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Regulation of hepatic innate immunity by hepatitis C virus

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Abstract

Hepatitis C virus (HCV) is a global public health problem involving chronic infection of the liver in over 170 million people. Chronic HCV causes liver disease and is linked with liver cancer. Viral innate immune evasion strategies and human genetic determinants underlie the transition of acute HCV infection to viral persistence and the support of chronic infection. Host genetic factors, such as sequence polymorphisms in *IFNL3*, a gene in the host interferon system, can influence both the outcome of infection and response to antiviral therapy. Recent insights into how HCV regulates innate immune signaling within the liver reveal a complex interaction of patient genetic background with viral and host factors of innate immune triggering and control that impart the outcome of HCV infection and immunity.

HCV is highly successful at establishing a chronic infection

Hepatitis C virus (HCV) is a major public health problem that infects approximately 170 million people worldwide[1]. Infection occurs within hepatocytes, the chief parenchymal cell of the liver. HCV typically causes a chronic infection, and is a major cause of liver disease, liver transplantation, and liver cancer[2]. The first line of immune defense against HCV relies on cell-intrinsic innate immunity within hepatocytes. This innate immune response serves to recognize HCV as non-self and induces local antiviral defenses in the infected cell and liver tissue which can function to recruit and modulate the actions of immune cells of the adaptive immune response. Hepatic innate immunity is therefore essential for controlling the outcome of HCV infection and immunity. However, the majority of those infected with HCV fail to mount a productive immune response that clears infection. Underscoring this problem is that HCV has evolved multiple mechanisms to regulate and evade innate immunity, thus providing a foundation for chronic infection. The standard of care therapy for HCV infection has been treatment with pegylated IFN-α plus ribavirin. As an antiviral cytokine, IFN is an innate immune therapeutic but is very unpleasant for the patient and for those infected with the difficult to treat HCV genotypes (1 and 4) it is only curative 40-50% of the time[3]. While first-generation direct acting antiviral drugs are now being introduced into the clinic, they are applied in combination with IFN to enhance efficacy and reduce chances for viral resistance breakthrough[4-5]. Moreover, underlying HCV infection outcome are polymorphisms in *IFNL3* (also referred to as *IL28B*), a human gene of the host system of innate antiviral defense, and these polymorphisms can influence both the outcome of infection and response to therapy[6-10]. Understanding how HCV regulates hepatic innate

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immune responses is essential for guiding the improvement of HCV therapy, developing effective vaccine applications, and designing new therapeutic strategies to control infection and suppress liver disease. In this perspective, we will describe how both virus and host control of innate immune factors contribute to HCV persistence in the liver and how these virus/host interactions impact the outcome of infection, immunity, and current IFN-based antiviral therapy.

The host response to HCV infection

HCV is a hepacivirus and member of the *Flaviviridae* family. The viral genome is a single copy of positive-sense RNA. After entry into hepatocytes, the virus uncoats and the viral genome is translated into a single polyprotein that is co- and post-translationally processed into structural and nonstructural proteins by a combination of host peptidases and two viral-encoded proteases. The HCV nonstructural proteins assemble as a replication complex onto modified intracellular membranes to replicate the HCV genome. This process involves the production of an antigenomic replication intermediate RNA and likely an accumulation of double stranded (ds) RNA intermediate products. The new viral genomes are packaged into viral particles by the viral structural proteins. The resulting virus is released from the hepatocyte in association with host lipoproteins, such that in the blood HCV is present as a lipoprotein-coated virus[11]. During the viral replication process HCV is sensed as non-self by pathogen recognition receptors (PRRs) in the host cell that identify and bind to pathogen associated molecular pattern (PAMP) motifs within viral products, leading to coordinated activation of the innate immune response and adaptive immune responses. Both the innate and adaptive arms of immunity, including cross-talk between liver-resident and infiltrating cells, such as hepatocytes, Kupffer cells, pDCs, natural killer (NK) cells, and other immune cells, contribute to the host's ability to resolve HCV infection[12-14]. However, despite these immune defenses HCV infection becomes chronic in about 70-80% of those who are acutely infected[2]. This outcome is due to a combination of host and viral factors that regulate the intracellular innate immune response against HCV. Importantly, inactivation of this intracellular innate immune response by HCV may also result in a non-functional adaptive immune response. Indeed, PRR signaling (specifically MAVS) is required for functional innate and adaptive responses during infection with West Nile virus, a related virus of the *Flaviviridae* family[15]. While outside of the scope of this perspective, a functional adaptive immune response is also critical to resolving HCV infection (reviewed in [14,16]).

Detection of HCV

A variety of PRRs sense viruses as foreign invaders within the host cell through specific PAMP recognition to activate innate immune signaling. The RIG-I-like receptors (RLRs), retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5), are cytosolic PRRs that sense RNA viruses. Toll-like receptors (TLRs) mediate virus sensing from within endosomal compartments to signal innate defenses, while Nod-like receptors (NLRs) serve to sense cytosolic viral products or viral metabolites to drive inflammatory responses. Activation of these PRRs drives the innate antiviral and proinflammatory responses that limit virus replication and spread while also serving to recruit adaptive immune cells and enhance their effector actions at the site of infection. A variety of other

nucleic acid binding proteins can also serve as putative PRRs where their engagement of viral nucleic acid results in their interaction with and regulation of specific PRR signaling pathways. Protein kinase R (PKR) is an example of these nontraditional PRRs, as its dsRNA binding activity promotes its interaction with mitochondrial antiviral signaling protein (MAVS) impart PRR signaling of innate immunity [17-18]

We have shown that HCV is recognized by RIG-I within hours of infection and activates downstream signaling prior to extensive viral protein synthesis[19]. RIG-I signaling during HCV infection is initiated upon its binding of PAMP RNA that includes an exposed 5' triphosphate and the 3' non-translated region of the HCV genome RNA rich in poly U/UC ribonucleotides[20-21]. This multi-component PAMP motif presents a non-self RNA signature that is detected by RIG-I to activate innate immunity. While the 5' triphosphate and poly U/UC region are at opposite ends of the viral genome, known intra-genome interactions between the 5' and 3' ends of the RNA[22] can bring both into proximity for RIG-I binding and activation by HCV replication intermediates. HCV PAMP could be presented to RIG-I either from incoming genomes after viral uncoating or during HCV genome amplification. HCV RNA binding induces a RIG-I conformational change that promotes oligomerization and translocation from the cytosol into intracellular membranes[23-25]. This process requires interactions with the chaperone protein 14-3-3ε and the E3 ubiquitin ligase TRIM25, which together with RIG-I comprise a translocon that facilitates the interaction of RIG-I with the signaling adaptor protein MAVS[24,26]. The RIG-I/MAVS interaction promotes the formation of a MAVS signalosome that propagates activation of downstream effector molecules, including the transcription factors IRF-3 and NF-κB, and a variety of proinflammatory cytokines[27] (Fig. 1).

TLR3 has also been implicated as a PRR that senses HCV, although its role in HCV detection and immunity is still not fully understood. TLR3 is an endosomal sensor of dsRNA expressed in a number of cell types within the liver, including hepatocytes and the liver resident macrophage Kupffer cells[28-29]. TLR3 signals are transmitted through the adaptor protein TRIF, which activates IRF-3 and NF-κB for the production of type I IFN, proinflammatory cytokines, and chemokines, as well as for apoptotic signaling[30-31] (Fig. 1). Whereas synthetic dsRNA ligands of TLR3 can induce IRF-3 dependent signaling in cells expressing ectopic TLR3 within 24 hours[28], HCV infection in these cells activates this signaling, including cytokine production, at later times (3-4 days) after infection[28,32]. The TLR3 ligand of HCV has recently been identified as HCV dsRNA replication intermediates that accumulate late during HCV replication and interact with the dsRNA binding domain of TLR3[32]. It is unlikely that incoming viral genomes or early replication products serve as TLR3 ligands, as TLR3-based cytokine production requires several days to be induced. Instead, the HCV ligands of TLR3 are probably present either following uptake of extracellular HCV dsRNA (either from dying cells or from the extracellular milieu) by scavenger receptors on nearby uninfected cells[33] or by accumulation of dsRNA viral replication intermediates exposed to TLR3 in either the endosome or autophagic vesicles[34]. Therefore, during HCV infection TLR3-mediated signaling could serve as a secondary innate immune detection or surveillance system for uninfected cells after initial RIG-I detection of HCV, possibly involved in setting up an antiviral state within regions of the liver or chemokine induction for recruitment of T cells to the liver during HCV infection[32].

Interestingly, the cytokine induction profile following TLR3 activation by HCV is distinct from that induced by the dsRNA mimic polyinosine-cytosine, suggesting a level of specificity attributed to the HCV/TLR3 interaction[28,32]. It is possible that the induction of the antiviral and proinflammatory responses by TLR3 and RIG-I could contribute to HCV-mediated pathogenesis, including liver inflammation and fibrosis progression during chronic infection, although the mechanisms of such immunopathogenesis are unknown. Additionally, the roles of TLR3 and RIG-I signaling in mediating HCV liver disease *in vivo* are not known.

Recent studies have now reclassified the well-known HCV antiviral protein kinase PKR as a genuine PRR for HCV that activates and contributes to innate immune signaling and IFN production[35]. PKR is a dsRNA binding protein whose kinase activity can be induced by binding to HCV dsRNA to phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α) to suppress host mRNA translation, but not HCV translation because HCV uses an internal ribosome entry site (IRES) translation mechanism that is insensitive to the levels of eIF2α phosphorylation that suppress Cap-dependent translation of host mRNAs[36-39]. It is now known that PKR binding to HCV dsRNA also activates a kinase-independent signal transduction cascade that drives induction of specific ISGs and IFN-β production by signaling through MAVS, TRAF3, IRFs, and NF-κB, all prior to RIG-I activation[17-18,35] (Fig. 1). The HCV ligand for PKR is the structured RNA at the IRES of the HCV RNA[35,39]. While the mechanisms governing PKR and RIG-I signaling cross-talk are still being defined, these studies reveal a role for PKR as a PRR that can cooperate with RIG-I to drive downstream gene expression that mediates antiviral defenses.

Innate immune effectors against HCV

Antiviral defenses are triggered by pathogen recognition and signaling that induces IFN and drives the expression of hundreds of ISGs encoding innate immune effectors that impart control of virus replication and spread. A recent study that directly compared the antiviral activity of nearly 400 ISGs has revealed that groups of ISGs or specific ISG “biosets” exhibit coordinate and synergistic virus-specific antiviral function[40]. For HCV, this bioset includes IRF family members, other signal transduction factors, and specific ISGs with potent viral regulatory activity, many with still undefined mechanisms of action (Table 1). While direct antiviral ISG action is important to control HCV replication[41], full restriction of HCV infection is mediated by additional actions of innate immune signaling amplification through IRFs and other virus-responsive pathways[42].

HCV evasion of innate antiviral immunity

Primary acute HCV infection can be cleared spontaneously when there is a high initial viremia[43], suggesting that high level and rapid PRR signaling and innate immune induction following sensing of HCV PAMPs can control acute HCV infection. However, despite the effective non-self detection of HCV by RIG-I, TLR3, and PKR to trigger innate immune programs, as many as 80% of people with acute HCV infection do not effectively control the virus and develop a chronic infection[2]. This high frequency of chronic infection reflects the fact that HCV has evolved several mechanisms to evade and suppress innate immunity,

resulting in HCV progression to chronicity (reviewed in[42]). The viral NS3/4A protease is a central component of the HCV innate immune evasion strategy. The multifunctional NS3/4A protease is required for HCV replication, during which it processes the HCV polyprotein at several sites to liberate the viral NS proteins[44]. The NS3/4A protease complex is anchored to intracellular membranes through the NS4A transmembrane domain and an amphipathic α-helix at the NS3 amino terminus that facilitates membrane association and cleavage of membrane-anchored substrates[45-46]. NS3/4A can block RIG-I signaling, because in addition to proteolytically processing the HCV polyprotein, NS3/4A targets and cleaves MAVS from intracellular membranes to prevent signal transduction[19,47-51] (Fig. 2). As MAVS must be anchored to membranes for downstream signaling, this cleavage event prevents activation of the RIG-I pathway during acute infection, abrogates IFN induction, and supports the progression to chronic infection. However, we note that other hepatotropic viruses, including hepatitis A virus (HAV) and GB virus B (GVB-B), encode proteases that also cleave MAVS[52-53]. While GVB-B can mediate a chronic infection in marmosets[54], HAV does not generally become chronic. Thus MAVS cleavage is probably necessary but not sufficient for viral chronicity.

NS3/4A cleavage of MAVS was previously thought to occur at the mitochondrial outer membrane, a primary site of MAVS localization[19,51,55-56]. However, we now know that the MAVS transmembrane domain also anchors the protein at diverse sites within an intracellular membrane network at peroxisomes and on mitochondrial-associated membranes (MAM), an ER membrane subdomain residing at junctions between the ER and mitochondria[57-58]. The NS3/4A protease complex localizes to all of these membranes during HCV infection[58]. However, rather than cleaving MAVS from the outer mitochondrial membrane, NS3/4A targets and cleaves the MAM-localized MAVS. This process abrogates RIG-I signaling despite a level of intact MAVS remaining on the mitochondria[58]. Therefore, during HCV infection, the MAM-resident MAVS likely transduces RIG-I pathway signaling. Cleavage of MAVS during HCV infection has also been shown *in vivo* in the liver of patients with chronic HCV infection[19,59]. Importantly, patients with cleaved MAVS exhibited lower levels of IFN pathway activation[59]. Thus, MAVS cleavage by the HCV NS3/4A protease disrupts RIG-I signaling of innate antiviral immunity and attenuates IFN production.

The HCV NS3/4A protease also proteolytically targets TRIF[60], the TLR3 signaling-adaptor protein. Cell culture studies have shown that TRIF is cleaved by NS3/4A *in vitro*[60]. While specific TRIF proteolytic fragments have not been detected during HCV infection, the relative abundance of TRIF protein is decreased, likely due to destabilization and degradation following cleavage[28]. NS3/4A cleavage of TRIF suggests that control of TLR3 signaling must be important for successful HCV infection, perhaps by preventing excess inflammation that could impart viral suppression or by blocking chemokine induction required for vigorous cytotoxic T cell (CTL) responses known to be important for HCV clearance[16]. TLR3-independent RNA sensing mechanisms that signal through TRIF to impart innate immunity and inflammatory responses have also been described[61], and NS3/4A targeting of TRIF may also contribute to HCV persistence by blocking their actions during infection.

HCV regulates PKR activity during viral infection; however this regulation is complex and need to be redefined in the context of PKR having seemingly opposing pro- and anti-HCV roles during infection[35-37]. PKR-mediated translational suppression of host mRNAs during HCV infection and IFN therapy can inhibit translation of host factors important for HCV replication and cellular growth (PKR functions as an antiviral molecule) but it also can inhibit translation of ISGs and IFN (PKR functions as a proviral molecule)[36-37]. Furthermore, PKR-kinase independent signaling during HCV infection to activate specific ISGs and IFN-β would be considered to be antiviral. We know that HCV has several PKR-inactivation strategies, both at the level of PKR/PRR signaling (NS5A/E2 direct actions on PKR; NS3/4A cleavage of MAVS) and at the level of PKR-regulated translational inhibition (NS5A/E2[62-64]) that likely contribute to viral persistence and/or IFN therapy responses, but the exact mechanisms, timing, and how they would support this viral persistence are still unclear (Fig. 2). It is possible that NS5A and E2 inactivation of PKR-translational suppression could function only at specific times during infection (for example, after NS3/4A suppression of MAVS-dependent signaling) to ensure the requisite synthesis of host factors required to maintain cellular growth, while supporting an environment for persistent HCV replication.

Innate immune regulation in the HCV patient might determine the response to IFN therapy

IFN-based therapies are the standard course of therapy for HCV. IFN is an antiviral cytokine that induces the expression of hundreds of ISGs, many of which have antiviral or immunomodulatory activities that can limit virus replication and spread, as well as prime the adaptive immune response to HCV infection[65]. In fact, in acutely-infected patients, IFN-therapy is extremely successful at preventing chronic infection[66]. However, in chronically infected patients, the response to IFN-based therapies is variable, and many patients maintain high levels of HCV viremia in spite of IFN treatment. We propose that the low effectiveness of IFN therapy in those chronically infected with HCV is attributed to a combination of viral and host factors that contribute to regulation of IFN action and treatment-induced clearance (Fig. 3). The viral factor that most highly predicts IFN treatment response is the viral genotype. There are 6 major HCV genotypes defined by their sequence conservation and variation that further divides them as subtypes and viral quasispecies[67]. Interestingly patients infected with different HCV genotypes have differential therapy responses. Patients infected with HCV genotypes 2 and 3 exhibit the highest response rates to therapy, with 70-80% of these patients achieving a sustained virologic response (SVR) while only 45-60% of patients with HCV genotype 1 or 4 infection achieve SVR (summarized in[68]). Indeed, HCV genotypes 1 and 4 induce high levels of hepatic ISG expression in the infected patient liver before therapy resulting in an IFN-insensitive phenotype that attenuates treatment responses[69-71].

What explains the viral genotypic differences in therapy responses? Further, why does HCV persist in the liver in spite of high levels of IFN signaling and elevated hepatic ISGs, many with known antiviral actions? Within the HCV-infected hepatocyte, HCV proteins, including Core, could be antagonizing IFN signaling through Jak-STAT pathway

inactivation (reviewed in[42]) or suppression of specific ISGs that could otherwise limit HCV infection (Fig. 3). Additionally, there are known genotypic differences in HCV-mediated immune regulation that likely contribute to the observed differences in therapy responses. For example, NS5A and E2 from different genotypes differentially bind and regulate PKR, with the IFN-resistant viral genotypes being better able to block PKR function[62-64]. Importantly, the viral sequences encoding E2 (PePHD/PKR inhibitory domain) and NS5A (PKR binding domain) show the most evolution between HCV genotypes, supporting the idea that E2 and NS5A are potential determinants of genotype-specific clinical outcome[68]. In addition, differential levels of MAVS cleavage among HCV genotypes were found in the livers of HCV patients in which those with fully cleaved MAVS (genotypes 2 and 3) exhibited lower levels of pre-therapy ISG expression[59]. Interestingly, higher pre-therapy genetic variability of HCV within the major genotypes, especially within the sequences of Core, NS3, and NS5A, correlates with a more successful therapy outcome[72]. Taken together, this data suggests that alterations in the sequences of these viral regulatory factors lead to increased immune regulation during HCV infection. This increased immune regulation, while beneficial to the virus, would be predicted to prevent pre-therapy induction of ISGs and thereby make this virus more susceptible to HCV therapy.

While IFN therapy induces many anti-HCV ISGs (see Table 1)[69], it is unlikely that any single ISG or its regulation by HCV completely governs the outcome of IFN therapy during HCV infection[73]. Further within the liver, there is no single ISG among all of those expressed prior to therapy that is associated with poor response to treatment but it is rather the overall composition of induced ISGs that associates with poor therapy responses[69,74-75]. Indeed, it is likely a combination of factors mediating control of innate immune and IFN signaling, along with viral and host genetic variability (to be discussed below), that all contribute to the differential responses to IFN-based therapy in HCV patients.

Host genetic components that determine the outcome of HCV infection and response to therapy

While viral genotype differences influence clinical outcome to IFN-based therapies, host genetic differences also play a role in this outcome. Genome-wide association studies (GWAS) have identified single-nucleotide polymorphisms (SNPs) upstream of the *IFNL3* locus that can predict both successful clinical outcome to HCV therapy[6-7,9-10] and spontaneous HCV clearance[8,10]. These SNPs associate with altered mRNA expression of *IFNL3*, which encodes the antiviral cytokine IFNL3, suggesting that IFNL3 expression levels are likely associated with HCV clearance and response to therapy[6-7,70]. We note that while several early studies failed to find altered mRNA expression of *IFNL3* associated with these SNPs[74,76], as the specificity of real-time PCR primers for *IFNL3* (that differentiate between *IFNL3* and the closely related *IFNL2*) has increased, it is now clear that these identified SNPs in the *IFNL3* locus do impact the expression of IFNL3 within the liver, peripheral blood mononuclear cells, and whole blood[6-7,70,77-78], with the minor/unfavorable allele/haplotype resulting in less in IFNL3 expression.

Detailed analyses of the SNPs within the haplotype/genomic block at the *IFNL3* gene locus have revealed candidate functional SNPs involved in both HCV natural and treatment-based

clearance (Fig. 4)[79-81]. However, studies of the function of these genetic elements have not yet defined the single causal variants and how these variations regulate *IFNL3* mRNA expression. The locations of the current candidate casual SNPs within the *IFNL3* gene suggest possible regulatory mechanisms (see Fig. 4). As the SNPs in *IFNL3* do appear to impact IFNL3 expression[6-7,70,77-78,82], it is unlikely that these SNPs are simply in linkage disequilibrium with some other gene involved in HCV pathogenesis. Defining how *IFNL3* polymorphisms enhance HCV therapy response rates will be important for the development of new therapeutic strategies to suppress HCV through treatment with recombinant IFN-λ and to better understand the role of IFNL3 in the host response to HCV infection. A recent study showed that patients with the unfavorable *IFNL3* genotype have depressed innate immune function, particularly with respect to NK cells[83], suggesting the decreased expression of IFNL3 affects immunity and therefore clearance of HCV.

Additionally, a dinucleotide polymorphism that can create or disrupt an open reading frame for a new gene, *IFNL4*, has been identified[84-85] (Fig. 4). In this case, it has been proposed that loss of expression of *IFNL4* is protective for HCV. Based on the current data, it is still not clear what the role, if any, is for *IFNL4* in the differential responses to HCV therapy, but it is clear that the IFN-λ locus is important for immune control of HCV infection.

The type III IFNs (IFN-λ) now include IFNL3 and the closely related cytokines IFNL1, IFNL2, as well as IFNL4. As IFNL4 only shares 29% homology with the other IFN-λ genes and functions in a slightly different manner[84], we will limit our discussion here to IFNL1-3. These cytokines have antiviral and immunomodulatory activity, similar to the type I IFNs. IFN-λ signals through a receptor consisting of the IL10R2 and IFNLR1 subunits that converges on the Jak/STAT pathway, induces similar ISG profiles as type I IFN, and can inhibit HCV replication *in vitro* (reviewed in[86]). However, the cellular producers and receptor proteins for type III IFN have a more limited distribution than type I IFN[87], suggesting a more localized role for type III IFN in immunity. Interestingly, the kinetics of ISG induction varies between these cytokines, with IFN-λ inducing a more sustained expression of ISGs than IFN-α[88]. Further, unlike IFN-α IFN-λ signaling does not become refractory to repeated applications[89], and it can function cooperatively with type I IFN to impart enhanced ISG induction and antiviral activity[90]. Therefore, we could speculate that in the innate immune response to HCV, IFN-λ provides a localized, sustained activation of ISGs in specific cell types in the infected liver, and primes an effective immune response, both at the innate and adaptive levels, for HCV clearance. In fact, IFN-λ has emerged as new candidate for HCV therapy with lower side effects than IFN-α treatment[86], likely due to its restricted receptor distribution. It is still not clear why the genetic polymorphisms associated with natural and therapy-induced control of HCV are found specifically in *IFNL3* (and now *IFNL4*), and not within the *IFNL1* or *IFNL2* loci, and why *IFNL3* specifically would play such a key role in the host response to HCV. More studies aimed at understanding how these very similar genes are activated and regulated during HCV infection, as well as determining both the cell types of production and the mechanism of induction of the IFN-λ cytokines during HCV infection are needed. In particular, new studies need to continue to carefully discriminate between the virtually indistinguishable *IFNL2* and *IFNL3* genes, both at the protein and mRNA levels. Moreover, there is a need to actually demonstrate the expression

IFNL4 (both protein and mRNA levels) and its regulation in patients, as well to understand the relationship of *IFNL4* on impacting levels of *IFNL3*. Only then will we have a foundation of understanding of the role of the IFN-λ cytokines in HCV immune control and response to therapy in the liver.

The polymorphisms in *IFNL3* associated with response to treatment are not wholly predictive of HCV therapy and infection outcome, as between 20-40% of those patients with the favorable *IFNL3* genotype do not respond to current IFN-based therapy[6,9-10]. Therefore, other factors must contribute to a successful outcome of therapy. In fact, the strongest predictor of response to therapy is dictated by the pretreatment expression pattern of ISG mRNA within the liver[69-70,91]. Patients with pre-therapy induction of ISG mRNA in hepatocytes achieve the lowest therapy response rates. While we do not know why this correlates to lower therapy response rates, it is possible that the pre-therapy induction of ISGs prevents further induction of ISG levels by IFN-α during therapy due to activation of negative regulators leading to refractoriness to signaling[89], or that PKR translational suppression of ISGs prevents their effector function, precluding effective therapy responses[37,89](see Fig. 2). Interestingly, in Kupffer cells, the resident liver macrophages, the pre-therapy induction level of ISGs is also a strong predictor of therapy response[71]. In Kupffer cells, the phenotype is opposite of that seen in hepatocytes, with virtually all non-responders lacking pre-therapy induction of ISGs, while responders have strongly-induced ISGs[91]. This suggests that during HCV infection, Kupffer cells (activated to induce ISGs, either by cytokine signaling or through phagocytosis and sensing of viral products[75,92-93]; Fig. 5) may play a key protective role for the host in both therapy responses and HCV clearance. Understanding how macrophages are activated and interact with hepatocytes for immune control during HCV infection and treatment will play a key role in determining the mechanisms driving this phenotype, as well as HCV pathogenesis.

Why do some patients have pre-therapy induction of ISGs in hepatocytes while others do not? It is known that host genetic differences, as well as HCV-induced ISG expression profiles in liver cell subtypes can predict therapy responses. However, they are often independent predictors of therapy responses meaning that the patient *IFNL3* genotype cannot definitively predict the hepatic ISG activation state[70,91,94]. Thus, the ISG expression profile of hepatocytes and macrophages is still the single best predictor of therapy response, where virtually all patients that lack pre-induced hepatic ISGs respond to therapy while those who lack pre-induced macrophage ISGs fail therapy[91]. Therefore, the host genotype at *IFNL3* or other host loci and viral genotype likely predispose the HCV infected patient to a particular therapy response outcome but it is a combination of factors, including cross-talk between liver cell subtypes and environmental factors (such as age, race, sex), which ultimately dictates the response to therapy (Fig. 3B).

Endogenous hepatic IFN regulates innate immunity to HCV

The hepatic pre-therapy ISG mRNA expression profiles of some patients with chronic HCV infection indicates that IFN (either Type I or Type III) is being produced endogenously during infection. The cell type of Type I or Type III IFN production could be the infected cell as well as bystander cells responding to virus exposure[33,75,95]. Importantly, the fact that

patients with the favorable *IFNL3* SNPs generally have higher levels of *IFNL3* and lower pre-therapy hepatic ISG levels, but higher macrophage ISG levels[91], suggest that other cell types within the liver besides the infected hepatocytes could be producing the IFN. Further, production of Type I and/or Type III IFN or response to the IFN at the level of the receptor or downstream signaling in the infected hepatocyte is likely regulated by HCV infection itself. We know this is the case for Type 1 IFN, and it is quite possible that this is also true for IFNL3 (see Figs. 2 and 3). Here, we propose a number of virus/host interactions in the context of the liver that could drive the hepatic production of IFN and proinflammatory cytokines (Fig. 5). Before viral proteins accumulate during infection, HCV activation of RIG-I and/or PKR actions in hepatocytes can stimulate IRF-3 activation, leading to an early, albeit transient, induction of IFN-β and ISG expression[35-36,96]. HCV can also induce type III IFN in primary liver cultures[97-98]. Resident or infiltrating liver myeloid cells, including plasmacytoid dendritic cells (pDC) and Kupffer cells, could produce type I or III IFN after stimulation by viral products from neighboring infected hepatocytes. In particular, exosomal transfer of HCV RNA from hepatocytes to neighboring pDCs can stimulate high level type I and type III IFN production through TLR7, as well as through RIG-I signaling[95,99], while Kupffer cells may be able to phagocytose HCV[92] to stimulate local type I and III IFN production. While this local production of IFN could serve an obvious antiviral function for suppression of acute HCV infection, the chronic production of IFN (particularly IFN-α) also regulates ISG expression and antiviral function that limits the efficacy of both the local hepatic IFN and IFN therapy[69,89]. During HCV infection, cytokines produced by pDCs, Kupffer cells, and infected hepatocytes within the liver could also impact the recruitment of immune cells to the liver, including myeloid cells, NK cells, and T cells. HCV regulation of this cytokine induction could play a key regulatory role in dictating activation of these immune cells for an effective adaptive immune response to HCV, resulting in changes in HCV-induced pathogenesis and varying outcomes of IFN therapy[14]. This cross talk between liver cell subtypes would be expected to be influenced by the patient *IFNL3* genotype, with those with the favorable genotype having increased immune cell function and better viral clearance, as has been suggested[83]. Understanding the cross talk between liver cell subtypes is now key to defining the complex nature of both type I and type III IFN actions, priming of the adaptive immune response, especially generation of virus-specific CTLs, therapy outcome, and HCV suppression.

Summary and insights

While the immune response can clear HCV[43], virus exposure often carries forward into a chronic infection. Chronic HCV is linked to dysregulation of innate and adaptive immune signaling and viral-induced cytotoxicity/apoptosis in the hepatic environment which together affect infection outcome, response to therapy, and likely contribute to liver fibrosis and cirrhosis. HCV accomplishes this immune dysregulation by evading the host innate immune response at multiple points, to prevent the initial coordination of innate immunity that primes the entire immune response, including adaptive immunity, leading to increased viral replication and contributing to viral persistence. Within the liver microenvironment, multiple cell types contribute to the immune response towards HCV, and interruption of any aspect of the innate response by HCV could lead to an unbalanced, ineffective immune

response, both in natural and treatment-induced HCV clearance, that could drive the progression to liver disease. A full understanding of how viral immune modulation imparts infection outcome requires us to define the spectrum of antiviral and immunomodulatory cytokines that govern the cross-talk between the various cell types within the complex liver tissue. For example, how do IFN-α and IFN-λ responses crosstalk to one another and with hepatic responses to proinflammatory cytokines, such as IL-1β, IP10, and others, implicated in hepatic inflammation? What are the key producer cells for these cytokines in the infected liver, and how is production of these cytokines stimulated and/or regulated during HCV infection? There may also be a role for hepatic stellate cells in mediating some of these processes as they have recently been implicated in driving inflammatory responses during HCV-infection of hepatocytes[100].

Clinical trials with IFN-λ are already showing great promise for its role as an effective treatment antiviral cytokine, with fewer side effects than observed in patients undergoing IFN-α-based therapy. While genetic studies have revealed insights of *IFNL3* polymorphisms and their linkage with an effective immune response that clears HCV, we note that *IFNL3* genotype is only one of the factors that predict therapy responses (Fig. 2B). While knowledge of one's *IFNL3* genotype could be used to guide therapy decisions, further genetic studies to define additional host factors that impact HCV clearance and/or disease progression will help us understand what dictates successful immune responses and treatment outcomes against HCV.

It is quite likely that an upcoming treatment era for HCV may fully embrace direct acting antivirals alone, such as NS3 protease, NS5A replication complex, or NS5B polymerase inhibitors, in the absence of IFN combination therapy. While these therapies are providing a major step forward in treating HCV infected patients, viral breakthrough and drug resistance will need to be considered and monitored carefully in these situations[101]. In addition, efficacy of these drugs for HCV genotypes other than genotype 1 still needs to be determined.

Research to continue onward in these exciting discoveries and developments will no doubt reveal the key factors required for successful immunity to HCV. In the coming age of personalized medicine, this knowledge could lead to individually tailored HCV therapies, based on knowledge of both one's host and viral genotype. An understanding of these detailed components of an effective immune response to HCV both at the innate and adaptive levels will be useful to guide the development of the long desired HCV vaccine.

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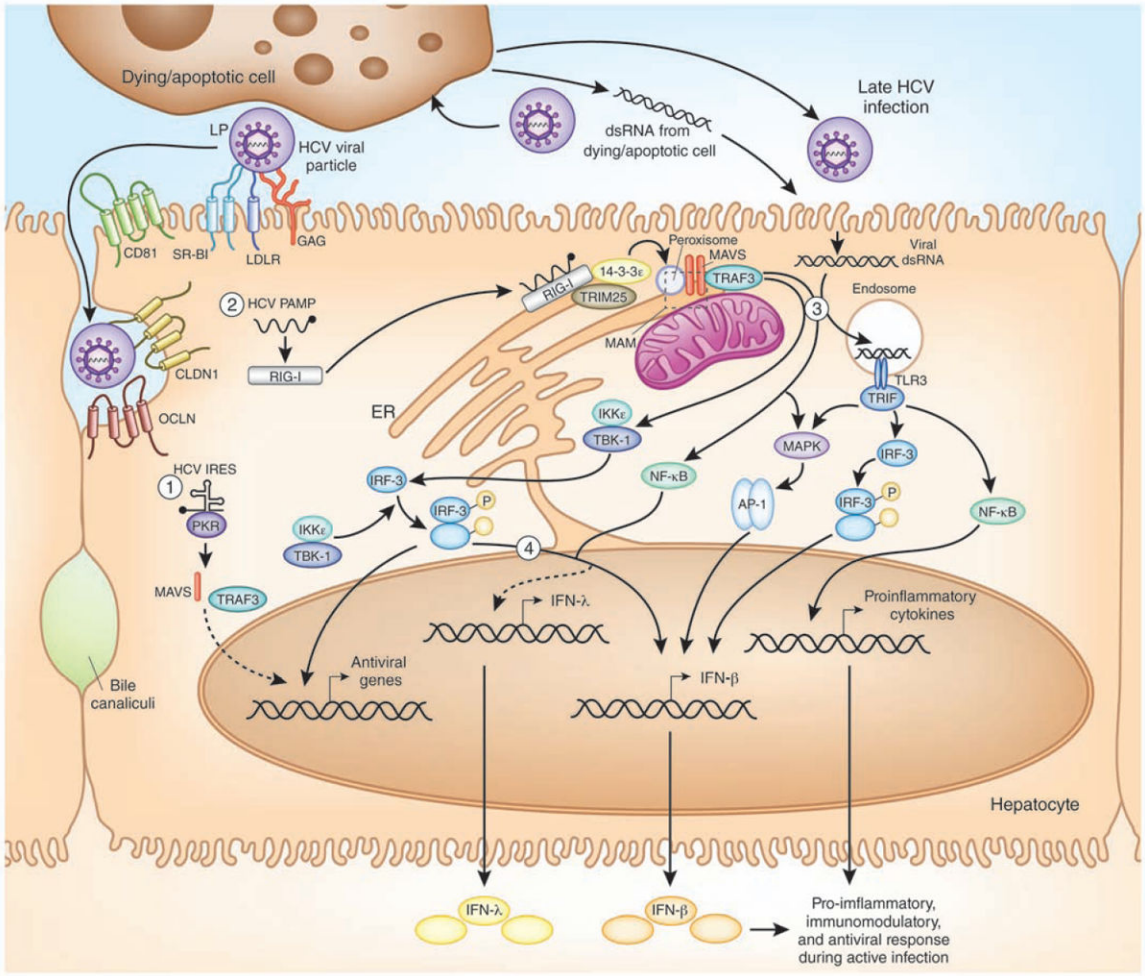


Figure 1. Sensing of HCV can activate innate antiviral defenses through IFN induction in hepatocytes

Innate sensing of HCV in the infected hepatocyte occurs through the combined actions of (1) PKR (2) RIG-I, and (3) TLR3. These proteins recognize specific features of HCV, including the dsRNA in the HCV IRES and the HCV poly U/UC PAMP early during infection; and dsRNA that accumulates following HCV infection (virus particle coated with apolipoprotein (LP) is depicted) or by uptake of HCV dsRNA from dying cells later during infection. This recognition leads to downstream signaling, as indicated by the arrows, that results in the induction of antiviral/immunomodulatory genes, IFN-β, and other proinflammatory cytokines. See the text for mechanistic details. The dashed box marks the location of the mitochondrial-associated ER membrane (MAM), a site of MAVS signaling. (4) The mechanisms leading to hepatic activation of IFN-λ during HCV infection have yet to be fully characterized.

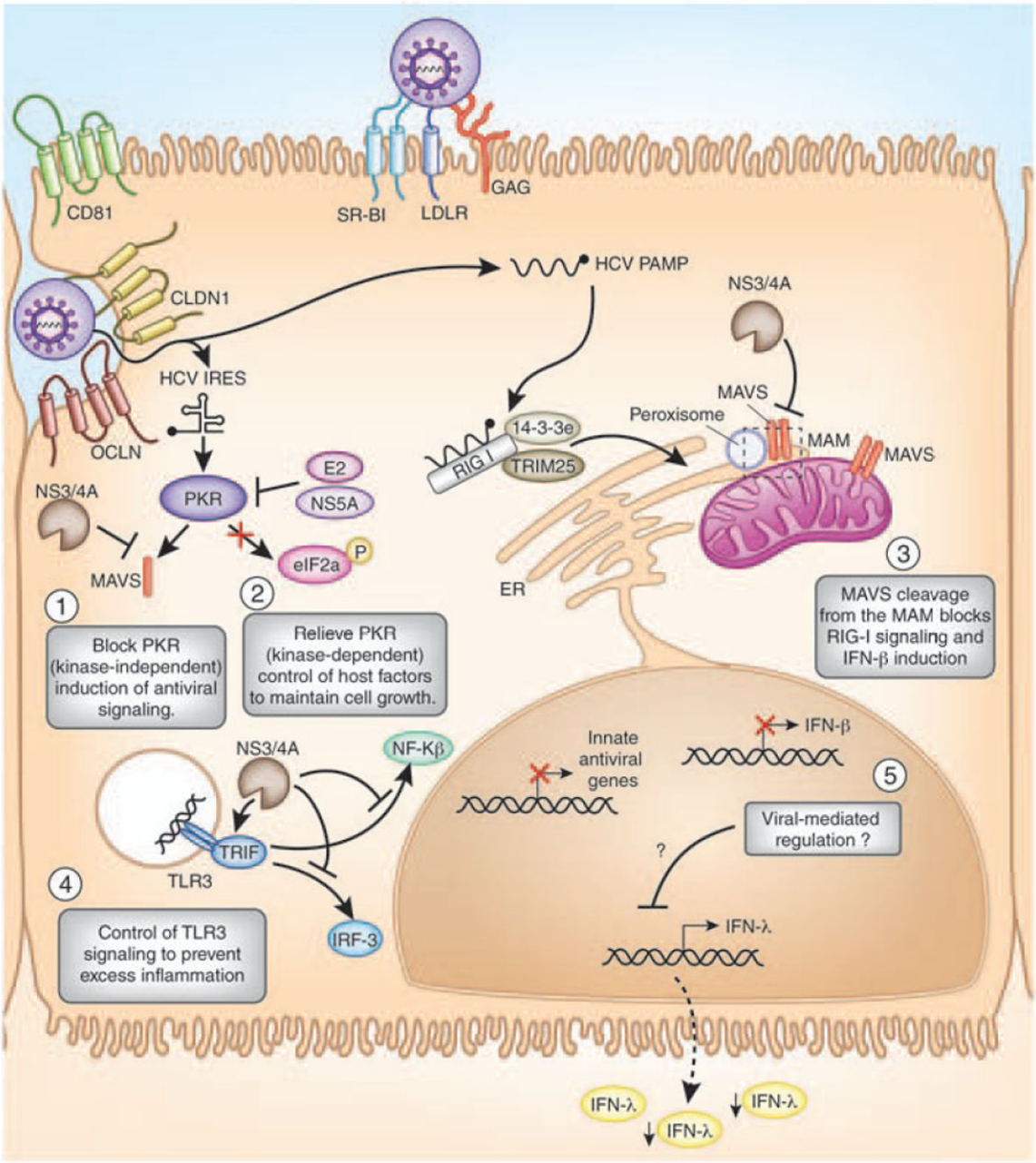
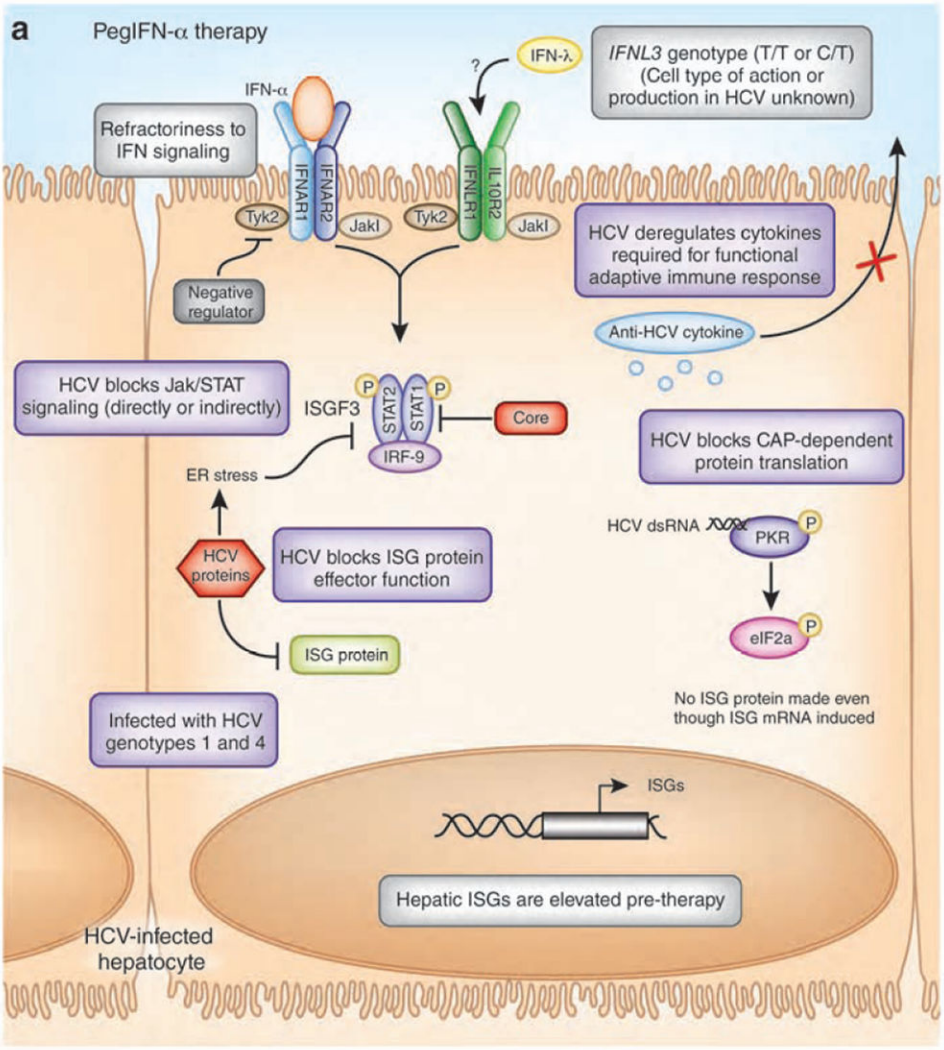


Figure 2. HCV control of IFN induction and immune evasion

Immune evasion by HCV in the hepatocyte occurs at several points during viral infection. The proposed regulation is shown here, where the HCV NS3/4A protease cleaves the signaling adaptors MAVS (on the mitochondrial-associated ER membrane (MAM; in the region indicated by the dashed box) and TRIF to inactivate (1) PKR, (3) RIG-I, and (4) TLR3 signaling pathways to prevent induction of immunomodulatory innate antiviral genes and IFN-β allowing for HCV replication. (5) HCV infection control of IFN-λ induction is not yet defined; (2) HCV E2 and NS5A proteins inactivate PKR kinase-dependent activation of the host translation factor eIF2α to reactivate protein translation during infection.



b

Elevated pretreatment ISGs

	HCV genotype	Hepatocyte	Macrophage	*IFNL3* genotype[b]
Responder (SVR)[a]	2 or 3	No	Yes	C/C
Non-responder	1 or 4	Yes	No	C/T or T/T

Figure 3. Factors that influence the host response to IFN therapy during HCV infection

(A) In the HCV-infected hepatocyte, a number of possible factors influence therapy responses to pegylated IFN-α. These factors include the patient *IFNL3* genotype, the viral genotype, and the activation status of hepatic ISGs prior to therapy, including ISGs that are negative regulators of IFNAR that make the cells refractory to IFN signaling. HCV itself could regulate the host response in the infected cell to prevent IFN therapy action by directly or indirectly blocking Jak/STAT signaling, ISG protein effector function, CAP-dependent protein translation, and cytokines required for functional adaptive immune responses (see purple boxes). (B) Table of key factors that determine HCV therapy responses.[[a](SVR: sustained virologic release); [b](the nucleotide SNP at rs12979860)]

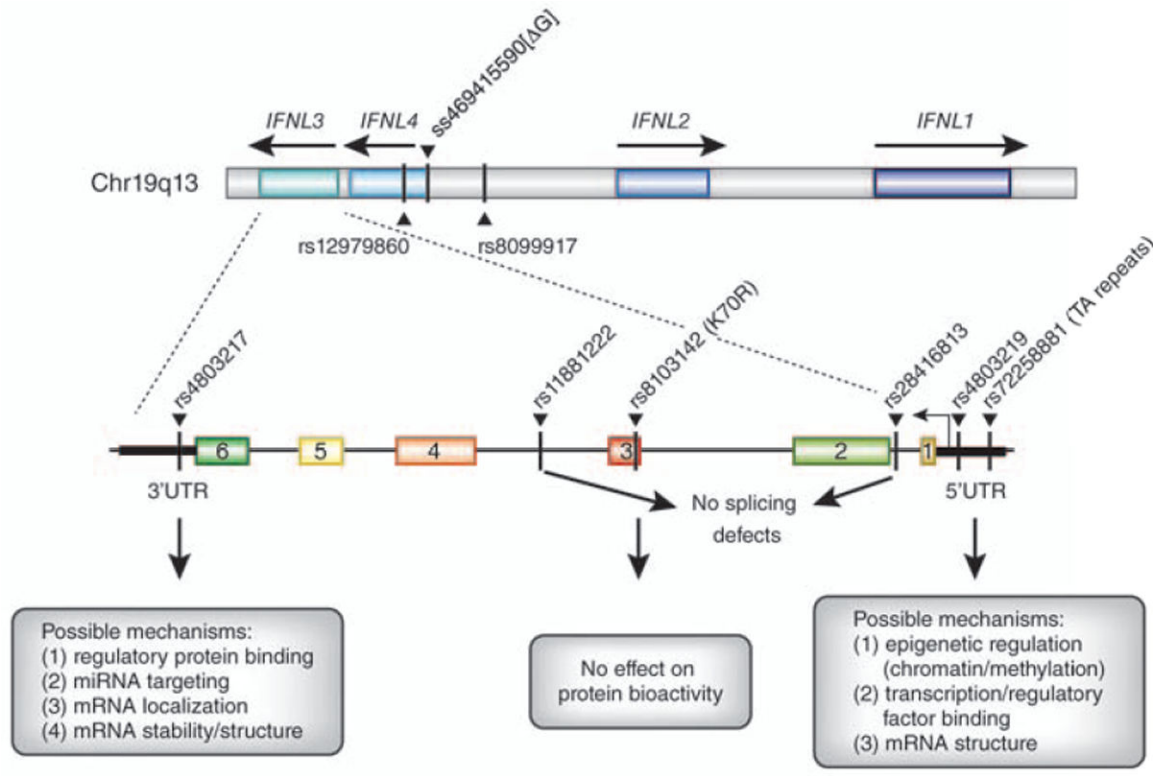


Figure 4. Single nucleotide polymorphisms (SNPs) in the *IFNL* gene locus

A schematic of the *IFNL1-4* gene locus in Chromosome 19q13 is depicted, based on the determined sequence of the 5' and 3' ends of the *IFNL3* mRNA[81]. The significant proximal SNPs around *IFNL3* associated with response to IFN therapy are shown in the map, as indicated by the red dash. Candidate functional SNPs near the *IFNL3* and *IFNL4* locus, along with possible regulatory mechanisms at these sites, are depicted. Several of these SNPs have been tested as candidate functional SNPs (rs4803219 and rs11881222, role in splicing; rs8103142 (K70R), role in protein bioactivity) and found to have no affect on IFNL3[81].

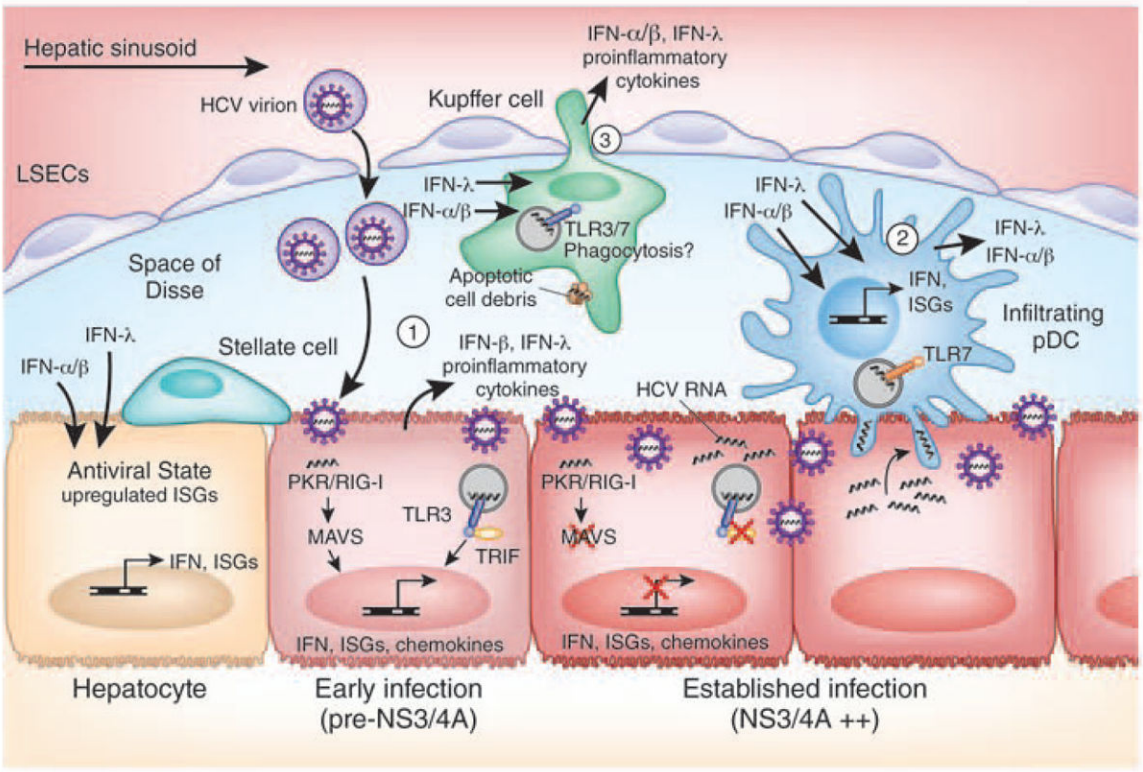


Figure 5. IFN induction by HCV in the liver

The liver contains different cell types that can secrete IFN upon stimulation by HCV infection, including (1) hepatocytes, (2) infiltrating plasmacytoid dendritic cells (pDCs), and (3) Kupffer cells. The activation of these cells is required to prime the adaptive immune response to HCV, which plays a major role in eventual viral clearance[16]. Both type I and type III IFN can act on uninfected hepatocytes to induce an antiviral state that limits virus spread. In the infected cell, HCV can evade IFN induction through viral-mediated processes, including NS3/4A-mediated cleavage of MAVS and TRIF, impacting HCV outcome of infection and disease progression. Cross-talk between hepatocytes and hepatic stellate cells during HCV infection has also been shown to induce inflammatory cytokines and chemokines (not depicted here)[100].

Table 1

Anti-HCV interferon stimulated genes

Gene Symbol	Proposed anti-HCV mechanism	Ref.
ADAR	editing viral RNA	41
DDIT4	unknown	41
DDX58 (RIG-I)	activates IFN-β pathway signaling, including IRFs	41,102
DDX60	enhances RIG-I signaling	41
EIF2AK2 (PKR)	inhibits protein translation (vie eIF2α); activates RIG-I	35,103-105
GBP1	unknown	106
IFI44L	unknown	41
IFI6	unknown	106
IFIT1	sequesters viral nucleic acids; suppresses translation	107-109
IFIT3	enhances MAVS/TBK1 signaling	73,110
IFITM1	inhibits HCV receptor-mediated entry	73,108,111
IFITM3	unknown, possibly inhibits intracellular trafficking	73,112-113
IRF-1	IFN induction, direct induction of ISGs	40,104
IRF-7	IFN induction, direct induction of ISGs	41
ISG12	unknown	106
ISG20	exonuclease activity	103
MAP3K14 (NIK)	unknown, possibly NF-κB pathway activation	41
MOV10	unknown	41
MS4A4A	unknown	41
MX1 (MxA)	unknown	106
NOS2	unknown	73
NT5C3	unknown	41
OAS1	activates RNaseL	106
OASL	unknown	40,114
PLSCR1	unknown, possibly prevents membrane rearrangements	73
RNAseL	cleaves viral genome, activates RIG-I signaling	73,115
RSAD2 (viperin)	interacts with NS5A and VAP-A to block viral replication	73,103,116-117
SSBP3	unknown	41
TRIM14	unknown	73