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Hepatitis C virus evasion of adaptive immune responses: a model for viral persistence

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Abstract Hepatitis C virus (HCV) infects over 170 million people worldwide and is a leading cause of cirrhosis and hepatocellular carcinoma. Approximately 80% of those acutely infected clear the infection, whereas the remaining 20% progress to chronic infection. Hepatitis C thus provides a model in which successful and unsuccessful responses can be compared to better understand the human response to viral infection. Our laboratory studies the strategies by which HCV evades the adaptive immune response. This review describes the impact of viral mutation on T cell recognition, the role of cell surface inhibitory receptors in recognition of HCV, and the development of antibodies that neutralize HCV infection. Understanding what constitutes an effective immune response in the control of HCV may enable the development of prophylactic and therapeutic vaccines for HCV and other chronic viral infections.

Keywords Hepatitis C virus · CD8 T cell · Viral immunity · Viral escape · Epitope variant

Hepatitis C cellular epidemiology

HCV infection is found in virtually every region of the world, and an estimated 170 million persons are infected with HCV worldwide [1]. In the United States, there are approximately four million persons with persistent hepatitis C infection and more than 10 thousand HCV-related deaths each year, with mortality from HCV expected to double in this decade and possibly surpass that caused by HIV [2–4]. Morbidity and mortality occur in the setting of chronic infection. Chronic HCV may cause cirrhosis and is the most common indication for liver transplantation in the United States. In addition, HCV is the major cause of hepatocellular carcinoma in the United States, Europe, Japan, Australia, and many other parts of the world [4–8]. Although HCV induces both antibody (Ab) and T cell responses,

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the virus evades them effectively in most cases, with 75% of those exposed becoming chronically infected.

Hepatitis C cellular immunology

Most viral infections induce successful immune responses and do not persist. HCV has developed mechanisms to evade immune elimination, thereby allowing it to persist in the liver in the majority of infected individuals. The immune correlates that determine whether a patient resolves infection or proceeds to chronic infection are not well defined. Previous studies have shown that spontaneous clearance of HCV infection occurs in association with a broadly specific and vigorous cellular immune response [9–12]. In contrast, chronic infection is characterized by low frequencies of specific CD8 T cells in peripheral blood [13–18]. Experiments performed with in vitro model systems have demonstrated mechanisms by which HCV subverts the innate antiviral responses needed to stimulate lymphocyte effector functions [19–21]. This may partially explain impaired generation of an effective immune response to HCV, but it is unclear how subversion of the innate immune response differs between hosts or how those differences would affect downstream adaptive immune responses.

CD4 T cell responses in humans are more frequently detected and more durable in those who control HCV infection than in those with chronic HCV infection, and CD4 T cell responses seen in those who progress to chronic infection have been associated with transient control of HCV RNA [11, 12, 22, 23]. Chimpanzee data support the importance of CD4 T cell responses in the control of infection [24]. CD8 T cells are also critical to the control of HCV, and the appearance of HCV-specific CD8 T cells in liver and blood is kinetically associated with control of viremia [25, 26]. While virus-specific CD4 and CD8 T cell responses play a role, generation of a cellular immune response does not ensure control of infection. A detectable cellular immune response is usually present in early infection regardless of outcome and that response may even persist in chronic infection [27]. It is unclear why those immune responses fail to control infection, but we and others have demonstrated that the responses generated in acute infection decline in subjects who remained persistently infected [11, 26–28]. Most subjects with detectable cellular immune responses during the acute phase of infection had gradual loss of responses, in both breadth and magnitude, during the chronic phase of infection. Despite ongoing viremia and ample evidence that HCV sequence varies during acute and chronic infection, those persistently infected did not develop new epitope specificities after the first 6 months of infection. Taken together, these results suggest that development of HCV-specific T cells is arrested during the first year of chronic infection.

Hepatitis C escape from the immune response

The decline in T cell responses to HCV is poorly understood, but escape is a likely contributing factor. Because immune responses develop over weeks and pathogen replicate on the order of hours or days, it is well recognized that immune escape mutations may blunt the effectiveness of the immune response [29]. Mathematical models of viral kinetics suggest that up to 10^{12} virions are produced each day in a chronically HCV infected human [30]. The high level of virion turnover, coupled with the absence of proofreading by the HCV RNA polymerase, results in frequent mutations within the viral genome. Mutation of class I or II

major histocompatibility complex (MHC) restricted T cell epitopes may alter the outcome of infection by preventing or delaying clearance of infected hepatocytes [31]. In the face of a vigorous multispecific cytotoxic lymphocyte (CTL) response, mutation of several epitopes, perhaps simultaneously, would be required for the survival of the virus. In the chimpanzee model, antibody-mediated CD4 T cell depletion prior to HCV infection does not prevent initial CD8 T cell responses in a previously exposed animal, but does impair viral control in association with epitope escape mutations in the viral sequence [24]. Longitudinal analysis during chronic infection demonstrated a very low rate of amino acid substitution in CTL epitopes, suggesting that CTL escape that occurs may be limited to early infection [32].

HCV sequence variability

A major challenge in the study of HCV immunology in humans is the high variability of the antigen, which varies not only from person to person, but also at any instant and over time within an infected individual. HCV exists in each infected host as a swarm of genetically related but distinct variants, collectively called a quasispecies [33–37]. This characteristically diverse set of viruses in an individual is not completely random, but rather appears to be driven by the host immune system and balanced by functional constraints [38–41]. As a result, each collection of HCV genomes in a quasispecies has a distinctive set of shared characteristics that make it distinct, allowing it to be distinguished from others [42]. The random generation of sequences results in mutations that may be deleterious, neutral, or offer a selective advantage to the virus because they increase replication efficiency or permit evasion of the immune response. Our laboratory has worked with that of Dr. Stuart Ray to discover the immunologic mechanisms underlying this evasion.

Selection of mutations within T cell epitopes with HCV sequence variation and T cell evasion

To test the hypothesis that immune escape occurs during progression to chronic infection, we compared sites of T cell epitopes with viral amino acid replacements [41]. For subjects with persistent viremia and T cell responses, amino acid substitutions were a median of 16.7-fold more likely to occur within T cell epitopes than outside epitopes (range 11.4–24.9). In general, substitutions in recognized epitopes tested resulted in decreased recognition by T lymphocytes compared to recognition of the sequence present at initial viremia, indicating escape. Therefore, virus-specific T cells select for mutants that are not recognized as well by those T cells.

Mechanisms through which mutations permit T cell evasion

Reduced recognition may result from changes in the epitope sequence itself or in flanking residues that are involved in antigen processing [43]. Evasion of the immune response via substitution within T cell epitopes occurs during HCV, HBV, SIV, and HIV infections via two known mechanisms: decreased MHC binding, and impaired antigen processing or presentation [Fig. 1; 41, 43–51]. Substitutions within T cell epitopes that do not decrease MHC binding or impair antigen processing or presentation have been observed in multiple chronic viral infections, and substitutions affecting T cell receptor (TCR) engagement have

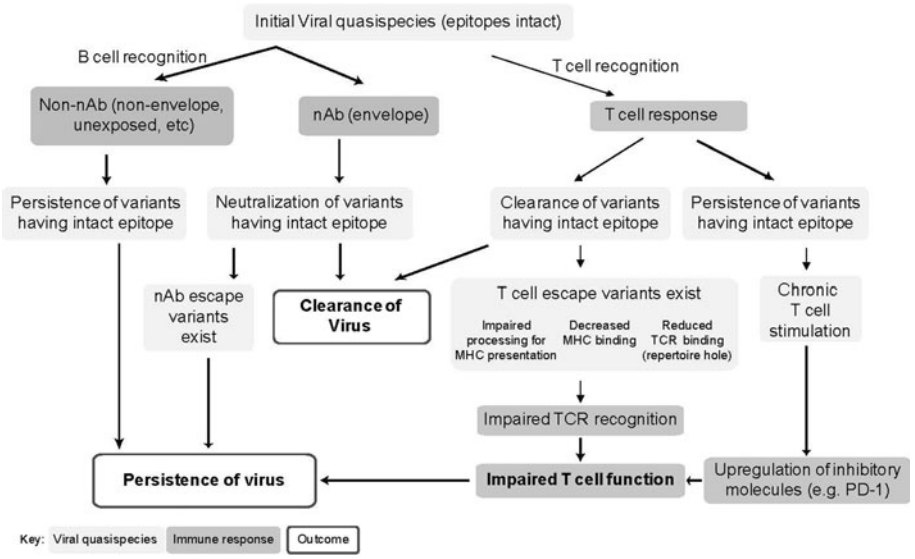

Fig. 1 Viral evasion from the adaptive immune response. The constraints placed on the virus due to immune pressure lead to clearance or select for mutations that successfully evade the immune response. nAb = neutralizing antibody. TCR = T cell receptor

been proposed to explain selection of these mutations. While impaired TCR engagement may allow immune escape, a new T cell repertoire should be available to recognize such neoepitopes. We recently identified a novel mechanism for impaired T cell recognition of a mutation involving a TCR contact residue in HCV [52].

A novel mechanism for impaired T cell recognition of a mutation involving a TCR contact residue

Substitutions in TCR contact residues would be expected to lead to only transient immune escape in immunocompetent hosts since the new epitope should be recognized by a distinct repertoire of T cells specific for the substituted epitope. In addition to identifying multiple mutations which decrease or abrogate HCV peptide binding to the human leukocyte antigen (HLA), we identified an amino acid substitution that reduces T cell recognition due to impaired engagement of the TCR with the variant peptide/A*0201 complex. The mutated peptide was not an antagonist, nor did it affect HLA-binding affinity, nor prevent antigen processing and presentation. However, it failed to expand T cells primed against the original sequence, poorly activated T cells specific for the original antigen to secrete cytokines, and weakly engaged the TCR when complexed to MHC [52]. We determined that the failure to mount a better response to the peptide observed later in infection represented poor intrinsic immunogenicity of this variant because of a 'hole in the repertoire'. This is reflected by an absence or paucity within the primary repertoire of T cells containing high-affinity TCR with specificity for that variant. There was a dramatic difference between the ability of the original and variant peptides to prime responses from the primary naïve T cell repertoires in HLA A*0201^{+} individuals never exposed to HCV. The variant virus is highly inefficient at priming naïve T cells specific for that region relative to

the virus at initial infection. Thus, in the setting of viral infection, the variant virus can exploit the relative hole in the T cell repertoire as an escape mechanism. This is the first example of a viral escape substitution, resulting in evasion of the T cell response via exploitation of a hole in the T cell repertoire.

While substitution within T cell epitopes and the resultant loss of antigen may partially explain the decline of T cell responses generated to HCV in the acute phase of infection with progression to chronic infection, T cell responses to HCV epitopes that do not undergo substitution also decline. Additional evasion mechanisms must be employed to account for the decline observed when escape does not occur and the antigen is preserved.

Mechanisms for evasion of T cell responses other than sequence variation

Detectable levels of CD8 T cell recognition do not always result in escape substitutions, and some CD8 T cell epitopes remain intact while others mutate within the same host [41, 53]. Reversion of immune escape mutations after transmission to a new host suggests that immune escape can be associated with a significant cost to the virus in terms of fitness [43]. It is therefore possible that some substitutions result in virus with such significantly reduced fitness that substitution at that position is not observed. With preservation of viral sequence, alternative mechanisms to evade T cell responses must prevail.

T cell inhibitory receptors

The growing family of inhibitory (or regulatory) receptors may be one of the most important categories of cell membrane receptors participating in the hyporesponsiveness of HCV-specific T cells. Although many of these were initially identified in CD4 cells, their expression on CD8 cells has been documented and their engagement appears to inhibit CD8 effector function. One example is programmed death-1 (PD-1). PD-1 is an ITIM-containing inhibitory receptor expressed on activated T cells that binds two known ligands: PDL-1 (also called B7-H1) and PDL-2 (also called B7-DC) [54–57.]. Anti-B7-H1/PDL-1 and anti-PD-1 antibodies partially restore activity in exhausted LCMV-specific T cells. PD-1 has recently been shown to be upregulated on HCV-specific T cells and blockade of signaling through it to enhance their function[58–60]. We found that surface PD-1 levels are significantly higher on HCV-specific T cells in individuals who fail to control infection versus T cells from those who control infection in both early (<180 days) and later (>180 days) infection. Although multivariant statistical analysis indicated PD-1 levels on HCV-specific T cells are directly correlated with HCV RNA levels, high PD-1 levels are also associated with progression to chronic HCV infection *independently of HCV RNA levels* [61]. Our comparative analyses of the outcome of infection (clearance versus persistence) and PD-1 levels during acute infection demonstrate that PD-1 expression in HCV-specific T cells varies tremendously during acute infection and suggests that it is one of the independent determinants of outcome, a conclusion that bears relevance to intervention with blocking antibodies.

Factors associated with PD-1 regulation

In our statistical analysis, we determined that HCV RNA levels and outcome of infection correlated with PD-1 levels, but not with subject gender or age [61]. Levels of PD-1

expression can also be dramatically affected by the presence versus absence of pro-inflammatory signals at the time of initial TCR engagement [62, 63]. In addition to inflammatory stimuli, the presence of antigen is necessary to maintain PD-1 expression. A recent study examining PD-1 expression on CD8 T cells specific from SIV infected macaques demonstrated that PD-1 expression gradually declined on CD8 T cells specific for an SIV-derived epitope that had undergone mutational escape [64]. We observed highly variable levels of PD-1 expression on tetramer + HCV-specific T cells during the chronic phase (>180 days infected) in patients who did not clear their infection.

Despite the association between high HCV RNA levels and high PD-1 levels, we observed T cells with low PD-1 levels in the setting of high-circulating HCV RNA levels, even at later time points once chronicity has been established. We performed serial viral sequence analysis in subjects and correlated with PD-1 levels on tetramer + HCV-specific CD8 T cells. Viral sequence was assessed at initial infection and at multiple subsequent time points at which T cells specific for known antigens were detectable. Where PD-1 levels decreased despite persistently high HCV RNA levels, we detected substitutions representing T epitope escape mutations in the circulating virus. In contrast, PD-1 levels remained high on virtually all of the T cells specific for HCV epitopes that did not undergo sequence substitution over the period of evaluation. Furthermore, restoration of intact antigen after escape was associated with an increase in PD-1 levels [61]. These results provide evidence that immune evasion mechanisms that allow HCV to persist either involve epitope escape or alternatively, signals that maintain high expression of inhibitory "checkpoint" receptors on virus-specific T cells (Fig. 1). Validation of this hypothesis requires defining the functional roles of these inhibitory receptors, which can in part be determined by assessing the effects of in vivo antibody blockade of the PD-1 and other inhibitory pathways.

Durability and protective nature of hepatitis C immunity

Although the vast majority of those infected will fail to clear hepatitis C virus, about 20–30% of individuals will clear the virus spontaneously. However, spontaneous control of HCV does not always result in sterilizing immunity. Reinfection with a new strain of HCV is possible and sometimes results in persistent infection. Reinfection provides another opportunity to query parameters of protective immunity. Sequential inoculation of convalescent chimpanzees over a period of 3 years with different HCV strains of proven infectivity resulted in viremic infection with the challenge virus [65]. Control of a second HCV infection in chimpanzees that had previously controlled their initial HCV infection was kinetically linked to rapid acquisition of virus-specific cytolytic activity by liver resident CD8 T cells and expansion of memory CD4 and CD8 T cells in blood [25]. The importance of memory CD8 T cells in control of HCV infection was confirmed by antibody-mediated depletion of this lymphocyte subset before a third infection. Virus replication was prolonged despite the presence of memory CD4 T cells primed by the two prior infections and was not terminated until HCV-specific CD8 T cells recovered in the liver. The same group also found that memory CD4 T cells are critical to long-term immunity by showing that antibody-mediated depletion of CD4 T cells before reinfection of two immune chimpanzees resulted in persistent, low-level viremia despite functional intrahepatic memory CD8 T cell responses [24]. These experiments demonstrate an essential role for T cells in long-term protection from chronic hepatitis C.

Demonstration of protective immunity following reinfection has been limited within humans due to the low incidence of reinfection and the stringent requirements for longitudinal follow up. However, a recent study suggests that reinfection with subsequent persistent viremia may not be uncommon [66]. In two epidemiological studies that controlled for infection risk factors, individuals with previous clearance of HCV infection were less likely to develop infection than those infected for the first time and may have a lower risk of acquiring HCV despite ongoing exposure [67, 68]. Another study found long periods of undetectable viremia in subjects who cleared infection even in the setting of ongoing injection drug use [6]. To examine the hypothesis that protective immunity could be achieved in humans, we identified 164 people in the ALIVE cohort [69] who had no evidence of previous HCV infection and 98 individuals who had been previously, but were not currently, infected with HCV[68]. We compared the incidence and persistence of HCV viremia in these two groups over four consecutive 6-month periods. Of participants without previous infection, the incidence of HCV infection was 21%. By contrast, people previously infected were half as likely to develop new viremia (12%), even after accounting for risk behavior (hazard ratio, 0.45; 95% CI 0.23–0.88). Furthermore, in HIV-1-negative people who developed detectable viremia, those previously infected were 12 times less likely than people infected for the first time to develop persistent infection (odds ratio 0.05, 95% CI 0.01–0.30), and the median peak HCV RNA concentration was two-logs lower. These data suggest that immunity against viral persistence can be acquired, and that vaccines should be tested to reduce the burden of HCV-related liver disease.

We have begun to investigate the cellular and humoral adaptive responses associated with protective immunity through examination of reinfected subjects. A major challenge in the study of HCV immunology in humans is the typically asymptomatic nature of acute HCV infection. We have in part overcome this barrier by longitudinally following injection drug users (IDU's) at risk for infection and with ongoing HCV exposure following control of the initial infection. Sequencing of the virus allows us to identify infection with heterologous virus. These two factors permit the identification of members of the cohort who have been reinfected and the opportunity to study the role of the immune system in long-term protection from chronic HCV. Between 1997 and 2007, we identified 113 people who seroconverted with sufficient follow up to evaluate the outcome of infection. Reinfection occurred in 50% of those who controlled their first infection with variable virologic outcomes of subsequent infections. Although viral clearance occurs in approximately 25% of patients with primary infections, spontaneous viral clearance was observed in 83% of reinfected patients. The incident reinfection rate in our group of cleared seroconverters was very similar to the incident primary infection rate previously reported in the same cohort [70], strongly suggesting that prior clearance of HCV infection does not provide sterilizing immunity against reinfection. However, the clearance rate of reinfection was almost reversed from the clearance rate of primary infection in our cohort, suggesting protection from chronic infection.

Further evidence for the existence of protective immunity against HCV infection comes from the analysis of infection kinetics during primary and reinfection. Frequent monitoring of HCV infection status allowed us to assess the kinetics of viremia during initial and subsequent infections within the same subjects. The duration of viremia during primary infection was nearly four-fold longer than in subsequent infections in reinfected individuals. Similarly, the maximum concentration of HCV RNA detected in blood during reinfection was approximately three-logs lower compared to initial infection even when persistent reinfections were included in the analysis. The lower duration and magnitude of viremia in the subsequent infections suggest that prior exposure to HCV provides

protective immunity against persistent reinfection since fixed genetic factors associated with the control of infection would not be expected to improve with each infection.

Cellular immune responses following exposure to a new virus

Because reinfection is associated with altered infection kinetics, we investigated whether reinfection altered cellular immune responses to HCV, and specifically the generation of new T cell responses not detected during the initial infection. In reinfected subjects, exposure to a new virus elicited new T cell responses in all subjects. In contrast, evolution of the same viral sequence in subjects with chronic infection resulted in new cellular responses in only one of 12 subjects tested, and new cellular responses were detected in only three out of nine subjects in whom multiple dominant viral sequences were detected during initial infection, significantly fewer than in reinfected subjects. These results suggest that exposure to a new virus is not sufficient to elicit new cellular immune responses, and that previous complete control of viremia is associated with enhanced generation of new cellular immune responses in response to a new HCV infection. However, one reinfected subject with new T cell responses developed a persistent reinfection, suggesting that development of new T cell responses does not provide absolute protection against persistence.

Humoral immune responses during reinfection

The development of new T cell responses was not associated with absolute protection from persistence and other factors must be important. Because the appearance of neutralizing antibodies (nAb) corresponds with clearance of initial infections in some individuals [71], we investigated the role of nAb in the control of reinfection. Given the absence of a culture system for circulating HCV strains, the capacity of serum antibodies to broadly neutralize HCV envelope protein binding and cell entry has been assessed using HCV pseudoparticles as previously described [71]. During acute infection, nAbs against heterologous viral pseudoparticles were detected in 60% of reinfected subjects. Those nAb drive sequence evolution in the envelope regions of the virus, allowing escape of viral variants that negate neutralization [Fig. 1; 72]. In contrast, cross-reactive nAbs are rarely detected in early infection in patients who progress to chronic infection [71]. Compared with the initial infection, HCV reinfection is associated with a reduction in the magnitude and duration of viremia, broadened cellular immune responses, and the generation of cross-reactive humoral responses. These findings are consistent with the development of adaptive immunity that does not sterilize but protects against chronic disease.

Future directions

Fully human anti-human monoclonal antibodies against PD-1 have been tested in patients with chronic HCV infection in a Phase I clinical trial that was completed in fall of 2009. As part of the immunologic monitoring of this trial, analysis of the effects on T and B cells of blockade of PD-1 in vivo is planned and should provide insight into the effects of PD-1 blockade in humans. Ongoing analysis of the immunologic parameters of HCV control in

acute infection continues in our laboratory, including in-depth analysis of the neutralizing Ab and T cells responses produced in response to repeated infection in those who control an initial infection but continue to expose themselves to HCV via high-risk behavior. Individuals who repeatedly control HCV infection may provide valuable insight into what constitutes a protective immune response to infection and a goal for parameters in successful vaccine development.

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