denotes a peptide epitope, and the size of the slice represents the proportion of total response. The size of the pie denotes the magnitude. A-H represent different subjects tested. Black dots on the viremia area denote time points when HCV viral level or genotype was tested.
Figure 1B. Reinforced Clear Subject 176
Figure 1C. Reinfected Clear Subject 144

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Figure 1D. Reinforced Clear Subject 112

![Graph showing HCV RNA levels over time with key for different proteins.]

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Figure 1E. Reinfection Clear Subject 26
Figure 1F. Reinfection Chronic Subject 553

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Figure 1G. Reinfect ed Chronic Subject 18

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HCV RNA (IU/mL)

Time since initial viremia (days)
Figure 1H. Reinfected Chronic Subject 133

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- HCV RNA (IU/mL) vs. Time since initial viremia (days)
Figure 2A. Magnitude of ELISpot response for all subjects. Dashed lines indicate infections that become chronic.
Figure 2B. Breadth of ELISpot response for all subjects. Dashed lines indicate infections that become chronic.
Figure 3A. Magnitude of ELISpot response for reinfected subjects from primary infection to first reinfection. Dashed lines indicate reinfections that become chronic.
Figure 3B. Breadth of ELISpot response for reinfected subjects from primary infection to first reinfection. Dashed lines indicate reinfections that become chronic.
Figure 4A. Ratio of the total SFC comparing first reinfection to primary infection.

Each dot denotes one subject comparing the total magnitude of ELISpot response of the first reinfection to primary infection.
Figure 4B. Ratio of number of epitopes recognized from first reinfection to primary infection. Each dot indicates one subject.
Figure 4C. Ratio of new epitopes recognized upon reinfection. Each dot denotes the ratio of epitopes recognized in first reinfection that were not recognized during the primary infection to the total number of epitopes recognized during the first infection.
Figure 5. Mapping of recognized epitopes along HCV polyprotein. Each dot denotes a location along the HCV genome that was recognized. Red dots indicate epitopes recognized during infections that become chronic.
IMMUNOPHENOTYPING OF HCV-SPECIFIC CELLS DURING REINFECTION

Introduction: Surface molecule definitions

Flow cytometry as a tool to examine phenotype

Characterizing HCV-specific cells based on activation, memory, and exhaustion status as well as T-cell subsets was performed with flow cytometry, "arguably the most powerful technology available for probing human immune phenotypes" (Maecker 2012). This technology allows for single cell analysis of multiple surface molecules, intracellular cytokines, and protein expression. Comprehensive phenotype of HCV-specific cells during reinfection was obtained using a twelve-color polychromatic panel examining cell surface protein expression. Figure 5 demonstrates the flow cytometry experiment performed.

Figure 6 Flow cytometry experiment outline (Adapted from Maecker 2012)
Markers of T-cell subsets

CD45RA is a transmembrane protein tyrosine phosphatase and the expression of various splice variants of this molecule indicates whether T cells have encountered antigen. T cells that have not yet encountered antigen express CD45RA, while T cells express the splice variant CD45RO upon activation. Terminally differentiated effector memory cells revert back to CD545RA expression (Wills 1999). Expression of CC-chemokine receptor 7 (CCR7) controls homing of T cells to secondary lymphoid organs such as the lymph nodes (Forster 1999). As CD45RA is re-expressed after a period of time since antigenic stimulation, the CD45RA+ subset includes both naïve and non-naïve T cells, thus the combination of CD45RA and CCR7 is useful to define T cell differentiation (Carrasco 2006). Moreover, the combination of CD45RA and CCR7 expression defines classical T cell subsets as: naïve T cells are CCR7+CD45RA+, central memory T cells are CCR7+CD45RA-, effector memory T cells are CCR7-CD45RA-, and terminally differentiated effector memory T cells or Temras are CCR7-CD45RA+ (Maecker 2012, Sallusto 2004, Appay 2008). These T cell subsets differ in their effector function potential and ability to home to secondary lymphoid organs. Circulating CD8+ T cells from healthy individuals range from 57-64% naïve, 7-15% central memory, 10-21% effector memory, and 9-26% Temras (Thome 2014, Geginat 2003). Central memory cells, defined as CCR7+CD45RA-, possess the ability to migrate to secondary lymphoid tissues and respond to antigenic stimulation with rapid proliferation and differentiation into effector cells (Sallusto 2004). Conversely, effector memory cells, CCR7-CD45RA-, respond to antigenic stimulation with rapid effector function including release of perforin and production of IFN-γ, interleukin (IL)-4, and IL-5, but have reduced proliferative
potential compared to central memory cells (Sallusto 2004). Temras, defined as CCR7-CD45RA+, are the most differentiated T cells with the greatest cytolytic capacity and carry the largest amount of perforin and granzyme B and express high levels of Fas ligand (Sallusto 2004, Hamann 1997). HCV-infected individuals have lower percentages of naïve CD8+ T cells and higher percentages of Temra cells compared to CD8+ T cells from healthy individuals (Shen 2010).

**Molecules of activation**

The ectoenzyme CD38, a defining molecule of activation, catabolizes nicotinamide adenine dinucleotide (NAD+), leading to increased intracellular calcium-mobilizing compounds (Malavasi 2008). Accumulation of extracellular NAD induces ADP ribosylation via ecto-mono-ADP-ribosyltransferases to initiate apoptosis due to decreased calcium influx of activated T cells (Han 2000, Krebs 2005, Seman 2003). Therefore, CD38 allows selective expansion of primed T cells. Upon CD38 ligation with its receptor, signal transduction occurs that produces cytokines such as IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and IL-10 (Malavasi 2008). Importantly, CD38 on T cells has interesting implications in chronic viral infections. For example, CD8+/HLA-DR+/CD38- cells are associated with undetectable HIV plasma viral load in the absence of therapy (Saez-Cirion 2007).

During chronic HCV infection, activation molecules CD38 and HLA-DR are upregulated on CD8+ T cells compared to CD8+ T cells from healthy controls, and the expression level of HLA-DR, but not CD38, negatively correlates with circulating HCV viral load (47 Shen). The mean fluorescence intensity (MFI), an indicator of the level of expression
of a molecule on individual cells, of CD38 at the first study time point during infection was higher on HCV-specific T cells in chimpanzees that subsequently resolved infection than on HCV-specific T cells in those chronically infected (Shin 2013).

**Molecules of exhaustion**

Chronic antigenic stimulation, as occurs in cancer and in chronic viral infections, results in T cell exhaustion. This dysfunctional T cell state develops in a stepwise manner with gradual loss of cytokines and effector molecules starting with IL-2 and with IFN-γ among the last lost (Wherry 2011). Additionally, T cell dysfunction is characterized by overexpression of inhibitory receptors, downregulation of TCR signaling molecules, decreased expression of metabolic and translational machinery molecules, and altered chemotaxis, migration, and adhesion gene expression (Wherry 2007). Phenotypically exhausted T cells are seen in many persistent infections, such as lymphocytic choriomeningitis virus (LCMV) in mice, and hepatitis B virus (HBV), HCV, and human immunodeficiency virus (HIV) in humans (Zajac 1998, Boni 2007, Bengsch 2010, Day 2006). Transcription signatures in T cell exhaustion differ from a state of anergy, which occurs when T cells recognize antigen via the TCR but lack costimulation (Wherry 2007).

Programmed cell death protein-1, also known as PD-1 and CD279, contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail, which, upon surface binding to its ligand PD-L1 or PD-L2, inhibits T cell activation and T cell receptor-CD28 signaling (Freeman 2000, Ishida 1992, Latchman 2001). The outcome of acute HCV infection has been associated with levels of PD-1 on HCV-specific cells in
some studies. Specifically, in the first six months of infection, subjects who cleared infection had lower PD-1 levels on HCV-specific cells than those who failed to clear infection, although these differences were less pronounced beyond six months after initial infection (Rutebemerwa 2008, Urbani 2006). However, additional studies demonstrated high PD-1 expression on HCV-specific CD8 and CD4 T cells during acute infection regardless of clinical outcome, but noted that the percent of PD-1+ cells declined after the acute phase of infection in spontaneous resolution, but not in those developing chronic infection (Kasprowicz 2008, Urbani 2006). This is in agreement with the proposal that PD-1 is an activation marker rather than an exhaustion marker during acute HCV infection because upregulation during acute infection did not inhibit clearance (Shin 2013). Additionally, lower levels of PD-1 were seen in spontaneous resolution of cleared reinfection (Abdel-Hakeem 2014).

While the function of T-cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) has yet to be fully elucidated, there is a correlation between T cell dysfunction in chronic infection and Tim-3 expression (Fujita 2014, Jones 2008, Golden-Mason 2009). In HIV, Tim-3 is upregulated on both CD4+ and CD8+ T cells from chronic infection compared to uninfected individuals, and although Tim-3+CD8+ T cells had higher levels of perforin, they were defective in their ability to degranulate and have decreased ability to secrete effector cytokines IFN-γ and IL-2 (Jones 2008, Sakhardi 2012, Zhang 2015). In chronic HCV infection, Tim-3 is overexpressed on total CD4+ and CD8+ T cells and with highest frequency on HCV-specific T cells containing decreased ability to secrete effector cytokines tumor necrosis factor (TNF)-α and IFN-γ (Golden-Mason 2009). Blockade of Tim-3 ligation, both in the presence and absence of PD-1 blockade, rescues
HCV-specific T cell dysfunction by augmenting proliferation and increasing cytokine production of IFN-γ and TNF-α (Golden-Mason 2009, McMahan 2010). Similarly, blockade of PD-1 and its ligand enhances proliferation of HCV-specific CD8 cells (Urbani 2006).

Coexpression of Tim-3 and PD-1 correlates with increased T cell dysfunction, with higher levels of other inhibitory receptors such as LAG3 and 2B4, with decreased proliferative capacity upon peptide stimulation, and with reduced ability to secrete effector cytokines IFN-γ, TNF-α, and IL-2 (Jin 2010). Dual expression of Tim-3 and PD-1 on HCV-specific CD8 T cells during acute infection is associated with viral persistence as infections spontaneously resolved were characterized by a lower frequency of dual positive HCV-specific CD8 T cells than dual negative compared to chronic infection (McMahan 2010).

**Figure 7. T cell exhaustion (Kahan 2015)**
**Molecules of activation/homeostasis**

The combination of coreceptor CD28 and homeostatic cytokine interleukin-7 (IL-7) receptor-α CD127 can discriminate dynamics of T cell function. More specifically, CD28+CD127+ indicates resting or unstimulated T cells, CD28+CD127- T cells are receiving IL-7 signals, CD28-CD127+ suggests a previous TCR-driven response, and CD28-CD127- is the phenotype of antigen-activated effector cells (Thome 2014). IL-7 is essential for regulating CD8 T cell homeostasis, and thus expression of its receptor CD127 is an indicator of cells more likely to survive and differentiate (Schluns 2000). During acute infection of subjects who develop chronic infection, the percent of CD127 expression was decreased in CD8+ T cells compared to uninfected controls, while CD127 levels on CD8 T cells in subjects who resolved HCV infection were not significantly different from controls (Golden-Mason 2006). A decrease in IL-7 receptor CD127 was observed on HCV-specific T cells during the reinfection that resolved but not on HCV specific T cells in reinfections that become chronic (Abdel-Hakeem 2014).

**Methods**

**Immunophenotyping using polychromatic flow cytometry**

PBMCs stored at -160° Celsius liquid nitrogen were rapidly transferred into a 37° Celsius water bath to thaw. Using the drip method, 15 ml of R50 was added to cells. Cells were centrifuged at 400g for 5 minutes and the supernatant was decanted. Cells were resuspended in 15 ml of R10 and 20 µl was removed for counting using a
hemocytometer. Cells were centrifuged at 400g for 5 minutes. After centrifugation, the supernatant fluid was decanted. Cells were washed with 1X PBS, centrifuged at 400g for 5 minutes, decanted, and resuspended so that the final cell concentration was 1 million cells per milliliter of 1X PBS. Staining occurred in four sequential incubations: aquaviability, pentamer addition, surface stains, and fixation. For aquaviability staining, 1μl of reconstituted fluorescent reactive dye was added to each milliliter of cells and incubated at 4°C Celsius for 30 minutes. The tube of cells was filled with FACS buffer (0.1% BSA, 0.1% sodium azide in 1X PBS), centrifuged at 400g for 5 minutes, decanted, and resuspended at 1 million cells per milliliter of FACS buffer. For each test, 1-2 million PBMCs were allocated into individual Falcon 5ml polystyrene round-bottom FACS tubes. Each tube was filled with FACS buffer to wash, centrifuged at 400g for 5 minutes, and decanted. Prior to multimer staining, each multimer was centrifuged at 4°C Celsius 14,000g for 7-10 minutes. The fluorotag was centrifuged at 4°C Celsius at 14,000g for 3 minutes. Multimer staining was performed for 10 minutes at room temperature in the dark. After washing twice for 5 minutes at 400g in FACS buffer, cells were pelleted and directly stained with the following antihuman antibodies: CD8-AlexaFluor700 (clone SK1, Biolegend), CD4-PE-CF594 (clone RPA-T4, BD Horizon), HLA-DR-BV421 (clone G46-6, BD Biosciences), CD38-APC-H7 (clone HB7, BD Biosciences), CD28-PerCP-Cy5.5 (clone CD28.2, BD Biosciences), CD127-FITC (clone eBioRDR5, eBioscience), CD45RA-PE-Cy7 (clone HI100, Biolegend), CCR7-BV711 (clone 150503, BD Horizon), Tim-3-PE (Clone 344823, R&D Systems), PD-1-BV605 (clone EH12.2H7, Biolegend) and APC fluorotag (ProImmune) for unlabeled pentamers in BV brilliant stain buffer for 30 minutes at 4°C Celsius. Multimers were obtained from Dr. Georg Lauer.
and ProImmune. After two further washes with FACS buffer, cells were fixed with 150ul of 2% formaldehyde for 1 hour at 4° Celsius. Cells were washed twice and resuspended in 150ul of FACS buffer. Multiparameter flow cytometry was performed using a Becton Dickinson LSRII equipped with red (635nm), violet (407nm), blue (488nm), and green (532nm) lasers using FACSDiva software. FCS data files were analyzed using FlowJo software version 10.0.7 for Windows 7 (Tree Star, Inc.). Gating was based on fluorescence-minus-one (FMOs), with a representative FACS plot shown in Figure 8.

Results

MFI of individual molecules reveals no highly pronounced changes in expression of molecules upon reinfection.

To determine differences in molecule expression on HCV-specific CD8 T cells upon reinfection, we examined the median fluorescence intensity (MFI) within an individual. Analysis of the MFI demonstrates the expression level of molecule on the population of cells. The MFI of pentamer+CD8+ T cells was divided by the MFI of total CD8+ T cells to give a ratio. Therefore, any number above one implies that the MFI on the HCV-specific T cells is greater than the MFI on total CD8+ T cells. Conversely, any ratio below one indicates that the MFI on HCV-specific T cells is lower than total CD8+ T cells. The results from individual subjects are summarized in Figure 9. Each dot indicates a ratio of pentamer+CD8+ T cells to total CD8+ T cells. The different colors denote the
different pentamers used. The vertical lines for each reinfected subject mark the first point of clearance and the first time point of new virus detected.

For T cell phenotypic analysis, three subjects who cleared every reinfection, one subject who became chronic upon reinfection, and two subjects chronically infected were analyzed. Therefore, phenotype of HCV-specific T cells can be compared in various ways. First, reinfections can be compared to the initial infection within a subject. This method reveals changes in adaptive immune responses upon reinfection. Second, acute infections can be compared between chronic and reinfected subjects. This comparison could lead to differences in the initial immune response that lead to failure to control virus. Third, the first reinfection of the subject who becomes chronic can be compared to the first reinfections of subjects that clear all reinfections. This final method of analysis will reveal deficits in response that result in inability to clear reinfection.

Molecules specifying T cell subsets, CCR7 and CD45RA, varied depending on presence of virus. CCR7 expression was greater on HCV-specific T cells compared to total CD8 T cells upon clearance of virus in most subjects that clear reinfections. Within subjects who clear all reinfections, the CCR7 on HCV-specific T cells tended to increase upon clearance of detectable virus and decreased upon reinfection. However, there was no apparent difference in CCR7 expression between reinfections within a subject as shown in Figures 9A-C. In comparison, CCR7 expression on HCV-specific T cells in chronic infections varied depending on the pentamer as shown in Figures 9D-E. Some HCV-specific T cells had much greater expression of CCR7 compared to total CD8 T cells.
throughout all available time points, while other pentamers had a lower or the same levels of CCR7 on HCV-specific T cells compared to total CD8.

Upon clearance of initial infection, CD45RA tended to increase on HCV-specific T cells but did not always return to levels present on total CD8+ T cells. In subjects who cleared reinfection, CD45RA fluctuated depending on presence of virus, although this variation was only maximum four times greater than total CD8+ T cells in one subject. When virus is present in subjects who clear reinfection, CD45RA had lower expression on HCV-specific CD8+ T cells compared to the total CD8+ T cell population. There was no apparent difference in CD45RA expression upon subsequent reinfection. However, for chronically infected individuals, CD45RA tended to remain lower on HCV-specific CD8+ T cells compared to total CD8+ T cells throughout the entire infection. The subject who became chronic upon reinfection also had decreased levels of CD45RA on HCV-specific T cells. As the combination of CD45RA and CCR7 reveals T cell subsets, analysis of these two molecules is extended later.

An indicator of HCV clearance is elevated expression of the maturation molecule CD127. In this study, CD127 usually increased on HCV-specific CD8+ T cells upon clearance of virus in subjects who clear reinfection. No changes in CD127 expression upon subsequent reinfection in subjects that clear reinfection was observed. Interestingly, CD127 expression remained greater on most HCV-specific T cells compared to total CD8 T cells upon chronic reinfection. However, CD127 expression on HCV-specific T cells remained similar to the expression on total CD8 T cells for both chronic infections and cleared reinfections.
There appeared to be no uniform pattern of CD28 expression on HCV-specific CD8 T cells compared to total CD8 T cells. In one subject who cleared multiple reinfections, CD28 levels on HCV-specific T cells were lower compared to total CD8 T cells for all but one point during follow-up. However in another subject who cleared multiple reinfections, CD28 expression was greater on HCV-specific cells compared to total CD8 T cells during periods of aviremia. Within each subject, CD28 expression increases upon subsequent clearance of reinfection even though the total CD28 expression was lower on HCV-specific cells than total CD8 T cells. HCV-specific T cells in during chronic reinfection also had increased CD28 expression compared to the primary infection. In comparison, the CD28 expression on HCV-specific T cells remained stagnant during chronic infection and when the reinfection becomes chronic. Although the CD28 expression on HCV-specific T cells compared to total CD8 T cells varies between the chronic subjects, the level remained the same within each subject's infection. In other words, there were only slight to no changes in CD28 expression on HCV-specific T cells in chronic infection, but for reinjected subjects the CD28 expression differs depending on the presence of virus and upon reinfection outcomes.

Next, we examined individual molecules of exhaustion on HCV-specific T cells compared to total CD8 T cells. In subjects that clear subsequent reinfections, PD-1 expression was higher on most HCV-specific T cells than total CD8 T cells. The level of PD-1 was high after clearance of reinfection, but decreased after extended period of no virus. In chronically infected subjects, expression of PD-1 on HCV-specific T cells was generally greater than total CD8 T cells. However, there were some exceptions and these were seen for the same HCV epitope throughout the course of infection. Interestingly, in
one chronically infected subject, the PD-1 levels remained higher on HCV-specific cells for one epitope, but in the other HCV epitope the PD-1 expression was increased initially but then decreased compared to total CD8 T cells. An explanation of this phenomenon is that the antigen is no longer intact, as lowered PD-1 levels on virus-specific cells is seen during chronic infections (Blattman 2009, Rutebemberwa 2008). Similarly, the subject who becomes chronically infected upon reinfection had differences in PD-1 expression based on the epitope recognized as shown in Figure 9F. While the HCV-specific T cells had higher-than-total CD8 T cell levels of PD-1, one epitope had a much greater difference in expression than the other epitope.

Tim-3 expression tended to be higher on HCV-specific T cells during most aviremic time points for reinfected subjects (Figures 9A-C). However, when virus is present, Tim-3 expression on HCV-specific T cells compared to total CD8 T cells was decreased in reinfected subjects. There was no trend upon subsequent infections for Tim-3. In one chronic infection, Tim-3 expression on HCV-specific T cells remained similar to total CD8 T cell expression and was stagnant throughout infection (Figure 9D). The other chronic infection subject was similar to the chronic reinfection subject where Tim-3 expression is initially the same between the two HCV pentamers studied, but as time progressed the Tim-3 expression between the two pentamers in both subjects varied greatly (Figure 9E-F). Therefore, in the chronically infected and the chronic reinfection subjects who recognize multiple HCV epitopes, there was a different Tim-3 phenotype for the different epitopes. Interestingly, as duration of chronic infection increases in both the chronically infected and the chronically reinfected subjects, the levels of PD-1 and Tim-3 on HCV-specific T cells were inversely correlated. For HCV-specific T cells with
greater Tim-3 expression than total CD8 T cells, the PD-1 expression was lower on HCV-specific T cells than total CD8 T cells.

Finally, we examined differences in activation between reinfected-cleared subjects and chronically infected subjects. Within subjects who clear all reinfections, HLA-DR was generally elevated on HCV-specific T cells compared to total CD8 T cells when virus was detectable. However, HLA-DR was at some time points higher during the period of aviremia than during the acute infection and in one instance remained high nearly 800 days after a reinfection was cleared. In the subject who develops chronic reinfection, HLA-DR levels remained elevated on T cells recognizing one HCV epitope but decreased on T cells recognizing another HCV epitope. In chronically infected subjects, HLA-DR expression on HCV-specific T cells was not consistent between different T cell epitopes recognized by the same subject, but generally remained similar to HLA-DR expression on the total CD8 T cell population. In one chronic infection, HLA-DR on HCV-specific T cells was similar to that on total CD8 T cells but in another subject, HLA-DR was elevated to nearly 20 times the level on total CD8 T cells.

The other activation molecule examined was CD38. Between reinfections, CD38 decreased during periods of aviremia but increased again once virus was detected. Upon subsequent reinfections, CD38 expression did not differ compared to acute infection. However, CD38 expression in the subject who developed chronic reinfection never decreased during the period of aviremia on the HCV-specific T cells. Additionally, levels of CD38 remained elevated on HCV-specific T cells compared to total CD8 T cells in all chronic infections and on T cells from the subject who developed chronic reinfection.
Although CD38 was elevated on HCV-specific T cells during the initial infection in both chronic and reinfected subjects, this elevation was only temporary for cleared reinfections.

*Dual expressions of molecule subsets on contour plots demonstrate shifting populations of HCV-specific T cells over the course of reinfection.*

An advantage of polychromatic flow cytometry is the ability to examine multiple molecules on one cell. In this study, we examined dual expression of exhaustion markers, activation markers, and classic markers associated with HCV outcome. Representative contour plots for dual expression of reinfected subjects and chronically infected subjects are in Figure 10A-C and Figure 11A-C respectively. Dual expression of Tim-3 and PD-1 is a sign of exhaustion. We examined if HCV-specific T cells have similar levels of Tim-3 and PD-1 upon reinfection as those measured during acute infection. In time points when virus was detectable, dual expression of Tim-3 and PD-1 was seen in only one subject who cleared reinfection. However, other subjects who cleared multiple reinfections expressed one exhaustion marker. Upon reinfection, Tim-3 and PD-1 dual expression remained minimal, but most HCV-specific T cells expressed one of the two exhaustion markers. In contrast, the majority HCV-specific T cells from chronically infected subjects had dual PD-1 and Tim-3 expression during acute infection. Interestingly, the proportion of total HCV-specific T cells that have dual expression of Tim-3 and PD-1 decreased as infection becomes chronic.

Activation markers HLA-DR and CD38 on HCV-specific T cells during reinfection were elevated to levels seen in initial infection. However, these levels remained elevated
throughout the reinfection regardless of presence of virus. Additionally, dual positive HLA-DR and CD38 HCV-specific T cells were detected during chronic infection. Due to the low number of pentamer+ cells and variation of dual expression of HLA-DR and CD38, contour plots do not represent coexpression well so MFI outputs from FlowJo were used. Coexpression of CD38 and HLA-DR was elevated not only compared to chronic subjects, but this activation status remained similar between reinfections. The proportion of HCV-specific T cells that dually express CD38 and HLA-DR from the total HCV-specific T cell population in chronically infected subjects were initially elevated but decreased over the course of infection.

Elevated expression of CD127 during acute infection and decreased expression of exhaustion marker PD-1 have been correlated with clearance of HCV infection in previous studies (Golden-Mason 2006, Rutebemberwa 2008). To determine whether this was true with reinfection, we examined dual expression on HCV-specific T cells. HCV-specific T cells in reinfected subjects maintained high levels of CD127, but PD-1 decreased as duration from viral clearance increased. In comparison, HCV-specific T cells from chronically infected subjects did not dually express CD127 and PD-1 but had a greater proportion of CD127-/PD-1+ T cells throughout the infection.

Examination of T cell subsets using CCR7 and CD45RA revealed no great differences between reinfections of the same subject with representative classifications shown in Figure 13. Upon initial viremia during a reinfection, there was a spike in HCV-specific effector memory T cell population, followed by an increase in central memory T cell population and Temras. In the subject who developed chronic infection upon reinfection,
naïve T cells dominated but there was also a high percent of central memory T cells even in the presence of virus. Interestingly, as the reinfection becomes chronic, the effector memory T cell population became the least represented T-cell subset. In subjects who developed chronic infection, most HCV-specific T cells were naïve followed by either central memory T cells or effector memory T cells, and then Temra as the least represented. In contrast, HCV-specific T cells from reinfected subjects fluctuate between effector memory T cells and Temras as the least represented population.
Figure 8. Representative gating to HCV-specific CD8 T cells

Cells were first gated on lymphocytes using side scatter (SSC)-area and forward scatter (FSC)-area. Single cells were gating using FSC-height and FSC-width. Doublets have double the width value while maintaining the same height. Stricter single cells gating was used with SSC-height and SSC-width. Live cells were gated using SSC-height against the AmCyan Aquaviability Live/Dead dye. CD8+ T cells were gating based on CD4 (PE-CF594) and CD8 (Alexa Fluor 700). Pentamer+ cells were gated using Pentamer (APC) versus CD8 (Alexa Fluor 700).
Figure 9A-F. Individual MFI for each surface marker for each subject with virus levels. Each dot denotes the ratio of MFI from pentamer+ to the total CD8 T cells. The different colors of the dots indicate different HCV epitopes recognized. Any dot above the line indicates HCV-specific T cells that have higher MFI than total CD8+ T cell population. Vertical lines indicate reinfection and clearance of virus for reinfected subjects. For chronic subjects (Figure 9D and 9E), vertical lines are for reference. The shaded grey indicates viremia.

### Key for epitope dots:

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<th>Epitope sequence</th>
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</table>
Figure 9A. Reinforced cleared subject 57
Figure 9B. Reinfected cleared subject 112
Figure 9C. Reinfected cleared subject 176
Figure 9D. Chronically infected subject 30
Figure 9E. Chronically infected subject 51
Figure 9F. Chronically reinfected subject 18
Initial Infection
224/+  586/+  1037/-  1168/-

First Reinfection
21/+  38/+  112/-

Second Infection
15/+  58/-  737/-

Third Reinfection
57/-  122/-

Fourth Reinfection
20/+  90/-  176/-

Figure 10A. Representative double positive contour plots of Tim-3 versus PD-1 in subject 112 who cleared four reinfections. The top row in each set is HCV-specific T cells while the bottom row is all CD8+ T cells from the same time point. The numbers below the infection label indicate the number of days after virus detected for either the initial infection or the respective reinfection. The +/- indicates whether virus was detected at that time.
Figure 10B. Representative double positive contour plots of HLA-DR versus CD38 in subject 112 who cleared four reinfections. Each new axes label indicates a new reinfection. The top row in each set is HCV-specific T cells while the bottom row is all CD8+ T cells from the same time point. The numbers below the infection label indicate the number of days after virus detected for either the initial infection or the respective reinfection. The +/- indicates whether virus was detected at that time.
Figure 10C. Representative double positive contour plots of CD127 versus PD-1 in subject 112 who cleared four reinfections. Each new axes label indicates a new reinfection. The top row in each set is HCV-specific T cells while the bottom row is all CD8+ T cells from the same time point. The numbers below the infection label indicate the number of days after virus detected for either the initial infection or the respective reinfection. The +/- indicates whether virus was detected at that time.
Figure 11A-C. Representative double positive contour plots of Tim-3 versus PD-1 in subject 30 who is chronically infected. The top row is HCV-specific T cells while the bottom row is all CD8+ T cells from the same time point. The diagrams are oriented so that time from initial infection is increasing. The arrows indicate where along the infection each column pair is located.
Figure 11B. Representative double positive contour plots of HLA-DR versus CD38 in subject 30 who is chronically infected.
Figure 11C. Representative double positive contour plots of CD127 versus PD-1 in subject 30 who is chronically infected.
Figure 12A-C. Representative HCV-specific T cell subset population analysis for subject 176 who clears multiple reinfections. Blue lines indicate effector memory T cells (CCR7-/CD45RA-), red lines indicate Temra cells (CCR7-/CD45RA+), green lines indicate naïve T cells (CCR7+/CD45RA+), and purple lines indicate central memory T cells (CCR7+/CD45RA-). Vertical dashed lines indicate new infection.
Figure 12B. Representative HCV-specific T cell subset population analysis for subject 18 who clears initial infection but becomes chronically infected upon reinfection.
Figure 12C. Representative HCV-specific T cell subset population analysis for a chronically infected subject 30.
DISCUSSION

As examined in earlier studies, the magnitude of the T cell response increased upon reinfection. However, there was no significant difference in the breadth of the CD8 T cell response comparing primary infection and first reinfection. Interestingly, there was a significant difference in the breadth of response at the latest time point available compared to the primary infection. Even upon continued exposure resulting in infection, there is a memory component enabling rapid viral clearance characterized by increased breadth and increased magnitude of response that continues to increase upon subsequent reinfections.

Successful control of other infection is associated in some cases with recognition of specific T cell epitopes, known as immunodominance. Although new epitopes are recognized upon reinfection, with HCV, there is no immunodominance. Additionally, within subjects the dominance of epitopes recognized shifts between reinfections. This suggests that there is no specific region along the HCV polyprotein that is recognized by subjects able to clear reinfection. Conversely, there is no dominant region along the HCV polyprotein that is recognized by subjects unable to clear reinfection.

In examining individual molecule expression on HCV-specific T cells compared to total CD8+ T cells in infected subjects, there were no pronounced differences in expression between reinfections and the initial infection. Interestingly, CCR7, PD-1, and Tim-3 varied within subjects depending on the specific HCV epitope targeted. This could indicate different function upon recognition of that epitope.
HCV-specific T cells in reinfections remained activated, as demonstrated by coexpression of HLA-DR and CD38 throughout follow-up regardless of the presence of virus. In contrast, dual-expressing activated HCV-specific T cells in chronically infected subjects were present initially at high levels relative to the general CD8 T cell population, but decreased over the course of the infection. This could be due to a viral escape mutation of recognized epitopes. Unfortunately viral evolution analysis was not performed in this study at every time point across all epitopes so this remains to be determined.

Analysis of T cell subsets in this study provided curious information regarding the percent of naïve T cells. In both chronically infected subjects and reinfected subjects, the naïve T cell population usually dominated the HCV-specific T cell population. Instrument set-up, voltages, and FMO gating were thoroughly analyzed to ensure that these were not the cause of a high percentage of naïve T cell subsets. The subsets of naïve, effector memory, central memory, and Temra HCV-specific T cells varied between reinfections and presence of virus as expected. However, in chronic infection, Temras become least represented among HCV-specific CD8 T cells.

In order to comprehensively grasp the cellular immune response, future studies need to performed. First, assessing the function of HCV-specific T cells would be beneficial to determine antiviral potential. Therefore, intracellular cytokine staining of HCV-specific T cells is planned. Additionally, deep sequencing of HCV epitopes would be useful and would determine if the antigen is intact at the time points tested. A change in phenotype and magnitude may not be a change in the T cell response to that epitope but rather a
viral escape of that epitope with no T cell engagement. Finally, more subjects need to be analyzed. Although there are multiple reinfections in the BBAASH study, cellular analysis is limited. Increasing the number of reinfections will increase the power of analysis.

There are many variables in this study that could affect outcome such as sampling time, subject characteristics, genotype of infection, T cell epitopes recognized, and even duration of cell storage in the liquid nitrogen freezers. Although some variables can be controlled, such as subject study characteristics, other variables cannot be, such as time storage in the liquid nitrogen freezers. Additionally, it is important to remember that many arms of the immune system work together to fight viral pathogens. In this study, we examined only CD8 T cells, but CD4 T cells, antibodies, and the innate immune recognition could all be potential factors in clearing HCV.

CONCLUSION

Epitope recognition analysis revealed that reinfections continue to maintain a robust immune response measured by magnitude and breadth of response. Although no dominant phenotype was noted among subjects who clear multiple reinfections, HCV-specific T cells fluctuate activation and exhaustion molecule expression, indicating that the T cell response is not static, but rather evolving continuously upon reinfection. Importantly, further studies need to be performed to determine the function of these HCV-specific T cells. However, the results from this study suggest that subjects able to clear multiple reinfections have HCV-specific CD8 T cells capable of increasingly robust responses upon reinfection.
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PUBLICATIONS AND PRESENTATIONS
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