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T Cell Responses in Hepatitis C Virus Infection: Historical Overview and Goals for Future Research

Lauren Holz and Barbara Rehermann

Immunology Section, Liver Diseases Branch, NIDDK, National Institutes of Health, DHHS, Bethesda, MD 20892, USA

Abstract

Hepatitis C virus (HCV)-specific T cells are key factors in the outcome of acute HCV infection and in protective immunity. This review recapitulates the steps that immunologists have taken in the past 25 years to dissect the role of T cell responses in HCV infection. It describes technical as well as disease-specific challenges that were caused by the inapparent onset of acute HCV infection, the difficulty to identify subjects who spontaneously clear HCV infection, the low frequency of HCV-specific T cells in the blood of chronically infected patients, and the lack of small animal models with intact immune systems to study virus-host interaction. The review provides a historical perspective on techniques and key findings, and identifies areas for future research.

Keywords

Hepatitis C virus; T cell; infection; immunological techniques; history

Introduction

Hepatitis C virus (HCV) is well known for its propensity to establish chronic infection in about 70% of acutely infected immunocompetent adults. HCV is an enveloped virus with a relatively small plus-stranded RNA genome of about 9,000 nucleotides. Its single open reading frame encodes a polyprotein that is spliced by host and viral proteases into 3 structural and 6 nonstructural proteins (Scheel and Rice, 2013). Unlike the human immunodeficiency virus (HIV), HCV does not integrate into the host genome and unlike the hepatitis B virus (HBV), HCV does not form a viral minichromosome to establish persistence. Instead, HCV's ability to persist is based on its ability to counteract, suppress or evade immune mechanisms that would normally be expected to eliminate virus-infected cells (Park and Rehermann, 2014).

Address for correspondence: Barbara Rehermann, MD, Immunology Section, Liver Diseases Branch, NIDDK, National Institutes of Health, DHHS, Bldg. 10, Room 9B16, 10 Center Drive, Bethesda, MD, 20892, Phone: (1) 301 402 7144, Fax: (1) 402 0491, Rehermann@nih.gov.

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This process starts in a long incubation phase of about 8 weeks, during which HCV maintains high titers in the blood despite innate immune activation (reviewed by Ireton et al. in this issue (Ireton and Gale, 2014)). HCV appears to be virtually unnoticed by the adaptive immune system until HCV-specific T cell and antibody responses appear 8–12 weeks after infection (reviewed in (Reherrmann, 2013)), and they are quickly incapacitated as the infection progresses to chronicity. However, in contrast to HIV, chronicity is not universal with HCV as about 20–30% of patients with acute HCV infection are able to spontaneously eradicate the virus. Spontaneous clearance is associated with single nucleotide polymorphisms (SNPs) near the IFNL gene (Prokunina-Olsson et al., 2013; Thomas et al., 2009). Spontaneous HCV clearance occurs almost exclusively within the first year and usually within the initial 6 months of infection, emphasizing again that the virus gains a crucial advantage over the host immune response as time passes.

Over the past 25 years, research laboratories throughout the world have collaborated to characterize the protective immune response of patients who spontaneously clear the infection with the goal to use it as a template for the development of a protective vaccine. This review provides a historical overview of the technical challenges that had to be overcome and the main findings that were made. A timeline of selected milestones in immunological research, relative to other advances in the field of HCV research is shown in Table 1. From the perspective of harnessing what was learned about adaptive immune responses for HCV vaccine development it is useful to subdivide the review based on the following questions:

1. Which HCV antigens are targeted by HCV-specific CD4 and CD8 T cells?
2. What are the strength and kinetics of successful CD4 and CD8 T cell responses?
3. What are the mechanisms of T cell failure in chronic HCV infection?
4. Can protective T cell responses be induced by vaccination?

1. Which HCV antigens are targeted by HCV-specific CD4 and CD8 T cells?

HCV was identified by Choo et al. in 1989 by molecular cloning (Choo et al., 1989). Whereas HCV-specific antibodies were detected in the blood of chronically infected patients shortly thereafter using recombinant proteins generated from the HCV sequence (reviewed by Ball et al. in this issue (Ball et al., 2014)) the detection of the HCV-specific T cells took much longer.

CD8 T cells are considered the main effector cells of the adaptive immune response. They recognize short 8–10 amino acid long peptides, called epitopes, that are bound to the cell surface major histocompatibility (MHC) class I molecules on antigen-presenting cells and target cells. The identification of T cell epitopes within the HCV sequence was difficult at the time, because high-throughput techniques to map virus-specific CD8 T cell responses were not established when HCV was cloned. For example, libraries of overlapping peptides were not readily available due to the high costs of peptide synthesis, which was compounded by the fact that HCV exists in many different genotypes that differ in sequence (Smith et al., 2014). Thus, the first demonstration of HCV-specific CD8 T cells was based on antigen-nonspecific cloning of lymphocytes from liver biopsies of chronically infected patients

(Koziel et al., 1993; Koziel et al., 1995; Koziel et al., 1992) and chimpanzees (Cooper et al., 1999; Erickson et al., 2001) via stimulation with anti-CD3-specific antibodies and high concentrations of interleukin 2 (IL-2). The generated T cell clones were tested for cytotoxicity against a panel of target cells that were either infected with recombinant vaccinia viruses encoding parts of the HCV sequence or transduced with HCV expression constructs. Once the most vigorously recognized HCV sequences were identified, shorter synthetic peptides were loaded onto target cells to map the specificity of the CD8 T cell clones. Thereafter, a panel of amino- and carboxy-terminally truncated peptides was used to identify the minimal optimal CD8 T cell epitopes. Finally, the analysis concluded with assays for MHC restriction using allogeneic peptide-loaded target cells that shared individual MHC class I molecules with the patients from whom the CD8 T cell clones were derived. While this approach resulted in the successful identification of the first CD8 T cell epitopes within the HCV sequence, it required labor-intensive long-term propagation of CD8 T cells clones and introduced a selection bias for T cells that grow well in vitro. It also required liver biopsies as a lymphocyte source, because antigen-nonspecific in vitro stimulation failed to expand HCV-specific CD8 T cells from the blood due to the low precursor frequency of such cells in the systemic circulation.

Several years later, an alternate approach was developed to identify HCV-specific CD8 T cell epitopes using peripheral blood mononuclear cells (PBMCs) as starting material. The HCV sequence was scanned for the presence of human leukocyte antigen (HLA) A2.1 binding motifs, i.e. sequences of 8, 9 or 10 amino acids with a leucine, methionine, or isoleucine in the second position, and a valine, isoleucine, or leucine at the carboxy-terminus. Peptides that contained such motifs were synthesized and tested in HLA-specific binding assays against a radioactively labeled reference peptide with high binding affinity (Cerny et al., 1995). HCV peptides with high binding affinity were then used to generate short-term T cell lines from PBMC of chronically infected patients. These lines were assessed for their ability to kill patient-derived autologous Epstein Barr virus (EBV)-transformed B cell lines that were either loaded with the same peptide or infected with recombinant vaccinia virus (Cerny et al., 1995). The advantage of this technique was its high sensitivity for low frequency T cell responses, because it combined an antigen-specific in vitro expansion of low-frequency HCV-specific CD8 T-cell populations from the blood with an assessment of their effector function. Disadvantages were the pre-selection of peptides based on specific HLA-binding motifs (most epitopes were identified in HLA-A2 positive patients) and the possible loss of low-avidity T-cells during in vitro expansion.

Of note, epitope identification was not a problem for the analysis of peripheral blood CD4 T cell responses. Unlike HLA class I restricted CD8 T cell epitopes, HLA class II restricted CD4 T cell epitopes do not need to be externally loaded onto the MHC of antigen-presenting cells. Instead, antigen-presenting cells generate these peptides from recombinant proteins they take up, process and present to CD4 T cells on their HLA class II molecules. This allowed the generation of CD4 T cell lines by directly adding recombinant HCV proteins to PBMC cultures without prior knowledge of CD4 T cell epitopes (Diepolder et al., 1995).

Further developments concerned the synthesis and use of large panels of overlapping 15- or 18mer peptides spanning individual viral proteins, which allowed the analysis of CD4 and

CD8 T cell responses against the entire HCV polyprotein in a single ex vivo assay and in the context of all HLA-alleles of a given patient. Arranging pools of peptides in matrix format, i.e. with any two peptide pools sharing exactly one peptide, allowed the identification of candidate epitopes in the same assay (Fig. 1) (Day et al., 2002; Lauer et al., 2002). While this approach had many advantages, such as the use of blood as starting material and the short assay time, it missed low frequency T cell responses and T cell responses that do not cross-react with the specific HCV sequence from which the peptides are derived. While no single approach can be used to identify all T cell epitopes, many epitopes were confirmed by multiple technical approaches, and both CD4 and CD8 T cell epitopes were identified in all HCV proteins. However, there was no subset of epitopes that was preferentially recognized early in the course of an infection by those who later-on cleared HCV. Furthermore, the nonstructural HCV antigens likely contain about the same number of epitopes as the structural HCV antigens when the size of the respective protein is taken into account. Nevertheless, certain CD4 and CD8 T cell epitopes in HCV nonstructural HCV antigens such as the CD4 T cell epitope NS3₁₂₅₁ VLVLNPSVA (Diepolder et al., 1997; Schulze Zur Wiesch et al., 2012; Tabatabai et al., 1999) and the HLA-A2 restricted CD8 T cell epitope NS3₁₀₇₃ CINGVCWTV (Cucchiari et al., 2000; Koziel et al., 1995) were targeted more frequently than other epitopes. The reasons for the frequent recognition of these epitopes is not clear and likely reflects the sum of several factors such as the conservation of the epitope sequence, the abundance of the peptide class I complexes on antigen presenting cells, the T cell receptor repertoire and for CD4 T cell epitopes, the ability to promiscuously bind the several HLA class II alleles. The above mentioned CD4 T cell epitope VLVLNPSVA has for example been reported to bind to DRB1*0401, DRB1 *1104, DRB1 *1001, DRB3*0101, DR12, DR13 and DR16 (Diepolder et al., 1997).

Furthermore, certain HLA class I alleles, such as HLA-A*0301, B*2701, B*5701, and Cw*0102 and DRB1*0101 may increase the likelihood of spontaneous HCV clearance (Kim et al., 2011; Kuniholm et al., 2010; McKiernan et al., 2004). In a single-source HCV outbreak the strongest association with HCV clearance was identified for HLA-B*2701 (McKiernan et al., 2004), an allele that also been associated with beneficial effects, i.e. lower viral titers and slower disease progression in HIV infection (Trachtenberg et al., 2003). Of note, HLA-B*2701 is also associated with autoimmune diseases such as ankylosing spondylitis, which suggests that it may present self antigens and contribute to loss of tolerance against these antigens (Lopez de Castro, 2005). HLA-B*5701 is a second protective allele shared with HIV infection (Fellay et al., 2007). Several HLA-B*2701- and B*5701-restricted CD8 T cell epitopes have been identified, that are recognized by a high percentage of patients who clear the infection and select HCV escape mutants in those who do not (Kim et al., 2011; Neumann-Haefelin et al., 2006). However, the mechanisms underlying the unique association between these specific alleles and the outcome of HCV infection are still not understood.

2. What are the strength and kinetics of successful CD4 and CD8 T cell responses?

After T cell epitopes were identified, quantifying the strength of the HCV-specific T cell response became the next technical challenge. Using an in vitro expansion method, this was first performed with limiting dilution cultures to estimate the frequency of cytotoxic CD8 T

cell precursors (Cerny et al., 1995). Later on, ex vivo techniques such as Enzyme-Linked ImmunoSpot analysis (ELISpot) and tetramer assays became available and allowed the direct ex vivo quantitation of virus-specific T-cells without in vitro expansion (He et al., 1999; Scognamiglio et al., 1999). These techniques were especially suitable for the analysis of cryopreserved and re-thawed lymphocytes, which typically show a reduced proliferative response compared to freshly isolated lymphocytes. The combination of tetramer technology with staining for intracellular cytokines and multicolor flow cytometry allowed the analysis of both phenotype and function of HCV-specific T cells at the single cell level. Combined, the use of these techniques demonstrated that CD4 and CD8 T cells against HCV were present at a much lower frequency in the blood of HCV-infected patients than T cells against HIV, EBV, cytomegalovirus (CMV) and influenza virus. HCV-specific CD4 T cells were present at 10–100 fold lower frequency than HCV-specific CD8 T cells (Gruener et al., 2001), which limited direct ex vivo tetramer staining to acute HCV infection (Schulze Zur Wiesch et al., 2012) and required in vitro enrichment using tetramer-coupled magnetic beads for a more detailed analysis of more cells (Ulsenheimer et al., 2006). Furthermore, a large percentage of HCV-specific tetramer+ CD8 T cells in the circulation did not produce cytokines in acute HCV infection. This phenotype was transient with cytokine production becoming detectable when HCV titer decreased (Lechner et al., 2000). In contrast, a lack of cytokine is largely irreversible in advanced chronic HCV infection where it is associated with an “exhausted” CD8 T cell phenotype due to chronic antigen stimulation (Bensch et al., 2010).

Another challenge was evaluating the role of the T cell response for HCV clearance. Because acute HCV infection is clinically asymptomatic in most patients it took years to establish patient cohorts with a prospective immune response analysis starting early after infection. Moreover, it was difficult to obtain data from the site of infection, i.e. the liver. The biopsy fragments that remain from diagnostic biopsies in the chronic phase of infection are typically too small to provide sufficient numbers of T cells for informative assays. While the first observation of HCV-specific T cells came from CD8 T cell clones expanded from biopsies in chronic HCV infection (Koziel et al., 1993; Koziel et al., 1995; Koziel et al., 1992), only few studies analyzed their frequency and function with ex vivo techniques (He et al., 1999; Kroy et al., 2014) and none studied the acute phase of infection. In this respect, the chimpanzee model of HCV infection (Bukh, 2012), which was phased out in 2013 based on an NIH moratorium (Wadman, 2013), provided a major advantage because liver biopsies can be performed during the incubation and acute phase HCV infection (Fig. 2). In addition to several fine-needle biopsies, a larger wedge biopsy can be taken. Using this model, research on the early phase of HCV infection revealed, regardless of outcome, a strikingly long incubation phase of 2–3 months, where antigen-specific T cells are undetectable, which is followed by a relatively mild acute phase with moderate increase in liver enzyme levels. The increase in liver enzyme levels coincided with an increase in the frequency of HCV-specific CD8 T cells in the blood, and with an increase in CD8 β and IFN- γ mRNA levels in the liver (Shin et al., 2011; Thimme et al., 2002). This suggests that the recruitment of HCV-specific CD8 T cells to the liver is responsible for the liver injury. Because type I IFN induced upregulation of chemokines occurs in the incubation phase of HCV infection, the delay in the appearance of T cells in the liver appeared to be due to a defect in induction rather than

recruitment (Shin et al., 2011). A similar delay is also observed for the HCV-specific humoral immune response (reviewed by (Ball et al., 2014) et al. in this issue). This kinetic of the adaptive immune responses may provide the virus with a survival advantage because it allows ample time for the generation of a wide variety of viral quasispecies that allows rapid selection of viral escape variants once the adaptive immune response sets in.

The association of HCV-specific T cell frequency and function with divergent outcomes of HCV infection have been studied in detail over the past decade and were the topic of a recent detailed review (Park and Reherrmann, 2014). Briefly, acute HCV infections that resolve are characterized by broad CD4 and CD8 T cell responses that are sustained whereas chronic infections are associated with transient responses. At the CD4 T cell level, simple in vitro proliferation assays performed prospectively during the acute phase of infection provided the first correlate of T cell responses with outcome of infection (Diepolder et al., 1995). HCV-specific CD4 T cell responses were readily detectable by in vitro proliferation assays in patients who cleared acute HCV infection but were mostly undetectable in those with persisting infection (Diepolder et al., 1995). Interestingly, a few patients were identified who completely controlled HCV temporally in the presence of strong HCV-specific CD4 T cell proliferation but developed recurrence of HCV viremia and ultimately chronic infection when CD4 T cell responses were not maintained (Gerlach et al., 1999). Several years later, the use of HLA class II tetramers and in vitro cultures demonstrated that HCV-specific CD4 T cell responses were indeed present early in almost all subjects with acute HCV infection, but that these cells were unable to proliferate without the addition of IL-2 (Schulze Zur Wiesch et al., 2012; Semmo et al., 2005). These early CD4 T cell responses were quickly and completely deleted from the blood in patients who developed HCV persistence (Lucas et al., 2007). This failure of the CD4 T cell response during acute HCV infection may be caused by a decrease in IL-21 producing Th17 cells and an increase of Galectin-9 producing Foxp3+ regulatory CD4 T cells (Kared et al., 2013).

At the CD8 T cell level, expression of the memory precursor marker CD127 on HCV-specific tetramer+ CD8 T cells correlated with the outcome of acute HCV infection in patients (Golden-Mason et al., 2006) and chimpanzees (Shin et al., 2013). Differential expression of CD127 was observed at the first time point at which tetramer+ CD8 T cells were detected, and it not related to the presence viral escape mutants (Shin et al., 2013). The frequency of CD127+ HCV-specific CD8 T cells increased further during and after HCV clearance likely because expression of CD127, the IL-7 receptor, enables these cells to respond effectively to minute amounts of IL-7 and to proliferate as antigen-independent memory precursors. This is consistent with the finding that CD127+tetramer+ T cells mounted better TNF- α and IFN- γ responses upon stimulation with their cognate peptide than their CD127- counterparts (Shin et al., 2013). Cytokines such as TNF- α and IFN- γ play an important antiviral role in HCV infection as recently demonstrated in co-cultures of HCV-specific CD8 T cells with hepatoma cells that express a subgenomic, luciferase-tagged HCV replicon (Jo et al., 2009). In fact, cytokine secretion of HCV-specific CD8 T cells down-regulated HCV replication to a greater extent than cytotoxic effector functions in this model (Jo et al., 2009). This would be consistent with the mild and clinically inapparent nature of most acute HCV infections.

3. What are the mechanisms of T cell failure in chronic HCV infection?

Chronic HCV infection is associated with a marked downregulation of the HCV-specific T cell response that results in a relatively low level of intrahepatic inflammation and frequently requires decades to progress to advanced liver disease, fibrosis and cirrhosis. The downregulation of the effector phase of the HCV-specific T cell response is mediated by both viral and host mechanisms. Because HCV exhibits high replicative fitness paired with a high error rate of its polymerase, many dominant T cell epitopes are lost due to HCV sequence mutations. The first HCV mutations in T cell epitopes were demonstrated in HCV-infected chimpanzees in 1995 (Weiner et al., 1995). Statistical evidence for CD8+ T cell selection pressure against class I epitopes was shown shortly thereafter when several chimpanzees were studied and the ratio of non-synonymous to synonymous base substitutions was found to be higher in regions encoding T cell epitopes than in their flanking sequences (Erickson et al., 2001). Extending these observations to humans was difficult because in contrast to experimental infections of chimpanzees the sequence of the infecting virus was rarely known. Therefore, the first studies of human populations compared the sequence of the infecting virus with published prototype HCV sequences. This strategy resulted in the identification of HCV variant sequences that encoded peptides with reduced MHC affinity, had decreased T cell recognition or competed with T cell recognition of the wild-type peptide (Fig. 3) (Chang et al., 1997). These variant sequences were more frequently found in the presence of prototype-specific T cell responses than in their absence thus supporting the notion that HCV escape may have occurred. Definite proof of this hypothesis was provided in a series of later studies in which T cell responses were studied in patient populations for which the sequence of the infecting virus was known and/or HCV sequence evolution was studied over time (Cox et al., 2005; Ray et al., 2005; Seifert et al., 2004; Tester et al., 2005; Timm et al., 2004).

The selection of HCV escape mutants occurs mostly in the acute phase of infection (Callendret et al., 2011; Fernandez et al., 2004; Fuller et al., 2010) and often triggers the secondary selection of additional compensatory mutations to maintain replicative fitness (Cox et al., 2005; Dazert et al., 2009). As a result, viral sequences isolated from HLA-matched patient populations often exhibit characteristic clusters ('footprints') of mutations within T cell epitopes or their flanking amino acids (Timm et al., 2007). CD8 T cells that target the wild-type sequence of such 'escaped' regions typically display a CD127+ memory phenotype and do not express activation and exhaustion markers (Kasprowicz et al., 2010) (Rutebemberwa et al., 2008). Similar to memory CD8 T cells from patients with resolved HCV infection, these T cells respond well in in vitro assays upon stimulation with their cognate (wild-type) epitope but are ineffective in vivo because they do not recognize target cells infected with the mutant virus.

If CD8 T cells recognize epitopes that do not allow HCV escape mutations, they become functionally exhausted in persisting HCV infection. Expression of high levels of PD-1 was one of the biomarkers of these CD8 T cells (Radziewicz et al., 2007; Rutebemberwa et al., 2008). While PD-1 expression can be transient and reversible in acute HCV infection (Shin et al., 2013) (Urbani et al., 2006) it is regarded as a marker of chronic antigenic stimulation in persisting infection (Penna et al., 2007). PD-1+ HCV-specific CD8 T cells in chronic

HCV infection tend to co-express Tim-3 (Golden-Mason et al., 2009), 2B4, CD160 and other inhibitory molecules (Bengsch et al., 2010), in particular in the liver (Kroy et al., 2014). Intrahepatic T cells displayed a more exhausted phenotype than those in the blood, and co-expression of TIM-3 was identified as an important feature of exhausted intrahepatic T cells in persistent HCV infection compared with resolved HCV infection (Kroy et al., 2014). At the functional level, an exhausted phenotype is characterized by decreased proliferation (Kroy et al., 2014), reduced cytotoxicity and defective production of IFN- γ and TNF- α (Penna et al., 2007). An expansion of Foxp3+CD4+regulatory T cells (Tregs) in chronic infection additionally contributes to the lack of effector cell response (Kared et al., 2013; Sugimoto et al., 2003).

A key question is whether the exhausted CD8 T cell phenotype can be reversed. This has been addressed using multiple approaches. First, blockade of PD-1 and other inhibitory molecules has been evaluated in vitro and shown to increase the response of peripheral blood-derived HCV-specific CD8 T cells to peptide stimulation (Bengsch et al., 2010; Nakamoto et al., 2009). However, PD-1 blockade failed to restore the function of fHCV-specific CD8 T cells that were isolated from liver biopsies and expressed higher levels of PD-1 (Nakamoto et al., 2008). Subsequent studies demonstrated that the restoration of intrahepatic T cell function required simultaneous blockade of several inhibitory molecules (Kared et al., 2013; Nakamoto et al., 2009).

Second, blockade of PD-1 signaling using anti-PD-1 antibodies was tested in vivo in both chimpanzees (Fuller et al., 2013) and in patients with chronic HCV infection (Gardiner et al., 2013). In the chimpanzee study, an increase in HCV-specific CD8 T cell responses and a significant but transient reduction in HCV viremia was only observed in one of three chimpanzees. This was the animal that had the strongest and broadest CD4 and CD8 T cell response prior to development of chronic infection, which suggests that PD-1 blockade alone is not sufficient to achieve viral clearance (Fuller et al., 2013). In the patient study, a single dose of the PD-1 blocking antibody was followed by a greater than 0.5 log₁₀ IU/mL reduction in HCV RNA titer in five of 45 (11%) patients. At the highest administered dose (10 mg/kg), a >4 log₁₀ IU/mL reduction in HCV RNA titer was observed in three of 20 (15%) patients. This suppression of HCV replication persisted for more than eight weeks in most patients (Gardiner et al., 2013).

Third, the recent development of direct acting antivirals (DAAs) provided the opportunity to evaluate whether a rapid decrease in HCV titer results in a phenotypic and functional recovery of HCV-specific CD8 T cell responses. Interestingly, both the frequency of HCV-specific CD8 T cells in the blood and their proliferative in vitro response increased in patients who responded to a combination therapy with an HCV protease inhibitor and a polymerase inhibitor but not in non-responders (Martin et al., 2014). Effective suppression of HCV replication was additionally associated with a qualitative change in the composition of the HCV-specific CD8 T cell population, i.e. with an increase in the frequency of memory CD8 T cells that express CD127. Viral sequence analysis confirmed that this increase was not due to an outgrowth of CD8 T cells that targeted variant HCV epitopes in chronic infection. This qualitative change of the HCV-specific T cell population resulted in improved antiviral activity upon co-culture of HCV-specific tetramer+ CD8 T cells with

hepatoma cells that harbor the HCV replicon. Further studies need to determine whether an improved CD8 T cell function is consistently reported in all patients with a virological response and whether long-term immune memory can be established. This would be of interest not only for immune protection against HCV but also for other diseases that are associated with T cell exhaustion due to chronic antigenic stimulation.

4. Can protective T cell responses be induced by vaccination?

While HCV-specific memory T cell responses have been shown to persist for decades after clearance of acute HCV infection (Takaki et al., 2000) the chimpanzee model provided the first, and to date sole proof, that such responses protect against a secondary infection (Figure 1). As in humans multi-specific T cells remain readily detectable in this model for years after clearance of a primary infection (Shoukry et al., 2003). Challenge with either homologous (Nascimbeni et al., 2003) or heterologous strains (Lanford et al., 2004) of HCV display a typical memory response where T cell responses are rapidly induced and viremia is reduced in both magnitude and duration. The secondary immune responses were characterized by enhanced cytokine production and by a change in T cell phenotype from a central memory to effector memory (Nascimbeni et al., 2003). However, when CD8 T cells were experimentally depleted after recovery from a primary HCV infection, clearance of the secondary challenge was delayed (Shoukry et al., 2003). When CD4 T cells were depleted, the secondary challenge resulted in a chronic infection (Grakoui et al., 2003).

The chimpanzee model also demonstrated the limitations of protective immunity. First, resolution of a primary HCV infection with high-level viremia is required to build protective T-cell mediated immunity. In contrast, T cell responses that are induced by repeated low-level exposure to HCV (in the absence of quantifiable systemic viremia and seroconversion) do not confer protective immunity upon subsequent high-level HCV challenge (Park et al., 2013). Rather than mounting a rapid recall response, these T cells disappeared from the circulation after challenge with a regular dose with HCV. In parallel, an increase in the frequency of regulatory T cells was observed, which suppressed the de novo T cell response that is typically observed at 2–3 months after challenge (Park et al., 2013). Second, even memory T cell responses that are induced during a primary HCV infection and proven to be protective upon secondary HCV challenge may undergo attrition during repeated re-challenges with diverse inocula (Bukh et al., 2008), potentially due to repeated stimulation with diverse viral sequences similar to what has been described in the mouse model of LCMV infection (Selin et al., 1999). Collectively, these results demonstrate the complex nature of induction and maintenance of T cell-mediated protective immunity.

To date, the prevalence of T cell-mediated protective immunity in the human population is not known. Epidemiological data show that intravenous drug users who had cleared a previous infection were 12-times more likely to clear a second infection than those who had not cleared a previous infection. Moreover, secondary HCV infection was associated with a lower HCV titer than primary HCV infection (Mehta et al., 2002). Immunological studies performed in a small number of prospectively followed intravenous drug users demonstrated that the apparent protection against a secondary challenge was associated with a strong T cell response (Abdel-Hakeem et al., 2014; Osburn et al., 2010) that was increased in both

magnitude and breadth as compared to the response in the primary infection. Furthermore, as in the chimpanzee model, a transient change from memory to effector T cell phenotype was observed (Abdel-Hakeem et al., 2014). However, while protective immunity is possible, it does not occur in all who cleared a primary infection. Only 5 of 9 intravenous drug users who cleared a primary acute infection also cleared a secondary infection. Their immune response during the secondary infection was stronger and broader than those of the 4 intravenous drug users who developed chronic hepatitis upon secondary infection. The breadth of the responses in those with protective immunity may have prevented the selection of HCV escape mutations that were observed in those who developed chronic infection (Abdel-Hakeem et al., 2014). Because the period of viremia was short in those who exhibited protective immunity upon secondary infection the study was likely biased towards identifying patients who developed chronic infection. Further studies with closer follow up need to be conducted to estimate the true prevalence of protective immunity in frequently exposed and infected high-risk populations.

Vaccination studies have been conducted in both mice and chimpanzees, but again only the chimpanzee model allowed an assessment of the induced T cell responses in challenge experiments. In a representative study, an adenovirus prime, DNA boost regimen induced strong HCV-specific T cell responses against HCV nonstructural antigens in chimpanzees, which then displayed a significant reduced duration of viremia and lower HCV titer without ALT elevation upon HCV re-challenge (Folgori et al., 2006). Key to this protective immunity was the induction and early expansion of HCV-specific T cells with a high degree of functionality and high levels of the memory precursor marker CD127 (Park et al., 2012). This is reminiscent of the expansion of CD127⁺ HCV-specific T cells with high functionality in chimpanzees that proceed to clear acute HCV infection (Shin et al., 2013). A similar T cell-based vaccine was assessed in healthy volunteers and shown to induce multi-specific and multifunctional HCV-specific CD8 and CD4 T cell responses that were sustained for at least a year (Barnes et al., 2012). A human vaccine strategy based on chimpanzee adenoviral and MVA vectors has recently been shown to prime, boost, and sustain functional HCV-specific T cell memory (Swadling et al., 2014). Based on these data, the vaccine is now being tested in populations with high exposure risk.

Conclusion and goals for future research

Despite much progress in our understanding of T cell responses against HCV over the past decade many important research questions remain to be answered. First, HCV infection provides a unique window into the interaction between innate and adaptive immune responses. Open questions are whether there is a causal link between the two strongest predictors of the outcome of HCV infection – the presence of single nucleotide polymorphisms near type III interferon genes (reviewed by Ireton et al. in this issue (Ireton and Gale, 2014)) and the maintenance of a strong adaptive immune response. In this context it is also of interest to understand the factors responsible for the delayed induction of adaptive immune responses despite high levels of viremia and (supposedly) high level of antigen expression.

Further, the induction and fate of HCV-specific CD4 T cell responses in acute infection and the mechanisms of their impairment in chronic HCV infection are still incompletely understood. A better understanding of CD4 T cell responses, in particular in the liver, and the mechanisms for their maintenance, may be key for inducing protective CD8 T cell and antibody responses by vaccination. The development of a prophylactic vaccine that induces protective immunity is still an important research goal. Treatment with antiviral regimens will likely not be affordable in countries where the prevalence of HCV and the incidence of new infections are highest. A T-cell based vaccine would also set an important benchmark for vaccine development in other diseases, e.g. vaccination against HIV infection, malaria and cancer. Vaccine development in these diseases is likely more complex because in contrast to HCV infection there is no natural protective immunity.

Finally, the recent development of DAAs can help us to answer the question to which extent and by which mechanisms T cell exhaustion can be reverted and whether all T cell effector functions can be recovered. In particular it should be determined whether DAA-mediated HCV clearance results in the long-term expansion of HCV-specific memory T cells.

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Highlights

- Hepatitis C virus-specific T cells are key factors in the outcome of acute HCV infection and in protective immunity upon reinfection. Maintenance of a strong HCV-specific CD4 T cell response promotes HCV clearance.
- HCV-specific CD4 and CD8 T cell recognize epitopes in all HCV proteins.
- Spontaneous HCV clearance occurs in a minority of patients, and almost exclusively within the first year of infection.
- HCV's ability to persist in most patients is based on its ability to counteract, suppress or evade immune mechanisms.

Fig. 1. Identification of T cell epitopes using pools of overlapping peptides arranged in a matrix format

A. A set of overlapping peptides (typically 15mer overlapping by 10 amino acids) are synthesized based on the HCV sequence.

B. A matrix of peptide pools is generated in which two pools share one peptide each. The figure shows how 30 peptides (1–30) are used to generate 11 peptide pools (A, B, C, D, E, F, AA, BB, CC, DD and EE).

C. PBMC from an HCV-infected patient are tested against each peptide pool in standard immunological assay (typically IFN- γ ELISpot assay or intracellular cytokine staining/flow cytometry). In this case, pools B and DD yield the strongest response, suggesting that the shared peptide #20 is recognized. In subsequent assays, peptide #20 should be tested separately from the other peptides along with shorter, amino- and carboxyterminally truncated peptides to determine the minimal optimal epitope within the 15mer.

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<p>Patient Cohorts (PBMC, liver biopsy)</p> <p>Studies</p> <ul style="list-style-type: none"> • Natural history, treatment and vaccination <p>Pro</p> <ul style="list-style-type: none"> • Most relevant <p>Con</p> <ul style="list-style-type: none"> • Genetic diversity of patients and viral strains • Ethical and safety considerations	<p>In vitro Models (Primary hepatocytes, immortalized cell lines, subgenomic replicon system)</p> <p>Studies</p> <ul style="list-style-type: none"> • Studies of host and virus factors required for viral entry, replication and release; neutralizing antibodies <p>Pro</p> <ul style="list-style-type: none"> • Low cost • Allow full virus life cycle • Rapid readout <p>Con</p> <ul style="list-style-type: none"> • Limited to permissive cell lines and few HCV strains • Limited life span of primary human hepatocytes • Lack of cell polarization
<p>Chimpanzee Model (PBMC, liver biopsy)</p> <p>Studies</p> <ul style="list-style-type: none"> • Analysis of immune responses at the site of infection and in the acute phase of infection, vaccination and challenge, immunotherapy <p>Pro</p> <ul style="list-style-type: none"> • Sole animal model to study infection with an intact immune system • Infection with defined HCV inocula • Analysis of early time points after infection • Analysis of intrahepatic responses <p>Con</p> <ul style="list-style-type: none"> • Ethical considerations • High costs	<p>Mouse Models (Liver, lymphoid tissues, blood)</p> <p>Studies</p> <ul style="list-style-type: none"> • Mechanistic studies using transgenic and knockout mice • Analysis of passively transferred antibodies <p>Pro</p> <ul style="list-style-type: none"> • Availability • Low cost <p>Con</p> <ul style="list-style-type: none"> • Do not support full life cycle of HCV • Require transplantation with human hepatocytes or modified to express entry factors on mouse hepatocytes • Vaccine results cannot readily be translated to nonhuman primates and to humans

Fig. 2. Model systems to study immune responses to HCV infection

An NIH moratorium in 2013 restricted research with chimpanzees in 2013 (Wadman, 2013).

Research performed prior to 2013 is described in this figure. PBMC, peripheral blood mononuclear cells.

A *Correct peptide processing, MHC binding and TCR stimulation* **B** *Mutation (M) in epitope flanking sequence, altered processing* **C** *Mutation (M) in MHC anchor site, no MHC binding* **D** *Mutation (M) in TCR binding site* **Fig. 3.** Effect of HCV mutations on CD8 T cell responses

A. In this simplified cartoon, a target cell (hepatocyte) is shown on the left and a CD8 T cell on the right. Within the HCV infected target cells, a peptide with the sequence of a CD8 T cell epitope is generated from the endogenously translated HCV NS4A polypeptide by the antigen processing machinery (proteasome depicted here as a representative antigen processing enzyme) and loaded onto MHC class I molecules. The two anchor molecules of the peptide (e.g. amino acid in position 2 and carboxyterminal amino acid for HLA-A*0201-restricted peptides) bind correctly to the MHC molecule. The middle part of the peptide is

recognized by the T cell receptor (TCR) of the CD8 T cells, resulting in activation and in effector functions such as cytokine production and lysis of the target cell. If CD8 T cells recognize epitopes that do not allow HCV escape mutations, they become functionally exhausted in persisting HCV infection and express high levels of inhibitory molecules such as PD-1 CTLA-4, CD160,, Tim-3, are CD127-negative and lack effector function.

B. An HCV mutation associated with an amino acid change in the flanking sequence of an HCV CD8 T cell epitope results in proteasomal processing of the polypeptide sequence and cleavage within the epitope sequence. The correct epitope is not generated.

C. The HCV CD8 T cell epitope is correctly processed but a mutation results in an altered amino acid in one of the two MHC binding sites. As a result the MHC binding affinity of the peptide is reduced and the epitope is not correctly presented to CD8 T cells. CD8 T cells that had been primed prior to the emergence of HCV mutations (B, C) typically display memorylike phenotype because they do not encounter their cognate antigen anymore. They express low levels of inhibitory molecules such as PD-1 CTLA-4, CD160,, Tim-3, express high levels of CD127 and display strong recall responses when stimulated with their cognate antigen in vitro.

D. The HCV CD8 T cell epitope is correctly processed and bound to MHC class I molecules but an HCV mutation alters the amino acid sequence that is recognized by the T cell receptor. The result is reduced or absent TCR stimulation. The peptide may also compete with TCR stimulation by the wild-type peptide against which the CD8 T cells were originally primed, and thus act as an antagonist.

Table 1

Chronology of key immunological findings relative to advances in other fields of HCV research and treatment

Year	Advances	
1989	Discovery of HCV	(Choo et al., 1989)
1990		
1991	FDA approves IFN- α treatment	
1992	HCV testing added to all blood screens	
	HCV-specific CD8 T cells cloned from liver biopsies of patients with chronic HCV infection	(Koziel et al., 1992)
1995	HCV-specific CD4 T cells detected in patients with acute HCV infection who clear the infection	(Diepolder et al., 1995)
	HCV-escape in CD8 T cell epitopes identified in HCV-infected chimpanzees	(Weiner et al., 1995)
1997	HCV infectious clone generated	(Kolykhalov et al., 1997)
	FDA approves ribavirin	
1998	CD81 identified as an HCV co-receptor	(Pileri et al., 1998)
	Subgenomic HCV replicons established	(Lohmann et al., 1999)
1999	MHC class I tetramers used to identify HCV-specific CD8 T cells	(He et al., 1999)
	HCV-specific memory T cells detected decades after recovery from HCV outbreak	(Takaki et al., 2000)
2000	FDA approves treatment with pegylated IFN- α	
2001	HCV replicates in mice with transplanted human hepatocytes	(Mercer et al., 2001)
	Statistical evidence for CD8 T cell selection pressure on HCV sequences demonstrated in chimpanzees	(Erickson et al., 2001)
	MHC class II tetramers used to identify HCV-specific CD4 T cells irrespective of their function	(Day et al., 2003)
2003	SR-B1 identified as an HCV co-receptor	(Bartosch et al., 2003b)
	Proof of protective T cell memory: CD4 or CD8 T cell depletion abrogates protection in chimpanzees	(Grakoui et al., 2003; Shoukry et al., 2003)
	Neutralizing antibodies detected with infectious retroviral pseudotypes bearing HCV glycoproteins	(Bartosch et al., 2003a)
	Regulatory CD4 T cells described in chronic HCV infection	(Sugimoto et al., 2003)
2004	HCV CD8 T cell escape mutants identified in single source HCV outbreak and in prospectively studied patients	(Seifert et al., 2004; Timm et al., 2004)
	Protective memory T cell responses against heterologous HCV challenge demonstrated in chimpanzees	(Lanford et al., 2004)
	Genetics: KIR/MHC compound haplotypes affect likelihood of HCV clearance	(Khakoo et al., 2004)
	HCV JFH-1 strain propagated in hepatoma cell lines	(Wakita et al., 2005; Zhong et al., 2005)
2005		
2006	Claudin-1 identified as an HCV co-receptor	(Evans et al., 2007)
	T cell vaccine induced protective immunity against HCV in chimpanzees	(Folgori et al., 2007)
2007	Transient strain-specific antibody responses detected during recovery from single source HCV outbreak	(Pestka et al., 2007)
	Selection of HCV CD8 T cell escape mutants seen at the population level in cohorts with shared HLA alleles	(Timm et al., 2007)
	Genetics: <i>IFNL</i> SNPs associated with spontaneous and treatment-induced HCV clearance	(Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009)
2008	Occludin identified as an HCV co-receptor	

Year	Advances	
2009	In vitro restauration of exhausted HCV-specific CD8 T cells by blockade of inhibitory receptors (PD-1)	
2010	Severely impaired phenotype of intrahepatic T cells with multiple inhibitory receptors described	(Bengsch et al., 2010)
2011	FDA approves combination therapy with PegIFN- α /ribavirin and direct acting antivirals	
2012	HCV life cycle completed in genetically humanized mice	(Dorner et al., 2013)
2013	In vivo PD-1/PD-L1 blockade tested in HCV-infected chimpanzees and patients with limited decrease in viremia	(Fuller et al., 2013; Gardiner et al, 2013)
	Subinfectious HCV exposure induces Tregs and suppresses T cell responses to subsequent primary infection in chimpanzees	(Park et al., 2013)
2014	Adenoviral/MVA vaccine primes and boosts HCV-specific memory T cells in healthy uninfected subjects	(Barnes et al., 2014)

Legend:

FDA, food and drug administration; IFNL, interferon lambda; JFH-1, Japanese fulminant hepatitis strain 1; KIR, killer immunoglobulin receptor; MHC, major histocompatibility complex; SNP, single nucleotide polymorphism; SR-B1, scavenger receptor B1.

Color code:
Selected immunological findings
Virological findings
Clinical testing and treatment
Mouse model systems
Genetics