Review

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Innate immunity and HCV

Markus H. Heim*

Division of Gastroenterology and Hepatology, University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland; Department of Biomedicine, University of Basel, Hebelstrasse 20, 4031 Basel, Switzerland

Summary

Hepatitis C virus (HCV) infections become chronic in the majority of infected individuals, and chronic hepatitis C (CHC) can lead to cirrhosis and hepatocellular carcinoma. The innate immune system is central to host-virus interactions during the entire natural course of the disease. The HCV NS3/4A protease efficiently cleaves and inactivates two important signaling molecules in the sensory pathways that react to HCV pathogen-associated molecular patterns (PAMPs) to induce interferons (IFNs), i.e., mitochondrial antiviral signaling protein (MAVS) and Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). Despite this viral escape mechanism, the innate immune system strongly reacts to HCV within the first days after infection. The sensory pathways, the type(s) of IFNs involved and the cellular source of IFNs are largely unknown. After 4–8 weeks, HCV specific T cells are recruited to the liver. IFN-γ-stimulated genes get strongly expressed in the liver. In about 30% of patients, the virus is eliminated during the acute phase of the infection by T cell-mediated antiviral mechanisms. In the remaining 70% of patients, HCV persists for decades. During this phase, T cell-derived IFN-γ cannot be detected any more in liver biopsies. Instead, in about half of the patients, hundreds of type I or III IFN-stimulated genes become again strongly expressed. However, this innate immune reaction is ineffective against HCV. Moreover, patients with constitutive IFN-stimulated gene (ISG) expression have a poor response to treatment with pegylated IFN-α (PegIFN-α) and ribavirin. The viral escape mechanisms that protect HCV from IFN-mediated innate immune reactions are not entirely understood, but might involve blockade of ISG protein translation at the ribosome, localization of viral replication to cells with refractory IFN signal transduction pathways or to cell compartments that are not accessible to antiviral IFN-stimulated effector systems. Recently, genetic variations near the *IL28B* (*IFN-λ3*) were found to be strongly associated with spontaneous clearance of HCV and response to treatment with PegIFN-α and ribavirin. The finding supports a central role of the innate immune response in host-viral interactions. The signaling pathways that link genetic variants of *IL28B* with immune answers to HCV remain to be elucidated. The present review article attempts to summarize current knowledge of some central aspects of the interactions of HCV with the innate immune system.

Keywords: Interferon; Hepatitis C virus; Innate immunity; Jak-STAT.

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* Address: Department of Biomedicine, University of Basel, Zentrum für Lehre und Forschung, Hebelstrasse 20, 4031 Basel, Switzerland. Tel.: +41 61 265 33 62; fax: +41 61 265 38 47.

E-mail address: markus.heim@unibas.ch.

Abbreviations: AHC, acute hepatitis C; CHC, chronic hepatitis C; DC, dendritic cell; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN-α receptor; IFNGR, IFN-γ receptor; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation antigen 5; NK cells, natural killer cells; NKT cells, natural killer T cells; pDC, plasmacytoid dendritic cell; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PHH, primary human hepatocytes; PIAS, protein inhibitor of activated STAT; RIG-I, retinoic acid inducible gene-I; TLR, toll like receptor; TRIF, Toll-IL-1 receptor domain-containing adaptor inducing IFN-β; USP18, ubiquitin specific peptidase 18; UBP43, ubiquitin-specific protease 43 kDa.

Introduction

Hepatitis C virus

Hepatitis C virus (HCV) infects 130–170 million persons worldwide [1]. HCV is parenterally transmitted, mainly due to injection drug use and unsafe transfusions and therapeutic injections [2]. Acute HCV infections (AHC) are often oligo- or asymptomatic [3]. In 70–80% of those infected, the virus persists and the infection becomes chronic. Spontaneous clearance of HCV is rare in the chronic phase of the infection. In most patients, chronic hepatitis C (CHC) leads to some degree of liver fibrosis, and in 15–25% of patients, cirrhosis develops after 10–40 years [4]. Patients with CHC and cirrhosis are at increased risk of liver failure and hepatocellular carcinoma development [5].

HCV is a positive-strand enveloped RNA virus belonging to the family *Flaviviridae*. HCV isolates are classified into 6 major genotypes (numbered from 1 to 6) that differ in the sequence of the 9.6 kb genome by 30–35% [6]. Within genotypes, subtypes (designated with small letters, e.g., 1a, 1b) differ in their sequence by 20–25%. HCV infects humans and chimpanzees. Hepatocytes are the main target cells of HCV. Virus entry into hepatocytes requires multiple cellular factors including scavenger receptor type B1 (SR-B1), CD81, claudin-1, and occludin [7]. Reliable and widely reproducible methods to detect viral RNA or proteins in liver samples of infected patients are lacking. It is therefore still a matter of controversy what percentage of hepatocytes is simultaneously infected at any given time point during acute and chronic infection with HCV. The HCV RNA is translated at ribosomes into a long precursor polyprotein that is then cleaved by

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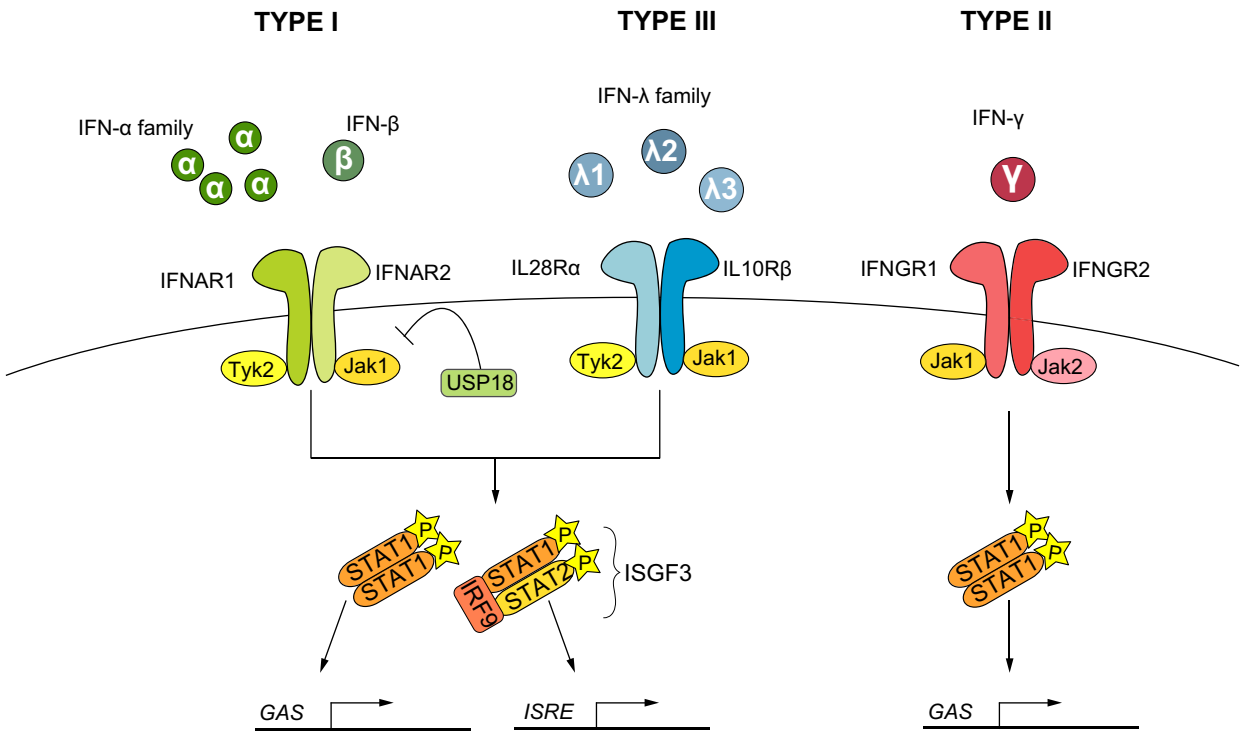

Fig. 1. IFN signaling through the Jak-STAT pathway. Type I (IFN-αs and IFN-β) and type III (IFN-λs) IFNs bind to distinct receptors, but activate the same downstream signaling events, and induce almost identical sets of genes mainly through the activation of IFN-stimulated gene factor 3 (ISGF3) and STAT1 homodimers. IFN-γ (the only type II IFN) activates STAT1, but not ISGF3, and induces a partially overlapping but distinct set of genes. Adapted from Ref. [116], with permission from Elsevier.

cellular and viral proteases into the mature viral proteins [8]. Inhibitors of the viral NS3/4A protease are used for therapy of HCV genotype 1 infections [9,10]. HCV replication occurs in the cytoplasm in specific membrane alterations named the membranous web [8]. Replication is very dynamic: an estimated 10^{12} virions are produced and cleared per day in an infected individual [11].

Innate immunity and interferons

Innate immune responses are the first line of defense against viral infections and interferons (IFNs) are the central cytokines responsible for the induction of an antiviral state in cells and for the activation and regulation of the cellular components of innate immunity such as natural killer (NK) cells [12]. Type I IFNs (comprising several IFN-α and one IFN-β) and type III IFNs (IFN-λ1, -λ2, and -λ3; also designated IL29, IL28A, and IL28B) are produced by cells infected with viruses and by key sentinel cells of the innate immune system: macrophages and dendritic cells (DCs). Type II IFN (IFN-γ) is produced by NK and natural killer T (NKT) cells as part of the innate immune response, and by antigen-specific T cells (both CD4+ Th1 and CD8+ cytotoxic T lymphocytes).

Two important pathways that detect viral genomes and induce type I and type III IFNs have been discovered and characterized during recent years: the toll-like receptor (TLR) dependent pathway [13,14] and the cytosolic pathway triggered by binding of viral RNA to the RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5) [15,16]. TLRs are a family of transmembrane pattern recognition receptors that recognize microbial pathogen-associated molecular patterns (PAMPs) and activate the expression of genes involved in inflammatory and immune responses [14]. There are at least 10 human TLRs, and 3 of them are involved in the recognition of viral infections: TLR3, TLR7, and TLR9. TLRs are expressed on various immune cells such as macrophages, dendritic cells (DCs), B cells, but also on fibroblasts and epithelial cells. While TLRs involved in the recognition of bacterial components are expressed on the cell surface, TLR3, TLR7, and TLR9 are localized in intracellular compartments such as endosomes. TLR3 recognizes double-stranded RNA [17], TLR7 detects single-stranded RNA [18,19] and TLR9 interacts with unmethylated DNA with CpG motifs [20]. TLR3 activation induces signaling cascades that mainly involve adapter molecules Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) and the kinase TBK1. TLR7 and TLR9 signal through MyD88 and the IRAK4-IRAK1-IKKα kinase cascade [21]. Both pathways converge on the activation of the key transcription factors NF-κB and interferon regulatory factor (IRF) 3 and 7. Importantly, macrophages and DCs do not have to be infected by viruses in order to produce IFNs. Instead, they constantly sample material from the outside, including virus containing remnants of apoptotic cells and intact viral particles. Degradation processes in the endosomes then expose viral nucleic acids to recognition by TLRs.

Viruses that avoid the endosomal entry pathway can be detected by the cytosolic pathways of type I and III IFN induction. These signaling pathways are initiated by the recognition of viral 5′ triphosphate RNA and double-stranded RNA by RIG-I and MDA5. Binding of viral RNA induces a conformational change of these sensors that results in the binding to mitochondrial

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antiviral signaling protein (MAVS, also designated Cardif, VISA, IPS-1), an essential downstream adaptor in the cytosolic pathway [22–25]. Through as yet unidentified mediators, MAVS then propagates the signal to the TBK1 and IKKi (IKKε) or the IKKα and IKKβ kinases that finally activate the transcription factors IRF3 and NF-κB, respectively. Activated IRF3 and NF-κB bind to response elements in the promoters of type I and III IFN genes.

All types of IFNs induce an antiviral state by the transcriptional activation of hundreds of genes. The specific set of genes differs between IFNs and target cell type. In general, IFN-αs and IFN-λs induce a largely overlapping set of genes in cells that express receptors for both IFN-α and IFN-λ [26], whereas the IFN-γ-induced gene set is more distinct [27,28]. The number of genes regulated by IFNs also differs between cells. For instance, pegylated IFN-α significantly induces 200 to 300 genes in the liver, but nearly 2000 genes in peripheral blood mononuclear cells (PBMCs) [29].

Type I and II IFNs are essential for the defense against virus. Knock-out mice that lack the receptors for IFN-α or IFN-γ, or components of the IFN signal transduction pathway succumb to otherwise harmless viruses [30,31], and infants with genetic defects of the IFN system die from viral infections despite best medical care [32]. Type III IFNs have a more restricted role, most likely in viral defense at epithelial surfaces in the respiratory and gastro-intestinal tract.

Interferon signal transduction

IFN-αs and IFN-β bind to the ubiquitously expressed IFN-α receptor (IFNAR). IFN-λs bind to a different receptor consisting of the ubiquitously expressed IL-10R2 chain (shared with the IL-10 receptor) and a unique IFN-λ receptor chain whose expression is mainly restricted to epithelial cells [33,34]. IFN-γ binds to the widely expressed IFN-γ receptor (IFNGR). All IFN receptors connect to the Jak-STAT pathway to transmit signals from the cell surface to the nucleus. Binding of IFNs to their cognate receptors activates members of the Janus kinase family that then phosphorylate tyrosine residues located on the intracellular receptor domains [35]. STATs are recruited to phosphorylated receptors through their SH2 domains, and a highly specific and selective interaction that determines specificity in Jak-STAT signaling [36]. Because of the presence of specific docking sites at their receptors, IFN-αs, IFN-β, and IFN-λs mainly activate signal transducer and activator of transcription (STAT) 1 and STAT2, whereas IFN-γ activates only STAT1. All IFNs induce STAT1 homodimers that bind to gamma activated sequence (GAS) elements in IFN-stimulated genes (ISGs). Type I and III IFNs additionally induce the heterotrimeric transcription factor IFN-stimulated gene factor 3 (ISGF3) that consists of STAT1, STAT2, and IRF9 and binds to IFN-stimulated response elements (ISRE) [35,37,38] (Fig. 1).

IFN signaling is controlled by a number of negative regulators such as SOCS, USP18, PIAS, and TcPTP. Suppressor of Cytokine Signaling (SOCS) proteins are important negative regulators of Jak-STAT signaling that are rapidly induced and provide an early negative feedback loop [39]. SOCS1 deficient mice develop severe inflammatory disease [40], but are very resistant to viral infections, most likely because of enhanced type I IFN signaling [41].

Ubiquitin specific peptidase 18 (USP18, also designated UBP43) is another important negative regulator in type I IFN signaling [42,43]. USP18/UBP43 was originally identified as a protease cleaving ubiquitin-like modifier interferon-stimulated gene

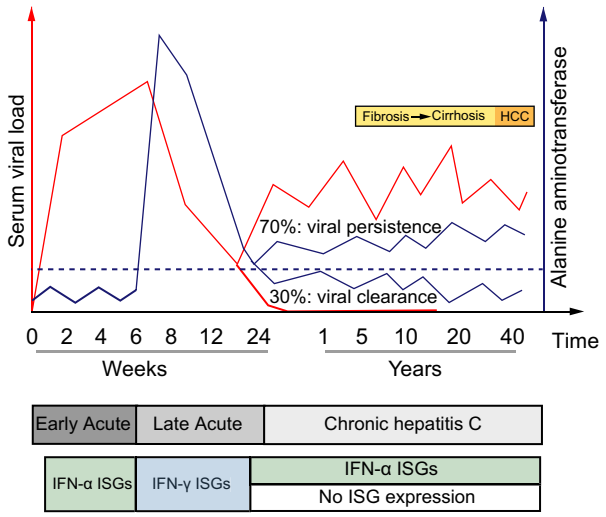

Fig. 2. Natural course of HCV infection. In the early phase of acute infection (the first 4–8 weeks), HCV induces a type I or III IFN response that restricts viral replication (green box). With the recruitment of HCV specific T cells in the late phase of AHC, the gene expression profile in the liver switches to an IFN-γ pattern (blue box). In late AHC, viral replication is strongly inhibited, and in about 30% of the patients, HCV is completely eliminated. In 70% of the cases, HCV persists and can induce again a type I or III IFN response in about half of the patients (upper green box). The other patients have little to no activation of ISGs in the liver (empty box). Changes in serum HCV load (red), alanine aminotransferase levels (ALT) (blue) and IFN-stimulated gene expression (top bar) are shown. The dashed line shows the upper limit of normal for ALT. Adapted from Ref. [116], with permission from Elsevier.

15 (ISG15) from target proteins [44]. Similar to ubiquitination, conjugation of ISG15 to target proteins (ISGylation) occurs through sequential enzymatic action of activating E1, conjugating E2, and ligating E3 enzymes [45]. ISGylation modulates signal transduction pathways and proteins involved in immunity and stress responses [46,47]. IFN-induced ISGylation has an important protective role against several viruses (e.g., influenza and *herpes simplex*) [48,49]. HCV seems to be an exception. ISGylation actually promotes HCV FL-J6/JFH virus production in HuH7.5 cells [50]. Since ISG15 also conjugates to STAT1, the negative regulatory role of USP18 on IFN signaling was originally attributed to its cysteine protease enzymatic activity. However, an active site cysteine mutant USP18 was found to inhibit IFN signaling as well, demonstrating that USP18 negatively regulates IFN-α signaling independently of its ISG15-deconjugating ability [51]. Indeed, USP18 was reported to inhibit the activation of Jak1 by interfering with its interaction with IFNAR2 [51]. USP18 deficient mice show a severe phenotype characterized by brain cell injury, poly-I:C hypersensitivity, and premature death [52,53]. Interestingly, they are resistant to otherwise fatal cerebral infections with lymphocytic choriomeningitis virus and vesicular stomatitis virus [54]. USP18 is a key mediator of refractoriness of liver cells to continuous stimulation with IFN-α [55]. USP18 is not induced by IFN-γ, and does not inhibit IFN-γ or IFN-λ signaling [56].

Protein inhibitors of activated STAT1 (PIAS1) and PIAS3 specifically bind to tyrosine phosphorylated STAT1 and STAT3, respectively, and inhibit the DNA-binding of STAT dimers [57]. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. As a consequence, PIAS1 deficient mice show increased protection against pathogenic infection [58].

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Review

Host-virus interactions in acute hepatitis C

Prospective studies with health-care workers with AHC after accidental needlestick injury, and with experimentally infected chimpanzees revealed an enormous replicative capacity of HCV [59–63]. Already within days after infection, high viral titers have been measured in the serum and liver of chimpanzees. After a very rapid initial increase, viral titers remain constant for the following 4–8 weeks, and then decline concomitant with the onset of an adaptive cellular immune response in the liver and a rise in serum transaminases [60–62,64]. The combined innate and adaptive host-response is often strong enough to eliminate HCV infections in chimpanzees. In humans, HCV infections become chronic in more than half of the infected individuals.

Key Points 1

- Hepatitis C virus (HCV) has a very high replicative capacity. Within days after infection, viral titers of >10^6 IU/ml can be measured in the serum
- The innate immune system reacts to HCV infections with the induction of interferon (IFN) stimulated genes in the liver. This initial type I and/or type III IFN driven response controls viral replication to some extent, but cannot eliminate HCV completely
- 4-8 weeks after infection, HCV specific T cells are recruited to the liver. HCV replication is inhibited by non-cytolytic (IFN-γ-mediated) and cytolytic mechanisms. In about 30% of the patients, the immune reaction during acute hepatitis C is strong enough to eliminate HCV infection
- In the acute phase of the infection, HCV is highly vulnerable to therapy with recombinant IFN-α. Over 90% of the patients can be cured with IFN-α monotherapy

Induction of interferon by hepatitis C virus

For the following discussion, the acute phase of HCV infection will be subdivided into an early acute phase prior to the activation and recruitment of HCV specific T cells in the liver, and a late acute phase characterized by the adaptive immune response (Fig. 2). The host reaction in the liver during the early acute phase of HCV infection has been exclusively studied in experimentally infected chimpanzees [60–62,64]. In this animal model, a strong host-response to HCV has been detected already days after infection. Transcriptome analysis revealed induction of type I IFN-stimulated genes [60,61]. The extent and duration of ISG induction showed a positive correlation with viral load [61]. This suggests that the most important regulator of ISG induction in the early acute phase is the amount of HCV-derived PAMP molecules that stimulate TLR dependent and/or RIG-I dependent sensory pathways. The precise nature of the HCV PAMP is a matter of debate. Several reports stress the importance of uncapped 5′-phosphate double-strand RNA, but the 3′ non-translated region of the HCV genome was also shown to induce the RIG-I pathway [65–68]. However, in all these studies, *in vitro* transcribed RNA was transfected into cells, and it is unknown if any of these RNA molecules are exposed to sensory pathways in HCV infected cells. Indeed, infection of RIG-I competent HuH7 cells with HCV does not stimulate the RIG-I pathway at all [69,70]. There have been several recent papers showing IFN induction in primary human hepatocytes (PHH) infected with HCV [71–73], but this experimental system is prone to contamination of the hepatocytes with non-parenchymal cells that could be the source of IFN production. It has been shown that plasmacytoid dendritic cells (pDCs) can produce large amounts of IFN-α when co-cultured with HCV replicon cells [74]. Therefore, it is conceivable that relatively few pDCs present in PHH cultures could be the source of IFNs produced after infection with HCV. Only experiments that directly visualize IFN mRNA *in situ* will clarify this issue.

There is another, indirect argument in favor of models that place IFN production in non-infected cells such as pDCs. It has been shown that the viral protease NS3/4A very efficiently blocks the activation of IRF3, a transcription factor that is essential for IFN induction [75]. NS3/4A interferes with both the TLR dependent and the cytosolic sensory pathways. NS3/4A cleaves and inactivates MAVS, an essential component of the RIG-I pathway [22], and TRIF, an adaptor in the TLR3 pathway [76]. It has not been investigated whether cleavage of MAVS or TRIF3 occurs in the early acute phase of HCV infection. In the chronic phase of HCV infection, cleavage of MAVS was detected in 62 out of 129 (48%) liver biopsies from patients with CHC [77]. MAVS cleavage most likely occurs in HCV infected hepatocytes, and could prevent IFN induction in those cells. IFN production in non-infected non-parenchymal cells such as pDCs would not be affected by cleavage of MAVS or TRIF.

It has to be stressed that all those arguments for or against hepatocytes *vs.* non-parenchymal cells as the source of IFN in early AHC rely on cell culture experiments that might not reflect the host-virus interactions *in vivo*. The clarification of the cellular source of IFN in the early acute phase of HCV will require *in situ* detection of IFN mRNA in liver biopsies of experimentally infected chimpanzees. Technical progress is also required to identify the type(s) of IFN responsible for the induction of ISGs. Studies with chimpanzees failed to detect induction of IFNs in early AHC [60,61]. More recent studies detected upregulation of mRNA of type III (but not type I) IFNs in liver biopsies [72,73], and an increase of type III IFN protein, primarily IFN-λ1 (IL29), in the serum of chimpanzees [73]. IFN-λ1 serum concentrations in 6 experimentally infected chimpanzees were in the range of 200 pg/ml during the first weeks of infection. In this early phase of AHC, all animals had a significant upregulation of ISGs in the liver. However, IFN-λ1 serum concentrations in the same range have been measured in healthy (not HCV infected) humans with presumably uninduced ISGs in the liver [78]. It is also not clear how well human hepatocytes respond to type III IFNs. Compared to IFN-α, IFN-λs are very weak activators of STAT1 phosphorylation in PHHs (Francois Duong and Markus Heim, unpublished). Finally, *in vivo* data on the effects of IFN-λ in the liver are completely missing. Therefore, the functional relevance of the observed induction of type III IFNs in early AHC remains speculative.

Antiviral efficacy of the early innate immune response

After a very rapid increase in the first days (to weeks) after infection, HCV viral loads remain stable for several weeks, until the

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emergence of the cellular immune response in the liver [60–62,64]. ISGs are strongly induced during this entire period, but this innate immune response is obviously not capable of clearing HCV infections. As discussed below, several potential mechanisms of viral interference with the IFN system have been explored, and there is mounting evidence that HCV inhibits the expression of functional antiviral effector proteins in infected cells. The co-existence of high viral loads and high ISG expression has been interpreted as proof that the innate IFN response is completely ineffective against HCV. However, the short duration of exponential increase of viral loads and the following permanent restriction of the viral load during the early phase of AHC could reflect an important role of the innate immune system in the containment of HCV.

Cellular immune responses in the late phase of acute hepatitis C

The late phase of AHC begins 6–10 weeks after infection and is characterized by the recruitment of HCV specific T cells. It lasts 2–6 weeks, is clinically characterized by elevated transaminases and sometimes icterus, and leads to clearance of the infection in about 30% of infected individuals. Portal and lobular CD8+ T cell infiltrates are, indeed much more prominent than in CHC [28], and several groups have shown that spontaneous elimination of HCV is associated with vigorous virus-specific CD8+ T cell responses targeting multiple epitopes (reviewed in [79]). CD8+ T cells inhibit viral replication through lysis of infected cells and, more importantly, through IFN-γ mediated non-cytolytic mechanisms [80]. Indeed, a recently published transcriptome analysis of liver biopsies of patients with AHC revealed a strong induction of IFN-γ stimulated genes [28]. Why does this IFN-γ-dominated response succeed in eliminating the virus in a considerable proportion of patients, whereas the activation of the type I or III IFN system in the early phase of AHC invariably fails? The number of ISGs and the expression level of ISGs seem not to be significantly different in the early *vs.* late phase of AHC. Is there an important qualitative difference in ISG induction? CD8+ T cell-derived IFN-γ could stimulate the induction of a specific subset of genes that are crucial for HCV elimination. It is also conceivable that IFNs alone are not sufficient and that the cellular immune response provides additional antiviral effector systems. However, such explanations are contested by the over 90% success rate of therapies with recombinant IFN-α in AHC patients. Obviously, type I IFNs can induce all the necessary antiviral effector system in HCV infected cells. The reasons for the failure of the innate immune system in early AHC remain unknown.

The infection period following the late phase of AHC, i.e., the early phase of CHC, has been studied only in chimpanzees. In animals that fail to clear HCV, viral load increases again after a transient decline in late AHC, and usually stabilizes at titers that are about 10 times lower than in the early phase of AHC [62]. Concomitantly, type I or III ISGs are again induced [81].

Interferon-stimulated gene expression in chronic hepatitis C

In humans who develop chronic infections, ISG induction varies considerably between individuals. In about half of the patients of Caucasian ethnicity, hundreds of type I or III ISGs are constantly expressed at high levels in the liver, whereas the other half has no detectable induction of the innate immune system. Apart from a strong association of the minor allele of the *IL28B* genotype with ISG induction [82–84], little is known about the factors that determine the activation level of the IFN system. Interindividual variability of MAVS cleavage by NS3/4A is probably involved. In liver biopsies of patients with CHC, the degree of MAVS cleavage inversely correlates with ISG expression, but the correlation is rather weak [77]. There are no systematic longitudinal observation studies, but our own unpublished observations revealed little variation of ISG expression levels between liver biopsies obtained from the same patients at different time points during CHC. Most likely, the extent of ISG induction remains rather stable over decades in a given patient with CHC.

Key Points 2

- In chronic hepatitis C (CHC), HCV escapes both innate and adaptive immunity by yet unknown mechanisms
- In a substantial proportion of patients, HCV infection induces an IFN-mediated innate immune response in the liver. The type of IFN (α, β, λ) responsible for continuous stimulation of IFN-stimulated gene expression and the cells that produce the relevant IFNs have not yet been identified
- Induction of the endogenous IFN system in the liver is not only ineffective in clearing viral infection, but also prevents response to therapies with pegylated IFN-α and ribavirin

Antiviral efficacy of the endogenous IFN system in chronic hepatitis C

The induction of the endogenous IFN system in the liver apparently has little antiviral efficacy. HCV persists for decades despite the expression of hundreds of ISGs [29,85,86]. Furthermore, there is no significant correlation between serum or intrahepatic viral loads with ISG expression levels [77]. Several mechanisms of viral interference with the hepatic IFN system are conceivable. (1) HCV could inhibit IFN-induced signal transduction through the Jak-STAT pathway. Indeed, experimental expression of HCV proteins in cells inhibits binding of activated STATs to IFN response elements [87]. Inhibition of Jak-STAT signal transduction was also observed in HCV transgenic mice [88], and in liver biopsy extracts from patients with CHC [89]. According to this first model, transcriptional induction of ISGs would occur only in uninfected hepatocytes, and would therefore have no effect on overall viral replication. (2) HCV could inhibit cap-dependent protein translation at the ribosomes. As a matter of fact, most reports on ISG induction in CHC relied on quantification of ISG mRNA expression levels [29,85,86]. In cell culture experiments, HCV infection triggers phosphorylation and activation of the RNA-dependent protein kinase PKR, which phosphorylates eukaryotic translation initiation factor eIF2a [90]. Because phosphorylated eIF2a inhibits cap-dependent translation, no proteins are produced from ISG mRNAs. Of note, HCV protein production is not impaired, because HCV RNA translation occurs through an internal ribosomal entry site (IRES) dependent mechanism that is not impaired by phosphorylated eIF2a [90]. This model is also supported by a recently published ISG overexpression screen that has identified

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translational inhibition as the key antiviral effector mechanism of numerous ISGs [91]. According to this second model, ISG mRNA expression would occur in both un-infected and infected hepatocytes, but ISG protein production only in un-infected cells. (3) HCV does not interfere with ISG transcription or translation. Both mRNAs and proteins of ISGs would be induced, but HCV replication occurs in subcellular compartments that are not accessible to antiviral proteins induced by IFNs. Presently, there is little experimental evidence to support the third model. (4) HCV does not interfere with ISG protein production (same as in the third model), but HCV proteins inhibit antiviral effector functions apart from translational inhibition. Indeed, it has been recently shown that USP18, a classical ISG, can be strongly expressed in all hepatocytes in liver biopsies from patients with CHC and an induced endogenous IFN system [28]. If confirmed with other ISGs, such uniform expression of ISG proteins would disqualify models 1 and 2, and favor models 3 or 4. Clearly, progress in this controversial field will only come with advanced imaging studies that allow detecting HCV RNA and proteins and ISG mRNAs and proteins on a single cell level in liver biopsies from patients with CHC.

Non-response to PegIFN-α in CHC patients with an activated endogenous IFN system in the liver

It is now firmly established that patients with an activated endogenous IFN system are poor responders to IFN-α-based therapies [29,85,86,92]. Analysis of paired liver biopsies obtained before treatment and 4 h after the first injection of PegIFN-α2 revealed that patients with an activated endogenous IFN system had hundreds of ISGs expressed at high levels already before treatment, and that PegIFN-α2 did not further increase the expression of these genes, i.e., was completely ineffective in the liver [29] (Fig. 3). In such biopsies, staining for the phosphorylated (activated) form of STAT1 revealed a faint staining in nuclei of hepatocytes in pretreatment biopsies, and no further increase of phosphoSTAT1 signals 4 h after PegIFN-α injections [29]. In contrast, no phosphoSTAT1 signals were detected in pretreatment biopsies of "responder" patients without constitutive induction of ISGs, but PegIFN-α injections induced a very prominent and strong activation and nuclear translocation within 4 h [29]. The reason for the apparent refractoriness of IFN-α-induced Jak-STAT signaling is not entirely clear, but there is evidence that USP18 is an important factor. USP18 was strongly expressed in a large number of hepatocytes in liver biopsies from patients with CHC and a pre-activated endogenous IFN system [28]. Moreover, there is convincing genetic evidence from knock-out mice experiments that USP18 is responsible for the long-term refractoriness of IFN-α signaling in the liver [55]. These observations generate an apparent paradox: since USP18 is not constitutively expressed in cells, but is only expressed after IFN stimulation, how then can its expression level be maintained at high levels despite complete refractoriness of IFN-α signaling? Or in more general terms: How can an IFN-α-induced negative regulator of IFN-α signaling be persistently induced?

The driving force of ISG expression in CHC

The subtype(s) of IFN that drive the permanent expression of ISGs in CHC have not been identified, and little is known about the cellular source of IFN(s) too. Expression of mRNA of IFN-αs, IFN-β, IFN-γ has not been consistently detected in liver biopsies from

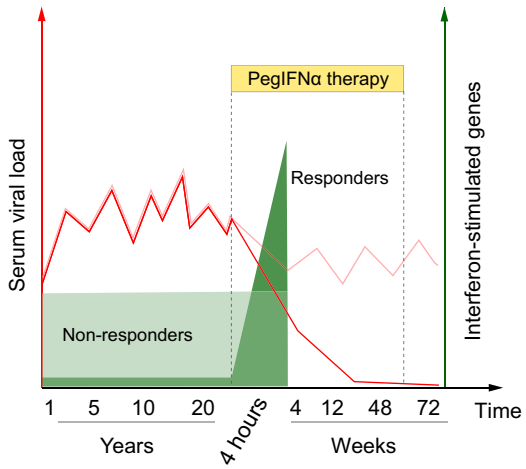

Fig. 3. Treatment of chronic hepatitis C with PegIFN-α and ribavirin. Patients with an activated endogenous IFN system have hundreds of ISGs upregulated already before treatment (light green). Application of PegIFN-α does not further increase ISG expression in the liver and continuous treatment with PegIFN-α and ribavirin rarely achieves a sustained virological response (light red line). Patients without a pre-activated IFN system show a massive induction of ISGs within 4 h after administration of PegIFN-α (dark green), and have a high chance of being cured by PegIFN-α/ribavirin therapy (dark red line). Adapted from Ref. [116], with permission from Elsevier.

patients with CHC, even in samples with very high expression of ISG mRNAs. IFN-γ can be further excluded as driver of ISG expression in CHC because the set of ISGs induced in CHC contains typical type I IFN-stimulated genes, but not type II-induced ISGs [28,29,81]. IFN-αs can be tentatively excluded because IFN-α signaling is subject to strong negative feedback inhibition, specifically by USP18, that would prevent long-lasting activation of ISGs [28,51,55]. One could only argue that the refractory state caused by USP18 is leaky and allows low-level STAT1 activation below the detection limit of phosphoSTAT1 Western blots or immunostaining techniques. However, there are more appealing alternative explanations. Interestingly, USP18 does not inhibit IFN-β signaling through the same receptor-kinase complex used by IFN-αs, and it does not inhibit IFN-λ signaling either [56]. Contrary to all other IFN subtypes, IFN-λ1, -λ2, and -λ3 mRNA can be detected in liver biopsies [82]. Admittedly, it is presently unknown if the low amount of mRNA detected produces enough bio-active protein to explain the strong induction of ISGs in CHC. No IFN proteins have been detected so far in liver biopsies of patients with CHC. However, considering the fact that IFN-λ signaling is not refractory, IFN-λs remain strong candidates as drivers of constitutive ISG induction in CHC patients with an activated endogenous IFN system.

Given the difficulties in identifying the subtype(s) of IFN responsible for the long-lasting induction of ISGs in CHC, it is not surprising that the cellular source(s) are not known. IFNs could be produced in HCV infected hepatocytes, but also in non-parenchymal cells such as pDCs.

IL28B genotype and innate immune responses to hepatitis C virus

The recent discovery of a strong association of genetic variants of the *IL28B* gene with response to PegIFN-α2/ribavirin combination treatment of CHC and with spontaneous clearance of HCV has been a major step towards a better understanding of the genetic

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factors that control natural history, host-virus interactions and IFN responsiveness in individual patients [93–97]. The association was discovered by genome-wide association studies (GWAS). GWAS make use of the ever-improving efforts to map the human genome to associate complex traits or disease outcome with inherited genetic variations. Single nucleotide polymorphisms (SNPs) are variations of one nucleotide in a genetic sequence. Common SNPs are defined as having a minor allele frequency of greater than 5%. An estimated 10 million common SNPs exist and are transmitted from generation to generation, often in blocks, allowing a few particular, or tag SNPs to be representative of a block (haplotype) [98]. Three SNPs close to the *IL28B* gene were found to be most strongly associated with response to Peg-IFN-α/ribavirin in patients with CHC: rs12979860 [93], rs8099917 [94,99,100] and rs12980275 [100]. rs12979860 is 3176 base pairs upstream (5′) of the *IL28B* gene, rs8099917 is 7554 base pairs upstream of the *IL28B* gene, and rs12980275 is 2488 base pairs downstream (3′) of the *IL28B* gene (Fig. 4). SNP rs12979860 detects a single nucleotide variation at position 39,738,787 of human chromosome 19. The major allele has a cytosine at this position, and the minor allele a thymine. Accordingly, the major and minor alleles are designated C allele and T allele, respectively. An individual patient can be homozygous for the C allele (C/C genotype), heterozygous (C/T genotype) or homozygous for the T allele (T/T genotype). For rs8099917 and rs12980275, the major/minor alleles are the T/G and A/G, respectively.

Key Points 3

- Genetic variants near the interleukin 28B (*IL28B*) gene are strongly associated with spontaneous clearance of HCV and with response to therapy with pegylated IFN-α and ribavirin
- The minor alleles (associated with poor response to therapy) are positively associated with IFN-stimulated gene expression in CHC
- The molecular mechanisms that link genetic variants near the *IL28B* gene to constitutive activation of the endogenous IFN system in the liver are presently unknown

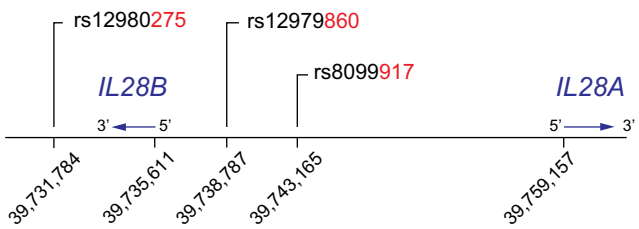

Fig. 4. *IL28B* gene locus on human chromosome 19. The 3 most strongly associated SNPs are in the genome regions flanking the *IL28B* gene. Nucleotide numbers on chromosome 19 are indicated for three SNPs and for the start of the coding region of the *IL28A* and *IL28B* gene.

Response to IFN-α therapy in chronic hepatitis C

The first published GWAS by Ge *et al.* reported a strong positive correlation of the C allele of rs12979860 with response to Peg-IFN-α/ribavirin treatment in patients with HCV genotype 1 infections [93]. In patients of European ancestry, the sustained virological response rate (SVR) was approximately 80% in patients with the C/C genotype, around 40% in patients with the C/T genotype, and around 35% in T/T patients. In African-Americans, SVR was lower in all genotypes compared to European-Americans, but the differences between *IL28B* genotypes were maintained. Interestingly, the allele frequencies of the major and minor rs12979860 variants show a wide variation between East Asians, European-Americans, Hispanics, and African-Americans [93]. In East Asians, the frequency of the major allele (C allele) was over 90%, in European-Americans and Hispanics around 70%, and in African-Americans around 40%. These findings correlate very well with the overall response rate to Peg-IFN-α/ribavirin treatments in the different groups, where East Asians have SVR rates of more than 70%, European-Americans and Hispanics around 50%, and African-Americans around 25% [93]. As mentioned above, additional SNPs near the *IL28B* gene were found to be associated with SVR in large patients' cohorts from Europe, Australia, and Japan [94,96,97]. The two most widely investigated SNPs, rs12979860, and rs8099917, are in strong linkage disequilibrium and are similarly informative.

Spontaneous clearance of HCV infection

The major alleles of the *IL28B* SNPs are not only associated with better response to treatment with PegIFN-α/ribavirin, but also with spontaneous clearance of HCV infections [94,95,101]. Spontaneous clearance is rare in the chronic phase of HCV infection, but occurs in 20–40% of the patients with AHC. The rate of spontaneous clearance varies according to *IL28B* genotypes: in patients with the rs12979860 C/C genotype, spontaneous clearance occurs in 50–60% of cases, compared to only 10–20% in the C/T and T/T group [95,101].

Molecular mechanism

The molecular mechanisms that link the genetic variants near the *IL28B* gene to spontaneous and PegIFN-α/ribavirin-induced clearance of HCV remain largely unknown. Since the *IL28B* genes encodes IFN-λ3, the most obvious explanations center on mutations in the promoter or the coding region of IFN-λ3. However, no such mutations have been identified despite extensive analysis.

There is a significant association between *IL28B* genotypes and ISG expression in pretreatment liver biopsies [82–84]. The activation of the endogenous IFN system in the liver is significantly more frequent in patients with one or two minor alleles. This could be elegantly explained by an increased *IL28B* gene expression from the minor allele. However, two studies reported the opposite, i.e., less intrahepatic *IL28B* expression in patients with the minor allele genotype [82,102]. Furthermore, serum levels IFN-λ1 (IL29) and IFN-λ2/3 were reported to be lower in carriers of the minor allele [78]. Finally, a stratification of patients according to response to treatment and *IL28B* genotype revealed that hepatic ISG expression correlates with treatment response

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irrespective of the *IL28B* genotype [82]. Therefore, IFN-λ does not seem to directly control the level of ISG induction.

In conclusion, the molecular mechanism linking *IL28B* genotype to treatment response remains to be elucidated. Interesting insights recently came from studies looking at treatment responses in patients with CHC that had received liver transplants. Liver grafts are universally reinfected, and post-transplant HCV infections often have a more severe course with rapidly progressive fibrosis. Recipients with the unfavorable *IL28B* rs12979860 T allele are at risk for more severe HCV recurrence and more rapid fibrosis progression [103,104]. Surprisingly, donor genotypes had the opposite effect: donor rs12979860 C/C genotype favored inflammation and fibrosis [103,104]. Similarly, the response to treatment with PegIFN-α/ribavirin in liver transplanted patients with CHC is also associated with the *IL28B* genotype of both the donor and the recipient [102,105–108]. This finding demonstrates that the molecular mechanism linking the *IL28B* genotype to response to treatment involves both the immune system or other organs in the body (the "recipient") as well as the resident liver cells (the "donor"). How this mutual crosstalk between recipient and donor cells is dependent on the *IL28B* genotype in both cell types remains entirely unclear.

Conclusion and perspectives

The study of host virus interactions in HCV infections has not only increased our understanding of the pathogenesis of one of the most important liver diseases worldwide, but has also made important contributions in basic innate immunity and immunobiology of chronic viral infections in general. The IFN system provides a powerful antiviral response, and in order to establish a persistent infection, viruses have evolved escape strategies. HCV is no exception to that rule, but its escape strategy seems to be unique. Most viruses inhibit either the induction of IFNs or IFN signal transduction, thereby preventing the transcriptional induction of antiviral genes [109]. In the early phase of infection, HCV cannot prevent the activation of the IFN system in the liver, at least not in experimentally infected chimpanzees. However, in over half of the infected patients, HCV persists despite a rapid and strong IFN response. The molecular mechanisms of escape remain elusive. In CHC, HCV can co-exist with a permanently active endogenous IFN system for decades. When such patients are treated with PegIFN-α and ribavirin, virological response is very rare. Not only is the endogenous IFN system not capable of eliminating HCV, it even inhibits response to therapeutically injected recombinant IFN-α. In another group of patients with CHC, HCV seems to be largely ignored or tolerated by the immune system. Those patients with an activated endogenous IFN system have a very good chance to be cured by treatment with PegIFN-α and ribavirin. The underlying molecular mechanisms are not known. Refractoriness of IFN-α signaling is an important factor, and restoration of a full response capability of IFN signaling pathways or circumvention of blocked signaling pathways might provide therapeutic opportunities. Both in AHC and in CHC, ISG induction is positively correlated with viral load. One of the most interesting results from a landmark study in chimpanzees with CHC was the finding that the inhibition of viral replication by anti-miR122 resulted in a simultaneous decline of ISG induction in the liver [110]. It is conceivable that current triple therapies with protease inhibitors or other direct acting antivirals achieve such high virological response rates by restoring IFN sensitivity in patients with pre-activated (refractory) IFN systems. Indeed, the finding that the major improvements of response rates are observed in patients with unfavorable *IL28B* genotypes (associated with high ISG expression) strongly supports such a model [111–115]. Refractoriness of IFN-α signal transduction pathways could be bypassed by PegIFN-λ. As outlined above, there is experimental evidence from cell culture and mouse experiments that IFN-β and IFN-λ signaling remains intact in presence of inhibitors of IFN-α signaling such as USP18. This has not yet been confirmed in humans, but if the concept is correct, PegIFN-λ would be especially favorable for the treatment of patients who did not respond to previous (peg)IFN-α/ribavirin therapies.

The development of potent new direct acting antivirals opens the perspective of IFN-free treatments for CHC, and with it, the perspective of a declining interest of the hepatology research community in host-virus interactions in HCV infections. However, even the best treatments should not detract efforts to develop a preventive vaccine. A thorough understanding of host-virus interactions is most likely a prerequisite for the rational design of a vaccine. Furthermore, the strong association of *IL28B* genotype with spontaneous clearance of HCV and response to treatments with (and without) IFNs is a landmark discovery in HCV (and GWAS) research. The elucidation of the molecular mechanisms that link single nucleotide polymorphisms near the *IL28B* (*IFN-λ3*) gene to basic host reactions, such as spontaneous virus control, and to the response to antiviral therapies remains one of the most interesting challenges in the field.

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Conflict of interest

The author declare that he does not have anything to disclose regarding conflict of interest with respect to this manuscript.

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