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Hepatitis C Virus. Strategies to Evade Antiviral Responses

Nandan S. Gokhale¹, Christine Vazquez¹, and Stacy M. Horner^{1,2,*}

¹Department of Molecular Genetics & Microbiology, Duke University Medical Center, Durham, NC 27710

²Department of Medicine, Duke University Medical Center, Durham, NC 27710

Summary

Hepatitis C virus (HCV) causes chronic liver disease and poses a major clinical and economic burden worldwide. HCV is an RNA virus that is sensed as non-self in the infected liver by host pattern recognition receptors, triggering downstream signaling to interferons (IFNs). The type III IFNs play an important role in immunity to HCV, and human genetic variation in their gene loci is associated with differential HCV infection outcomes. HCV evades host antiviral innate immune responses to mediate a persistent infection in the liver. This review focuses on anti-HCV innate immune sensing, innate signaling and effectors, and the processes and proteins used by HCV to evade and regulate host innate immunity.

Keywords

HCV; RIG-I; MAVS; NS3/4A; Immune Evasion; IFN; ISGs

Introduction

Hepatitis C virus (HCV) affects up to 2% of the world's population, with an estimated 130–170 million infected people worldwide, at the rate of 3–4 million new infections per year [1, 2]. HCV is a bloodborne pathogen that is primarily transmitted through exposure to contaminated blood and often through intravenous drug usage. HCV can establish a chronic infection within hepatocytes of the liver, leading to liver cirrhosis and hepatocellular carcinoma. The liver disease caused by HCV leads to over 350,000 deaths per year globally [1]. Previously, hepatitis C patients were treated with pegylated interferon (IFN)- α in conjunction with ribavirin, a therapy that produced an effective virological response against HCV genotype 1 in only about half of those treated [3]. In recent years, there have been major advances in HCV therapies, with several direct acting antivirals available on the market or finishing testing in clinical trials [4, 5]. The RNA-dependent RNA polymerase inhibitor Sofosbuvir, taken with ribavirin, has been approved for use in patients infected with HCV genotypes 2 and 3 [5]. Although this newly developed therapy is incredibly effective, it comes with a prohibitive cost of treatment, and viral resistance will likely emerge [4, 5]. Furthermore, the lack of an effective vaccine for HCV ensures hepatitis C

*Corresponding Author: Stacy M. Horner, Ph.D., Duke University Medical Center, 213 Research Dr., Box 3053 DUMC, Durham, NC 27710, Tel: 919-684-1921, Fax: 919-613-8646, stacy.horner@duke.edu.

will remain a significant global health issue. This fact is further compounded by the co-morbidity of HCV and human immunodeficiency virus, which can share a common route of transmission in intravenous drug users [6].

HCV, a member of the *Flaviviridae* family, is a single-stranded, positive-sense RNA virus. Upon infection of hepatocytes by HCV, the viral genome is released into the cytoplasm and gets translated into a single polyprotein that is processed by host and viral proteases into the structural and nonstructural proteins of the virus [7]. The viral genome is replicated through the orchestrated actions of the nonstructural HCV proteins in association with host intracellular membranes. During its life cycle, HCV is sensed by the host innate immune system by proteins called pattern recognition receptors (PRRs) that detect specific features within HCV to activate the antiviral innate immune response. A robust response by both the innate and adaptive arms of the immune system is required for effective immune clearance of HCV [8]. However, in spite of an activated immune response, HCV establishes a chronic infection in approximately 70–80% of infected patients [2]. The complex host-pathogen interactions that determine the divergent outcomes of HCV infection, as well as responses to therapy, are not yet fully understood, though it is known that human genetic variation, for instance at the locus of the genes encoding the type III IFNs, IFN- λ 3 and IFN- λ 4, is a critical factor that determines both natural and treatment-induced outcomes [9, 10]. It is therefore imperative that we gain a greater insight into the immune responses to HCV and the strategies used by the virus to evade these responses in order to identify the key features of protective immunity to HCV.

In this review, we highlight recent advances in our understanding of the innate immune response to HCV. We will focus on how infected cells detect HCV as non-self and signal to activate antiviral IFN systems. We will discuss how HCV can evade these cellular antiviral systems by modulating the function of innate immune proteins, including the signaling adaptor protein mitochondrial antiviral signaling protein (MAVS). Furthermore, we will describe recent advances in how type III IFNs are activated and regulated during HCV infection, as well as the mechanisms by which anti-viral effector proteins may restrict HCV infection. Taken together, this review will outline the major interactions at the interface of the host and HCV that likely contribute to the diverse outcomes of hepatitis C infection.

HCV is sensed as non-self by RIG-I and other PRRs

Tightly coordinated innate immune detection pathways provide the first line of host defense against HCV. Upon HCV infection, different PRRs can detect pathogen-associated molecular patterns (PAMPs) of HCV in a parallel, non-redundant manner to produce a type I and type III IFN response and activate the expression of IFN-stimulated genes (ISGs) (reviewed in [11]). These include the canonical PRRs, the RIG-I-like receptors (RLRs), the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), and other double-stranded RNA (dsRNA) sensing proteins, such as the dsRNA-activated protein kinase R (PKR). The main pathways for HCV sensing in hepatocytes are illustrated in Figure 1. The proper regulation and function of these PRRs is important for an effective intracellular innate immune response to HCV, which is required to prime the functional and robust adaptive immune response necessary for HCV clearance [8].

Both the cytosolic viral sensors retinoic acid-inducible gene-I (RIG-I) and melanoma-differentiation antigen-5 (MDA5) have now been shown to be important for the innate immune response to HCV [12, 13]. RIG-I, MDA5, and another RLR, laboratory of genetics and physiology 2 (LGP2) belong to the DExD/H-box family of RNA helicases [14]. Both RIG-I and MDA5 contain two caspase activation and recruitment domains (CARDs) at their N-terminus, a carboxy-terminal domain (CTD) that contains a repressor domain (RD), and a DExD/H helicase core separating the CARDs and CTD [14]. RIG-I binds short dsRNA containing either a 5' triphosphate motif or a 5' diphosphate motif and has sequence-specificity for RNA containing pU/UC regions, such as that found in the HCV 3' untranslated region (UTR) [15–19]. The presence of either a diphosphate or triphosphate motif at the 5' end of viral RNA enables the host to specifically recognize viral RNA from cellular RNA for initiation of immune signaling. Crystallographic studies of RIG-I have revealed the detailed mechanisms underlying RIG-I activation [20–22]. In the absence of viral ligand, RIG-I is in an auto-inhibited signaling state, with the helicase and RD in an open and flexible conformation [21, 22]. In this state, the RIG-I CARD domains are sequestered by the helicase domain, masking the ability of the helicase domain to bind RNA and holding the protein in this auto-repressed state. RIG-I signaling activation begins when the RD senses and captures the 5' triphosphate in PAMP RNA, inducing a coordinated conformational change within the helicase-RD that forms a compact, closed structure around the dsRNA, likely stabilized by ATP binding and hydrolysis [21–26]. This ordered activation and compaction of the helicase-RD of RIG-I results in exposure of the two RIG-I CARDs and the ATP-dependent formation of RIG-I filaments for interaction with downstream signaling adaptor proteins [22, 24]. Further, this RIG-I conformational change has been proposed to be enhanced by Riplet, an E3 ubiquitin ligase [27]. Riplet poly-ubiquitinates RIG-I at its RD via Lysine (K) 63-ubiquitin linkages, thereby releasing the auto-repression of the RIG-I CARDs and facilitating the interaction of RIG-I with tripartite motif-containing protein 25 (TRIM25), another E3 ubiquitin ligase that ubiquitinates the RIG-I CARDs [27]. The TRIM25-mediated K63-linked ubiquitination event on the RIG-I CARDs plays an important role in assembling the “lock-and-washer” helical tetramer of RIG-I that is required for full RIG-I activation [25]. This RIG-I tetramer conformation facilitates the interaction of the ubiquitinated RIG-I CARDs with the CARD of the innate immune adaptor protein, MAVS [25, 28]. Dephosphorylation of the RIG-I CARDs by the phosphatase PP1 is also required for downstream signaling [29].

Although RIG-I and MDA5 have similar protein domains, they detect different RNA PAMPs, with MDA5 preferentially recognizing long dsRNA or even higher order RNA structures that are likely viral RNA replication intermediates [30–32]. Upon binding to viral RNA, MDA5 forms a filament of repeating MDA5 dimers along viral RNA [33]. This, along with a dephosphorylation event at the CARD of MDA5 by the phosphatase PP1, results in the interaction of MDA5 with MAVS for downstream signaling [29]. Although MDA5 is known to be a viral RNA sensor, its exact roles in HCV sensing and PAMP recognition are still not completely understood. However, a recent study has now found that MDA5 does indeed sense HCV to activate an innate immune response that limits HCV replication, supporting earlier studies that showed an increase in HCV replication when MDA5 signaling was inhibited by the paramyxovirus V protein, and that over-expression of

MDA5 inhibits HCV replication [13, 34, 35]. Further emphasizing a role for MDA5 in the innate immune response to HCV infection is the fact that polymorphisms in the gene encoding MDA5 are associated with spontaneous resolution of infection [36]. Therefore, similar to West Nile virus, HCV is sensed by both MDA5 and RIG-I [37]. Understanding how HCV is sensed by both RLRs, how this sensing contributes to resolution of HCV infection, and how this sensing is regulated during infection are all be important questions to address in the future.

Upon activation, the RLRs must translocate from the cytoplasm to intracellular membranes to interact with MAVS, which is localized on the membranes of mitochondria and peroxisomes, as well as the mitochondrial-associated ER membranes (MAM) [38–40]. For RIG-I, this translocation is accomplished via a protein complex that contains TRIM25 and the molecular chaperone protein 14-3-3- ϵ [41]. A similar translocon-mediated complex has yet to be described for MDA5. RLR interaction with MAVS results in MAVS oligomerization and formation of a MAVS signalosome that recruits proteins to transduce signals of the innate immune response [42–45]. These signaling cascades that culminate in the transcription of IFN are driven by the concerted activity of multiple transcription factors, including interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). Activator protein 1 (AP-1), stimulated by mitogen-activated protein kinase signaling, also has an important function in the expression of IFNs [45]. MAVS can also signal to induce type III IFNs, thus highlighting the multi-faceted role of MAVS in innate immune signal transduction [46, 47].

In addition to being sensed by the RLRs, HCV infection can also be sensed by TLRs. Both of the endosome-localized proteins TLR3 and TLR7 have been proposed to coordinate protective immunity against HCV [48–50]. Furthermore, in plasmacytoid dendritic cells (pDCs), which are highly abundant in an HCV-infected liver, TLR7 can sense HCV RNA in exosomes released from infected hepatocytes to induce a type I IFN response [51, 52]. While other TLRs have been proposed to sense HCV proteins during infection, further *in vivo* studies are required to understand their relative contribution to a protective immune response to HCV (reviewed in [53]).

HCV can also be sensed by NLRs to activate inflammatory signaling [54–56]. Upon entry of HCV RNA into macrophages (which does not result in a productive infection), HCV RNA is sensed by TLR7 to activate the NLRP3 inflammasome resulting in caspase 1 cleavage and subsequent processing and secretion of the inflammatory mediators IL1 β and IL18 [54, 55]. This HCV-induced inflammatory cascade occurs in Kupffer cells, the liver-resident macrophages, and the primary immune response activated in these cells is inflammasome signaling and not IFN production. Importantly, up-regulation of these inflammasome-activated pro-inflammatory cytokines during HCV infection may contribute to the excessive liver inflammation and damage seen in HCV-infected patients [54, 55].

The interferon-induced, dsRNA-activated protein kinase PKR also senses HCV infection, especially at early times, to initiate antiviral signaling through MAVS [57]. Prior to detection by RIG-I, PKR binds to HCV RNA and signals independently of its kinase domain to activate ISG transcription and an innate immune response, which is expected to inhibit

HCV replication [57] (see later sections for more detail). HCV RNA can also activate the kinase domain of PKR, leading to PKR-dependent phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF2 α) and an inhibition of host cap-dependent mRNA translation. As HCV uses an internal ribosome entry site (IRES) that does not require eIF2 α for RNA translation initiation, its translation is insensitive to eIF2 α -phosphorylation. This allows the virus to replicate despite PKR activation; thus, in this capacity, PKR is likely not functioning in an antiviral fashion towards HCV [58, 59]. We note that PKR plays an important role in the induction of antiviral stress granules, which contain RLRs and antiviral effector proteins and have been implicated in innate immune sensing of RNA viruses [60]. Interestingly, PKR and RIG-I co-localize in stress granules, and HCV infection results in stress granule formation in hepatocytes, dependent on PKR [60–62]. However, it has yet to be determined if the mechanism used by PKR to sense HCV in stress granules activates an antiviral innate immune response or if it plays some other role in the HCV life cycle. Indeed, several stress granule proteins relocate to HCV assembly sites at lipid droplets and, in fact, function as pro-viral factors for HCV [61, 63, 64].

The HCV NS3/4A protease interferes with innate immune signaling

While HCV can be sensed by multiple PRRs of the innate immune system, the virus has evolved mechanisms to block several facets of the innate immune response. This innate immune regulation by HCV could contribute to its remarkable success as a pathogen. HCV encodes several proteins that block the innate immune response, including Core, NS3/4A, NS4B, and NS5A [53, 65]. Here, we focus on how the viral NS3/4A protease acts as a critical immune regulator during HCV infection.

NS3/4A is comprised of a viral protein complex consisting of the 54 amino acid NS4A protein and the NS3 protein, which contains an NTPase/RNA helicase domain and a serine protease domain. NS4A is the targeting subunit of the complex as it anchors the NS3 to intracellular membranes [66]. NS3/4A has been described to have multiple roles in the HCV life cycle in addition to targeting immune factors, such as viral replication, viral polyprotein processing, and virion assembly [67]. Interestingly, NS3/4A has several different subcellular locations, including at the mitochondria, peroxisomes, ER, and MAM [39, 67, 68]. It is possible that the differing actions of the viral protease in the HCV life cycle are regulated at the level of subcellular localization, although so far no regulatory mechanism for these diverse localizations has been identified. Through its protease domain, NS3/4A cleaves at several sites in the HCV polyprotein to liberate the nonstructural proteins from the polyprotein. NS3/4A is likely a key player in HCV pathogenesis as it cleaves several host proteins [27, 67, 69]. Importantly, three of these proteins (Riplet, MAVS, and TRIF) are known innate immune signaling proteins, highlighting the key role of NS3/4A as an innate immune evasion protein for HCV.

The first host protein found to be targeted by NS3/4A was MAVS, the central signaling adaptor protein in the RLR signaling pathway [70, 71]. Importantly, cleavage of MAVS by NS3/4A has been detected in the infected human liver, demonstrating the likely importance for this cleavage for HCV infection *in vivo* [72, 73]. NS3/4A cleavage of MAVS blocks innate immune signaling during infection as it releases MAVS from intracellular membranes

and the resulting cytoplasmic MAVS is unable to transduce RIG-I/MDA5 signals (reviewed in [67, 74]). While MAVS is localized to mitochondria, in more recent years, we and others have shown that MAVS is also localized on the MAM and on peroxisomes [38, 39]. NS3/4A cleavage of MAVS was originally thought to take place on mitochondria, however, immunoblot analysis of biochemically-purified subcellular fractions during HCV replication has now revealed that the MAM-localized MAVS is cleaved by NS3/4A, while surprisingly, the mitochondrial-MAVS remains uncleaved during HCV replication [39]. While it is yet unknown if NS3/4A cleaves peroxisomal-MAVS during infection, the fact that NS3/4A is localized to peroxisomes [39] suggests that it could also cleave MAVS from peroxisomes. This cleavage could abrogate peroxisomal-MAVS signaling, recently suggested to induce type III IFNs [46]. We still do not fully understand why NS3/4A does not cleave MAVS on the mitochondria or if HCV uses other mechanisms to target MAVS on mitochondria, but as MAM/mitochondrial interactions are important for regulated signaling to IFN- β [39], it could be that NS3/4A cleavage of the MAM-associated MAVS prevents these interactions to block signaling to IFN- β . In support of this idea, MAVS oligomerization, which occurs through the CARD domains, is also blocked by NS3/4A [42–44]. Therefore, NS3/4A cleavage of MAVS could also prevent interactions between the MAM-associated MAVS and the mitochondrial-associated MAVS, which might be important for regulated antiviral signaling to HCV.

Riplet, the E3 ubiquitin ligase that regulates the RIG-I signaling pathway upstream of MAVS, is also cleaved by NS3/4A during infection [27]. Riplet contains canonical NS3/4A serine protease cleavage sites, and mutation of these sites prevents cleavage by NS3/4A both in over-expression studies and also during HCV infection [27]. Importantly, NS3/4A cleavage of Riplet restricts Riplet-mediated ubiquitination of the RD of RIG-I, preventing the interaction of TRIM25 with RIG-I and subsequent downstream signaling. Indeed, knockdown of Riplet results in increased HCV replication, demonstrating that Riplet is required for the antiviral response to HCV [27]. Understanding why NS3/4A targets Riplet, when it already targets MAVS which is downstream of Riplet in the signaling cascade, and the mechanisms that regulate this differential targeting will be exciting areas of future research. We note that Riplet is also inactivated during influenza infection, highlighting its importance as a general antiviral innate immune signaling factor [75].

In addition to targeting proteins involved in the RIG-I/MAVS signaling pathway of innate immunity, NS3/4A also cleaves and inactivates TRIF, the TLR3-signaling adaptor protein. NS3/4A cleavage of TRIF blocks TLR3-dependent signaling, and this has been demonstrated both *in vitro* and during infection [50, 76]. TRIF has also been implicated as an adaptor protein for TLR3-independent signaling [77], and therefore NS3/4A cleavage of TRIF may prevent this non-canonical TLR3-independent innate immune signaling during HCV infection. It is important to note that other viruses use similar strategies to antagonize innate immune signaling; for example, hepatitis A virus also targets both MAVS and TRIF through virally encoded proteases, although unlike with HCV, infection with this virus is not chronic [78, 79].

HCV induces cellular biological changes in the infected cell that may impact IFN induction during infection. It is known that HCV infection induces a rearrangement of intracellular

membranes into a membranous web comprised of double membrane vesicles that are proposed sites of HCV replication [80]. Once these HCV replication centers are established, it is likely that they sequester any HCV RNA PAMPs from detection by the cytosolic PRRs. HCV infection also affects normal cellular mitochondrial dynamics, whereby during infection both mitochondrial fission and mitophagy are increased, resulting in decreased HCV-mediated apoptosis [81]. Mitochondrial dynamics have been previously implicated as playing a critical role in the innate immune response to RNA viruses, with mitochondrial fission suppressing the innate immune response [82]. Indeed, inhibiting HCV-induced mitochondrial fission leads to increased IFN signaling in infected cells, implicating HCV regulation of mitochondrial dynamics as being an important immune evasion strategy by the virus [81]. Further, MAVS, through association with mitochondrial fusion and fission regulators, such as mitofusin-1, could contribute to the remodeling of mitochondrial morphology during infection [82]. Therefore, cleavage of MAVS by NS3/4A may also alter mitochondrial dynamics as an additional level of innate immune regulation during HCV infection.

HCV activation of type III IFNs (IFN- λ) contributes to the antiviral response

While we know a great deal about the molecular mechanisms that contribute to the activation and regulation of type I IFNs during HCV infection, relatively little is known about how HCV activates and regulates type III IFNs during infection. The type III IFNs, also known as the IFN- λ s, are secreted antiviral cytokines that stimulate the production of ISGs, similar to type I IFNs. Like the type I IFNs, type III IFNs are directly antiviral towards HCV [83]. They comprise a family of 4 cytokines (IFN- λ 1–4) that signal through their receptors to the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, resulting in ISG transcriptional activation (reviewed in [84]). While these IFN families have many similar properties, there are a few general differences between the type I and type III IFNs. IFN- λ signals through a different receptor, comprised of IL10R2 and IFN λ R1 [85]. Further, while the distinct ISGs activated by the type I and type III IFNs do not seem to differ, type III IFN signaling drives a more sustained antiviral response than type I IFN, and in fact, type III IFN is the predominant IFN made during acute HCV infection in hepatocytes [84, 86–89].

The importance of the type III IFNs for control of HCV infection was firmly established by the pioneering genome wide association studies (GWAS) that identified single nucleotide polymorphisms (SNPs) in and around the gene encoding IFN λ -3 that predict both natural and treatment-induced clearance of HCV [9, 10, 90–92]. While initially controversial, multiple studies have now found that the favorable SNP at *IFNL3* correlates with increased IFN λ -3 expression [91–96]. Additionally, HCV-infected primary human hepatocytes with the favorable *IFNL3* SNP have an increased ISG response that limits HCV replication, supporting the idea that IFN λ -3 is a critical cytokine for control of HCV infection [97]. Previously, we have discussed how genetic variation within this region may impact HCV clearance [11]. Several candidate functional SNPs that may regulate IFN λ -3 function have been identified, including one within the 3' UTR of *IFNL3* (described in greater detail below), one within the promoter region, and one that causes a frameshift in the newly described *IFNL4* gene [98–100].

Interestingly, one of the original marker SNPs identified near *IFNL3* is actually within an intron of the gene encoding the newly discovered *IFNL4*. This marker SNP (rs12979860) identified by GWAS is in strong linkage disequilibrium with another SNP (rs368234815) that causes a frameshift in *IFNL4* (*IFNL4*-TT) preventing its expression. In this case, loss of IFN λ -4 correlates with increased natural and treatment-induced clearance of HCV [99, 101]. As both IFN- λ 3 and IFN- λ 4 signal through the same receptor, induce similar ISGs, and have similar antiviral activities [102], it is not entirely clear how both loss of IFN- λ 4 expression and increased expression of IFN- λ 3 could result in a better antiviral response to HCV. It has been hypothesized that expression of IFN- λ 4, which is not secreted, could make cells refractory to the IFN signaling required to eliminate the virus, or inhibit signaling by the other IFN- λ s through interactions with its receptor [102].

A greater understanding of the pathways that activate IFN- λ production during HCV infection is required, especially given the relevance of this IFN family for the control of HCV infection. Neither the molecular mechanisms underlying how sensing of HCV leads to production of IFN- λ , nor the most relevant or critical cell types that drive expression of IFN- λ have been fully elucidated. Likewise, the relevant PAMPs within HCV that induce expression of the type III IFNs during infection are not completely understood, although over-expression of the HCV PAMP (the poly U/UC region in the 3' UTR of HCV), does induce expression of the IFN- λ s in pDCs [103]. We now know that similar to the activation of type I IFN, the signal transduction cascade that activates type III IFNs in response to HCV RNA in hepatocytes is dependent on MAVS [47]. However, Huh7 cells, the liver hepatoma cell lines that support HCV replication in cell culture, do not make a robust type III IFN response to HCV infection [87]. Recently a new HepG2 cell line engineered to express the HCV entry factor CD81 and the liver-specific microRNA miR-122 that supports HCV replication was described [13]. In these cells, as compared to Huh7 cells, HCV replication was attenuated, and the critical difference between these cell lines was that type III IFNs were induced by HCV in the engineered HepG2 cells, which subsequently restricted HCV replication [13]. It appears that many of the cell lines that have been used primarily to study HCV replication (Huh7-based cell lines), lack some aspect of the innate immune response that signals to the type III IFNs during HCV infection, and future studies would benefit from using this engineered HepG2 line or primary human hepatocytes as model systems to study the role of type III IFNs in HCV infection.

Although there was an early activation of the IFN- λ s during HCV infection in these HepG2 cells, HCV replication actually suppressed IFN- λ induction over the long-term, and it may be possible that this also occurs in the infected hepatocyte *in vivo* [13]. We can hypothesize two possible mechanisms of how this might be occurring. First, NS3/4A itself could directly block IFN- λ induction during HCV infection through cleavage of MAVS, similar to how it blocks IFN- β induction. MAVS signaling from peroxisomes leads to induction of IFN- λ during viral infection [46], and as a portion of NS3/4A is localized to peroxisomes [39], it is likely cleaves MAVS from this subcellular localization to block MAVS-peroxisome signaling to IFN- λ during infection. A second strategy used by HCV to inhibit IFN- λ expression is through infection-induced transcriptional induction of two host myosin genes, *MYH7* and *MYH7B*, that encode intronic miRNAs called 'myomiRs' (miR-208b and

miR-499a-5p) that target the 3' UTR of *IFNL3* mRNA resulting in loss of expression of the IFN- λ 3 protein and subsequently increased HCV replication [98]. We note that in addition to these miRNAs being induced in cell lines infected with HCV, they are also induced in patients with chronic hepatitis C [98]. Importantly, there is a SNP (rs4803217) within the miRNA targeting site in the 3' UTR of *IFNL3* that is in strong linkage disequilibrium with the marker SNP identified by the original HCV GWAS (rs12979860), and the presence of the favorable SNP at this site prevents miRNA-targeting, as well as AU-rich element-mediated decay, of the *IFNL3* mRNA during HCV infection, resulting in increased levels of IFN- λ 3 [98]. The fact that HCV has multiple mechanisms to block IFN- λ induction and/or expression supports the idea that viral evasion of IFN- λ is critical for HCV persistence [86, 97].

The products of ISGs can restrict HCV infection

IFNs produced during viral infection induce the expression of hundreds of ISGs (reviewed in [104]). The proteins encoded by ISGs are thought to drive the anti-viral state, and subsets of ISGs can act together in an antiviral fashion against different classes of viruses [34, 105]. In spite of the fact that HCV utilizes several strategies to interfere with the signaling pathways that activate IFN, ISG mRNA expression is paradoxically upregulated in the livers of infected patients [106, 107]. These findings have also been reported in studies in cell culture models *in vitro*, as well as *in vivo* models of HCV infection in chimpanzees ([108, 109] and reviewed in [104]). This ISG signature indicates a predominant role for type I and type III IFNs in the antiviral response to HCV [87, 104], although type II IFN-induced ISGs have also been shown to restrict HCV *in vitro* [105]. Contrary to what one might expect, an elevated ISG signature within livers of infected patients prior to antiviral therapy correlates with reduced efficacy of treatment with ribavirin and pegylated IFN- α [106]. Specifically, patients with poor treatment outcomes display an elevated pre-treatment ISG signature within hepatocytes [93, 110, 111]. This inconsistency underlying why patients with upregulated ISGs do not respond well to IFN-based therapies might be a result of increased production of specific ISGs that negatively regulate signaling from IFN- α receptor IFNAR, such as USP18, thereby rendering cells refractory and unresponsive to the antiviral effects of IFN [112]. As opposed to non-responders, treatment responders show elevated ISGs in Kupffer cells, suggesting that crosstalk between different cell types within the liver may be important for positive therapeutic outcomes [110].

The exact cell types in the liver that produce IFNs during HCV infection, and consequently the mechanisms that result in ISG induction, are not fully understood. HCV appears to infect only a small subset of hepatocytes within the livers of chronic hepatitis C patients [113, 114]. Using a multiplexed fluorescence *in situ* hybridization in liver biopsies from chronically infected hepatitis C patients, Wieland and colleagues recently demonstrated higher transcript levels of the ISG *IFI27* within infected hepatocytes and their neighbors, with lower levels of *IFI27* transcripts in uninfected cells [107]. This spatially-constrained pattern of ISG expression may suggest that infected hepatocytes are a source of IFN during chronic infection, but it is also possible that resident immune cells such as Kupffer cells or other immune cells such as pDCs are recruited to these sites of HCV replication to secrete the IFNs that activate these ISGs [51, 115].

Of the hundreds of ISGs upregulated by IFNs, the results of several over-expression or RNA interference screens have identified a few key ISGs that play a role in restriction of HCV replication (see Table 1) [34, 105, 116, 117]. Among the ISGs with anti-HCV activity are members of the oligoadenylate synthetase (OAS)/ribonuclease L (RNaseL) pathway. OAS1 senses HCV dsRNA produced during viral replication, and this sensing stimulates the enzyme RNaseL to cleave cellular and viral mRNAs into fragments that may serve as PAMPs for RIG-I [104, 118, 119]. Interestingly, the HCV NS5A protein has been proposed to impair OAS1 function, thus counteracting the antiviral effect of this pathway [120]. At least two members of the IFN-inducible transmembrane (IFITM) family, IFITM1 and IFITM3, are antiviral towards HCV *in vitro* [105, 121–123]. IFITM1 blocks HCV entry by binding to and preventing the interaction between the HCV co-receptors CD81 and occludin at tight junctions in hepatocytes [122]. IFITM3 has been proposed to function as an anti-HCV ISG by impairing HCV IRES-mediated translation [123]. However, it could also affect the HCV life cycle by perturbing cholesterol- and lipid-related biology within the cell. In other viral infections, IFITM3 negatively regulates the interaction between the lipid and cholesterol homeostasis proteins vesicle-associated-membrane protein-A (VAP-A) and oxysterol-binding protein (OSBP) to repress viral entry by preventing endosomal fusion [124]. As both VAP-A and OSBP are known pro-viral factors for HCV, inhibition of their function by IFITM3 could similarly repress HCV entry by sequestration of HCV virions within endosomes [125, 126]. Another ISG that regulates HCV replication at the level of VAP-A is viperin, which localizes to lipid droplets and prevents VAP-A and HCV NS5A interactions required for replication [127, 128].

While the ISG PKR was originally thought to have direct antiviral effector activity towards HCV through PKR phosphorylation of eIF2 α and translational suppression of HCV RNA (reviewed in [104]), it is now clear that HCV IRES-mediated RNA translation is insensitive to PKR-mediated translation inhibition [58, 59, 129]. In fact, during HCV infection, activation of the PKR kinase domain results in a global translational suppression of ISGs, and so by preventing ISG expression, PKR could be acting as a pro-viral factor [58, 129, 130]. In spite of this apparent pro-viral role for PKR in HCV infection, both the HCV NS5A and E2 proteins do antagonize PKR function, supporting the idea that PKR does have some antiviral role during HCV infection, perhaps by preventing translational suppression of critical HCV-host factors or by inducing innate immune signaling [57, 131, 132]. Clearly, more study is required to reveal the complicated role of PKR in the HCV life cycle, and it may be that activation of PKR by different HCV PAMPs at specific times during infection could activate either a pro- or anti-HCV function of PKR. In addition to either directly blocking the function of anti-HCV ISG effectors or preventing their translation through PKR-mediated translation suppression, HCV has also been proposed to inhibit the IFN response by directly suppressing IFN signal transduction (reviewed in [133]). Thus, HCV possesses several mechanisms by which it can evade the IFN response program to establish persistent infection.

Conclusion and Future Perspective

HCV can be sensed by multiple PRRs for activation of an antiviral innate immune response. Within hepatocytes, we now know that both RIG-I and MDA5 play roles in the innate

immune detection of HCV. TLRs and NLRs also sense HCV as non-self, especially in immune cells like pDCs and Kupffer cells. The immune signaling cascades triggered by these PRRs following HCV detection activates the production of IFNs and other pro-inflammatory cytokines. While type I IFNs have been traditionally associated with antiviral responses, the more recently described type III IFNs are emerging as critical players during HCV infection, with genetic variations at the *IFNL3* and *IFNL4* loci being important determinants of natural and treatment-induced outcome in hepatitis C-infected individuals. For its part, HCV has evolved multiple strategies to evade the innate immune system, centered around the actions of the viral NS3/4A protease, which cleaves several host proteins to block antiviral signaling. Additionally, other HCV proteins may also evade innate immunity by inhibiting the function of antiviral effector ISGs that restrict HCV at different stages in its life cycle. Such antagonism of the innate immune system by HCV, both at the level of innate immune signaling and effector function of ISGs, may be one of the factors that contributes to viral persistence and chronic infection. Importantly, even a partial block in the IFN induction and response pathways by HCV during infection could give the virus enough of an advantage to evade immune signaling and establish a persistent infection.

Although extensive research over the past 25 years has illuminated many features of the complex interplay between the innate immune system and HCV, several questions remain unanswered. The importance of type III IFNs in hepatitis C has only recently been appreciated, and we anticipate that future work will focus on these critical cytokines, revealing further the mechanisms of their antiviral function and how they are targeted by HCV during infection. Additionally, the spatial and temporal dynamics of HCV infection *in vivo* require more study, as the reason why only a subset of hepatocytes are infected by HCV in patient livers is yet to be determined. It will be important to define the underlying differences between hepatocytes that make them permissive or non-permissive to infection, and the role of the timing of both antiviral signaling and viral antagonism in this permissiveness to HCV infection. While a few ISG effector proteins do have anti-HCV function, the increased expression of ISG mRNA in the livers of hepatitis C patients does not imply successful viral clearance. Although the expression of ISGs at the transcriptional level has been analyzed, sensitive measures to detect ISG protein expression in the infected liver on a per cell basis have not yet been reported, and so whether increased ISG mRNA levels translates to increased levels of ISG protein products *in vivo*, or whether this translation is suppressed during HCV infection (for example, through PKR) is still a mystery. Moreover, the pro-inflammatory cytokines generated from cross-talk between pDCs and Kupffer cells with hepatocytes in the liver and their role in fine-tuning the antiviral IFN responses, as well as their roles in priming the adaptive immune system *in vivo*, following HCV infection still needs to be explored. A better understanding of the interaction of HCV with the immune system gained through addressing such questions will be important in the development of new therapeutics or vaccines targeting the virus.

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Executive Summary

Hepatitis C virus (HCV) is sensed as non-self by RIG-I and other pattern recognition receptors (PRRs)

- HCV pathogen-associated molecular patterns (PAMPs) are sensed by RIG-I-like-receptors in infected hepatocytes to activate downstream signaling to interferon (IFN).
- Signaling molecule complex formation, such as that involving MAVS, is required for antiviral responses.
- Other PRRs, including Toll-like receptors, NOD-like receptors, and PKR can sense HCV as non-self to activate antiviral responses.

The HCV NS3/4A protease interferes with innate immune signaling

- Multiple HCV proteins can block antiviral innate immune signaling.
- The viral NS3/4A protein evades innate immune signaling through cleavage of cellular factors, including MAVS, Riplet and TRIF.
- The intracellular sites of immune evasion by HCV implicate MAVS with specific subcellular localization as important for the antiviral response.

HCV activation of the type III IFNs (IFN- λ) contributes to the antiviral response

- IFN- λ has potent antiviral activity against HCV.
- Single nucleotide polymorphisms at the IFN- λ gene locus influence variation to hepatitis C outcomes in patients.
- The mechanisms of HCV induction of IFN- λ and the relevant cell types involved are currently being investigated.
- HCV-induced microRNAs play a role in modulating expression of IFN- λ 3.

The products of IFN-stimulated genes (ISGs) can restrict HCV infection

- An IFN response is activated in the liver during HCV infection despite multiple strategies used by HCV to block PRR signaling in infected cells.
- Certain ISG effector proteins inhibit the HCV life cycle.
- HCV can counteract the antiviral function of the products of some ISGs.
- HCV may block the IFN response by directly inhibiting IFN signaling and through activation of PKR to suppress host ISG translation.

Figure 1.

Antiviral innate immune sensing of hepatitis C virus (HCV) in hepatocytes. Following entry of HCV into hepatocytes, viral PAMPs can be sensed by PRRs such as RIG-I (PAMP: poly U/UC tract in the 3' UTR of HCV RNA), TLR3 (PAMP: dsRNA in endosomes), and PKR (PAMP: dsRNA). RIG-I detection of viral PAMPs results in a protein conformational change that release its CARDs from the repressor domain (RD), aided by K63-linked ubiquitination of the RD by Riplet. K63-linked ubiquitination of the RIG-I CARD by TRIM25 enables the now-activated RIG-I to form a signaling complex to interact with MAVS on the mitochondrial-associated ER membrane (MAM) in signaling synapses between MAM, mitochondria, and peroxisomes. Innate immune signaling induction through RIG-I, TLR3, and PKR activates the production of type I and type III IFNs, other pro-inflammatory cytokines, and antiviral effector proteins through the action of transcription factors such as IRF3, AP-1, and NF- κ B. HCV proteins evade antiviral signaling, and one key examples is the viral NS3/4A protease which cleaves MAVS, Riplet, and the adaptor of TLR3 signaling, TRIF. Abbreviations: PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene-1; TLR3, Toll-like receptor 3; PKR, protein kinase R; CARD, caspase activation and recruitment domain; RD, repressor domain; TRIM25, tripartite motif-containing protein 25; MAVS, mitochondrial antiviral signaling protein; MAM, mitochondrial-associated ER membrane; IFN, interferon; IRF3, interferon regulatory factor 3; AP-1, activator protein 1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells.

Table 1

Anti-HCV interferon-stimulated genes and their proposed antiviral mechanisms

ISG	Anti-HCV mechanism	Reference
<i>ADAR1</i>	Viral RNA editing	[34, 134]
<i>BST2</i> (Tetherin)	Prevents viral release	[135]
<i>DDX58</i> (RIG-I)	PRR, activates IFN signaling pathways	[34]
<i>DDX60</i>	Enhances RIG-I signaling	[34]
<i>EIF2AK2</i> (PKR)	Inhibits host protein translation, activates IFN signaling pathways	[57, 132, 136]
<i>GBP1</i>	Unknown	[136]
<i>IFIT1</i>	Binds viral RNA and prevents translation	[121, 137]
<i>IFIT3</i>	Assists in IFN signaling pathway	[104, 105, 138]
<i>IFITM1</i>	Inhibits cellular entry of HCV	[105, 121, 122]
<i>IFITM3</i>	Inhibits translation, affects lipid homeostasis	[105, 123]
<i>IRF1</i>	IFN and ISG expression	[34, 139]
<i>IRF2</i>	IFN and ISG expression	[34]
<i>IRF7</i>	IFN and ISG expression	[34]
<i>ISG20</i>	Exonuclease	[140]
<i>MAP3K14</i>	Unknown, may be involved in NF- κ B signaling	[34]
<i>IFIH1</i> (MDA5)	PRR, activates IFN signaling pathways	[34]
<i>NOS2</i>	Unknown, reactive nitrogen species	[105]
<i>OAS1</i>	Activates RNase L	[141]
<i>RNASEL</i>	Cleaves viral genome	[118, 119, 141]
<i>RSAD2</i> (Viperin)	Competitively binds VAP-A and HCV NS5A	[105, 127, 128]
<i>TRIM14</i>	Unknown	[105]

HCV, hepatitis C virus; IFN, interferon; ISG, interferon stimulated gene; *ADAR1*, adenosine deaminase RNA-specific 1; *BST2*, bone marrow stromal cell antigen 2; *DDX58*, DEAD box polypeptide 58; RIG-I, retinoic acid-inducible gene I; PKR, dsRNA-activated protein kinase; *DDX60*, DEAD box polypeptide 60; *EIF2AK2*, eukaryotic initiation factor 2- α kinase 2; *GBP1*, guanylate binding protein 1; *IFIT1*, interferon-induced protein with tetratricopeptide repeats 1; *IFIT3*, interferon-induced protein with tetratricopeptide repeats 3; *IFITM1*, interferon induced transmembrane protein 1; *IFITM3*, interferon induced transmembrane protein 3; *IRF1*, interferon regulatory factor 1; *IRF2*, interferon regulatory factor 2; *IRF7*, interferon regulatory factor 7; *ISG20*, interferon stimulated gene 20; *MAP3K14*, mitogen-activated protein kinase kinase kinase 14; *IFIH1*, interferon induced with helicase C domain 1; *MDA5*, melanoma-differentiation antigen 5; *NOS2*, nitric oxide synthase 2, inducible; *OAS1*, 2'-5'-oligoadenylate synthetase 1; *RNASEL*, ribonuclease L; *RSAD2*, radical S-adenosyl methionine domain containing 2; *TRIM14*, tripartite motif containing 14.