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Basic

Innate and adaptive immune responses in HCV infections

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Summary

Hepatitis C virus has been identified a quarter of a decade ago as a leading cause of chronic viral hepatitis that can lead to cirrhosis and hepatocellular carcinoma. Only a minority of patients can clear the virus spontaneously during acute infection. Elimination of HCV during acute infection correlates with a rapid induction of innate, especially interferon (IFN) induced genes, and a delayed induction of adaptive immune responses. However, the majority of patients is unable to clear the virus and develops viral persistence in face of an ongoing innate and adaptive immune response. The virus has developed several strategies to escape these immune responses. For example, to escape innate immunity, the HCV NS3/4A protease can efficiently cleave and inactivate two important signalling molecules in the sensory pathways that react to HCV pathogen-associated molecular patterns (PAMPs) to induce IFNs, i.e., the mitochondrial anti-viral signalling protein (MAVS) and the Toll-IL-1 receptor-domain-containing adaptor-inducing IFN-β (TRIF). Despite these escape mechanisms, IFN-stimulated genes (ISGs) are induced in a large proportion of patients with chronic infection. Of note, chronically HCV infected patients with constitutive IFN-stimulated gene (ISG) expression have a poor response to treatment with pegylated IFN-α (PegIFN-α) and ribavirin. The 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 cell compartments that are not accessible to anti-viral IFN-stimulated effector systems, or direct antagonism of effector systems by viral proteins. Escape from adaptive immune responses can be achieved by emergence of viral escape mutations that avoid recognition by antibodies and T cells. In addition, chronic infection is characterized by the presence of functionally and phenotypically altered NK and T cell responses that are unable to clear the virus but most likely contribute to the ongoing liver disease. In this review, we will summarize current knowledge about the role of innate and adaptive immune responses in determining the outcome of HCV infection.

Keywords: Interferon; Hepatitis C virus; Innate immunity; Jak-STAT; CD8+ T cells; T cell exhaustion; Viral escape.
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Abbreviations: AHC, acute hepatitis C; CHC, chronic hepatitis C; HCV, hepatitis C virus; IFN, interferon; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; MAVS, mitochondrial anti-viral signalling protein; Mda5, melanoma differentiation antigen 5; NK cells, natural killer cells; PAMP, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cell; PHH, primary human hepatocytes; PIAS, protein inhibitor of activated STAT; RIG-1, retinoic acid inducible gene-1; TLR, toll like receptor; USP18, ubiquitin specific peptidase 18.

Introduction

Hepatitis C virus

Hepatitis C virus (HCV) infects 130 to 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 to 40 years [4]. Patients with CHC and cirrhosis are at increased risk for liver failure and for developing hepatocellular carcinoma [5].

Innate immunity and interferons

Innate immune responses are the first line of defence 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 [6]. 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). 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. Type II IFN (IFN-γ) is produced by NK and natural killer T cells as part of the innate immune response, and by antigen-specific T cells (both CD4+ Th1 and CD8+ cytotoxic T lymphocytes).

Virus infections are sensed by the toll-like receptor (TLR) dependent pathway [7,8] and the cytosolic pathway, triggered by binding of viral RNA to the RNA helicases retinoic acid inducible gene-1 (RIG-1) and melanoma differentiation antigen 5 (Mda5) [9,10]. Both pathways converge on the activation of the key transcription factors NF-κB and the interferon regulatory factor (IRF) 3 and 7. 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 [11], whereas the IFN-γ-induced gene set is more distinct [12,13]. 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) [14].

Type I and II IFNs are essential for the defence against virus. Knockout mice that lack the receptors for IFN-α or IFN-γ, or components of the IFN signal transduction pathway succumb to otherwise harmless viruses [15,16], and infants with genetic defects of the IFN system die from viral infections despite best medical care [17]. Type III IFNs have a more restricted role, most likely in the viral defence at epithelial surfaces in the respiratory and gastro-intestinal tract [18,19].

Interferon signal transduction

IFN-αs and IFN-β bind to the ubiquitously expressed IFN-α receptor. IFN-λs bind a different receptor, consisting of the ubiquitously expressed IL-10R2 chain (shared with the IL-10 receptor) and a unique IFN-λ receptor 1 chain whose expression is mainly restricted to epithelial cells [20,21]. Off note, IFN-λ receptor 1 expression is very low in control liver biopsy samples, but significantly increased in the setting of chronic viral infections [22]. IFN-γ binds to the widely expressed IFN-γ receptor. IFN receptors connect to the Jak-STAT pathway to transmit signals from the cell surface to the nucleus [23,24]. All IFNs activate STAT1 to form homodimers that translocate into the nucleus and 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) [23,25,26] (Fig. 1). Signalling through the Jak-STAT pathway is regulated by inhibitors such as SOCS, USP18, PIAS, and TcPTP. Suppressor of cytokine signalling (SOCS) proteins are rapidly induced by activated STATs and provide an early negative feedback loop [27–29]. Ubiquitin-specific peptidase 18 (USP18, also designated UBP43) is another important negative regulator in type I IFN signalling [30]. USP18 is a key mediator of the refractoriness of liver cells to continuous stimulation with IFN-α [31]. USP18 is not induced by IFN-γ, and does not inhibit IFN-γ or IFN-λ signalling [32].

Host-virus interactions in acute hepatitis C

Key Points 1

- Hepatitis C virus (HCV) has a very high replicative capacity. Within days after infection, viral titres 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 can not 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 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 patients can be cured with IFN-α monotherapy

Innate immune responses in early acute HCV infection

Prospective studies with health-care workers with AHC after accidental needlestick injury and with experimentally infected chimpanzees revealed an enormous replicative capacity of HCV [33–37]. Already within days after infection, high viral titres have been measured in the serum and the liver of chimpanzees. After a very rapid increase in the first days (to weeks) after an infection, HCV viral loads remain stable for several weeks, until the emergence of the cellular immune response in the liver [34–36,38]. 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 or the function of antiviral effector proteins in infected cells. The co-existence of high viral loads and high ISG expression has been interpreted as a proof that the innate IFN response is completely ineffective against HCV. However, the short duration of the 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.

For the following discussion, the acute phase of HCV infection will be sub-divided in 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 infections has been studied in experimentally infected chimpanzees [34–36,38] where a strong host-response to HCV has been detected already days after infection. Transcriptome analysis revealed induction of type I IFN-stimulated genes [34,35]. The extent and duration of the ISG induction showed a positive correlation with viral load [35]. 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-1/Mda5-dependent sensory

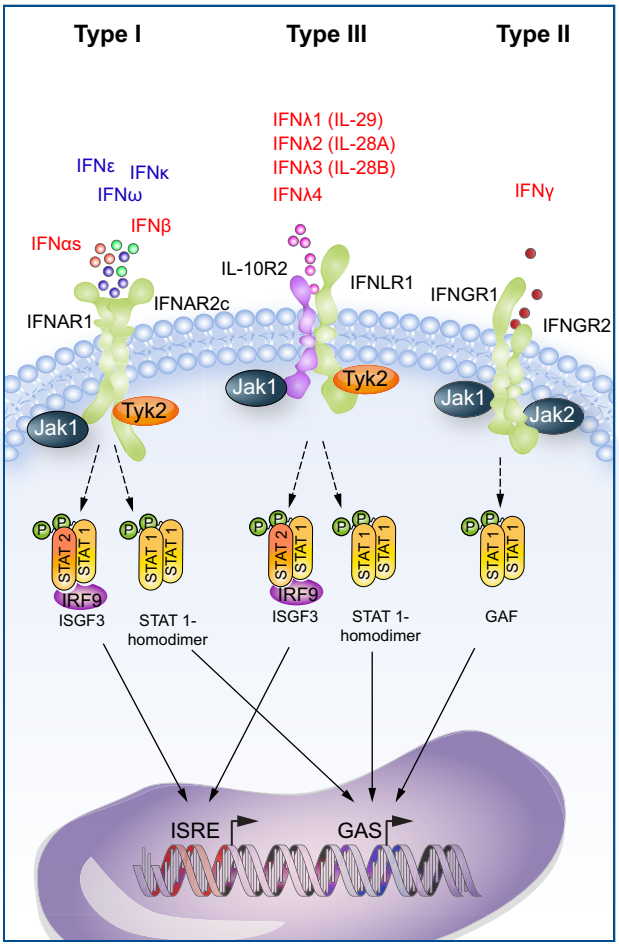

Fig. 1. IFN signalling through the Jak-STAT pathway. Type I (IFN-αs and IFN-β) and type III (IFN-λs) IFNs bind to distinct receptors, but activate the same downstream signalling events, and induce almost identical sets of genes mainly through the activation of IFN-stimulated gene factor 3 (ISGF3) and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

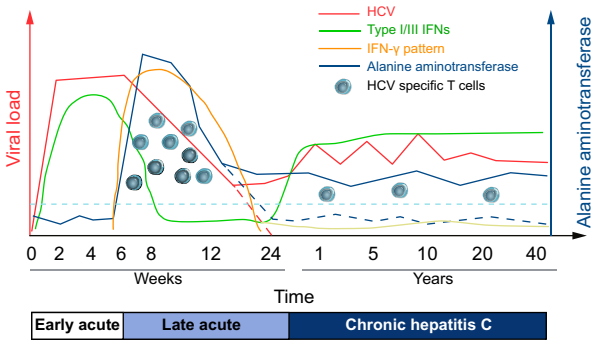

Fig. 2. Natural course of HCV infection. Within days after infection, viral load rapidly increases to a plateau of 10^5–10^7 IU/ml (red line) (IUs approximately correspond to genome equivalents). In this early phase of acute infection (the first 4–8 weeks), an innate immune response driven by type I or type III IFNs (green line) might restrict viral replication. 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 (yellow line). At the same time, alanine aminotransferase levels increase (blue line) and some patients get icteric. In late AHC, viral replication is strongly inhibited, and in about 30% of patients, HCV is completely eliminated (dashed red line) and alanine transaminase levels return to normal (dashed blue line). In 70%, HCV persists (solid red line), and alanine transaminase remains elevated (solid blue line). In the chronic phase of HCV infection, cellular infiltrates persist at a lower level, but IFN-γ driven ISG expression disappears. However, in about half of the patients, type I or type III IFN-stimulated genes are again strongly induced (green line). The other patients have little to no activation of ISGs in the liver (light green line). The light blue line shows the upper limit of normal for alanine transaminase.

pathways. The cellular source and the specific type of IFN(s), responsible for ISG induction in this phase of HCV infections, remain to be clarified. Several recent papers report IFN-λ induction in primary human hepatocytes (PHH) infected with HCV [39–41]. However, others have proposed that plasmacytoid dendritic cells, stimulated by cell-cell contact with infected hepatocytes, are the main producers of IFNs [42]. 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. Earlier studies with chimpanzees failed to detect induction of IFNs in early AHC [34,35]. More recent studies detected upregulation of mRNA of type III (but not type I) IFNs in liver biopsies [40,41], and an increase of type III IFN protein, primarily IFN-λ1 (IL29), in the serum of chimpanzees [41]. 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 [43]. It is also not clear how well human hepatocytes respond to type III IFNs. In one report, IFN-λ induced ISG expression in PHHs to a similar extent as IFN-α [44]. Different results were published in another report, where compared to IFN-α, IFN-λs were very weak activators of STAT1 phosphorylation in PHHs, because the specific chain of the IFN-λR, IFN-λR1, was found to be expressed at very low levels in uninfected, unstimulated hepatocytes [22]. However, IFN-λR1 could be rapidly induced by IFN-α [22]. The rapid induction of ISGs in early AHC might well be the results of a combined effect of type I and III IFNs.

Role of NK cells in the early phase of HCV infection

Natural killer (NK) cells are large granular lymphocytes that account for the majority of innate immune cells in the human liver [45,46]. Indeed, they are significantly increased in the liver compared to the peripheral blood although this becomes especially evident in chronic HCV infection. NK cells play an important role in the control of viral infections. They have direct antiviral as well as regulatory effects [46,47]. The direct antiviral effects are mediated by direct cytolytic (e.g., TRAIL or perforin-mediated) or non-cytolytic (e.g., IFN-γ mediated) effector functions. Several lines of evidence support a relevant role of NK cells in acute HCV infection. For example, genetic studies have demonstrated that genes encoding the inhibitory NK cell receptor KIR2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand directly influence resolution of HCV infection in patients homozygous for these genes [48]. Furthermore, $KIR2DL3^{+-}$ $NKG2A^{-}$ NK cells have been suggested to control early HCV infection prior to seroconversion and may thus result in an apparent state of "natural resistance" to HCV in persons who inject drugs

[49]. A possible role of NK cells in HCV immunobiology is further supported by the finding that they are activated in acutely infected subjects, as determined by an increased expression of the activating receptor NKG2D that is accompanied by an increased production of IFN-γ and cytotoxicity [50]. NK cell responses are also linked with T cell responses, e.g., increased degranulation of natural killer cells during acute HCV has been shown to correlate with the magnitude of virus-specific T cell responses [51,52]. Also, an activated multifunctional NK cell response, i.e., cytotoxicity and IFN-γ production, has been reported early after HCV exposure in healthcare workers who do not develop acute infection, suggesting an important contribution to the prevention of high level viremia [53,54].

Adaptive immune responses during acute HCV infection

In contrast to the innate immune responses that are induced within hours to days after infection, there is a striking and so far not understood delay of approximately 6–8 weeks before adaptive immune responses become detectable [35,38,46,55,56]. Different components of the adaptive immune system are involved in viral clearance, including humoral antibody and T cells responses (Fig. 3A) [57]. Indeed, most acutely HCV-infected individuals produce antibodies against epitopes within the structural as well as non-structural proteins. Most of them, however, have no relevant antiviral activity, and only a small fraction of antibodies is able to inhibit virus binding, entry, or uncoating. These antibodies are called 'neutralizing antibodies' [58]. Results obtained with a well-characterized and homogenous group of young women infected by an HCV-contaminated anti-D immunoglobulin preparation, containing the same virus inoculum (strain AD78), suggest the development of neutralizing antibodies in the early phase of infection in the majority of patients with resolving HCV infection [59]. In contrast, patients with a chronic course of infection showed a delayed induction of neutralizing antibodies [59]. Although this study links an early neutralizing antibody response with HCV clearance, it remains unclear whether the neutralizing antibody response really mediates viral clearance. Indeed, it is noteworthy that virus control and clearance has also been observed in the absence of neutralizing antibodies and even in hypoglobulinaemic individuals.

There is general consensus, however, that HCV elimination is associated with strong and sustained CD4+ and CD8+ T cell responses that target multiple epitopes within the different HCV proteins [33,38,60–64] and that remain detectable long after resolution of infection [64]. Several lines of evidence support the important role of both T cell subsets in controlling HCV infection: a clear temporal association between the onset of peripheral and intrahepatic virus-specific T cell responses and HCV clearance [33,38,60–64]; a strong association between certain class I (e.g., HLA-B27) and class II (e.g., DRB1*1101) alleles and spontaneous elimination of the virus [65]; and finally, the pronounced impact of CD4+ and CD8+ T cell depletion on the course of HCV infection *in vivo* [66,67]. Indeed, antibody-mediated depletion of CD8+ T cells prior experimental infection of previously protected chimpanzees led to HCV persistence until CD8+ T cell response recovered and an HCV-specific CD8+ T cell response emerged [66]. Depletion of CD4+ cells in previously protected chimpanzees led to HCV persistence and the emergence of CD8+ escape variant [67]. Collectively, these findings indicate that CD4+ T cells are central regulators, while virus-specific CD8+ T cells primarily function as the key effectors. The important role of CD4+ T cell responses has been recently further supported by a study showing an association between viral clearance and the strong expansion of CD161+CCR6+CD26+CD4+ T cell responses that produce IL-17 and IL-21 [68]. Insights into virus-specific CD8+ T cell effector functions were forthcoming from *in vitro* studies. Indeed, by using a subgenomic replicon-containing cell line that was stably transduced with the common MHC class I allele HLA-A2 gene it was shown that HCV-specific CD8+ T cells exert strong antiviral effects primarily by IFN-γ and only to a lower extent by cytolytic effector functions [69]. Of note, the possible role of IFN-γ during acute infection has also been supported in transcriptome analyses of liver biopsies of patients and chimpanzees with acute HCV infection that revealed a strong induction of IFN-γ stimulated genes [13,38]. It is currently unclear why this IFN-γ dominated response succeeds 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 fail. The number of ISGs and the expression level of ISGs seem not to be significantly different in the early *vs.* the 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 effectors in HCV infected cells. Clearly, the reasons for the failure of the innate immune system in early AHC remain unknown.

Host-virus interactions in chronic hepatitis C

Interferon-stimulated gene expression in chronic hepatitis C

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 the viral infection, but also prevents response to therapies with pegylated IFN-α and ribavirin

In humans who develop chronic infections, ISG induction varies considerable between individuals. In about half of 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

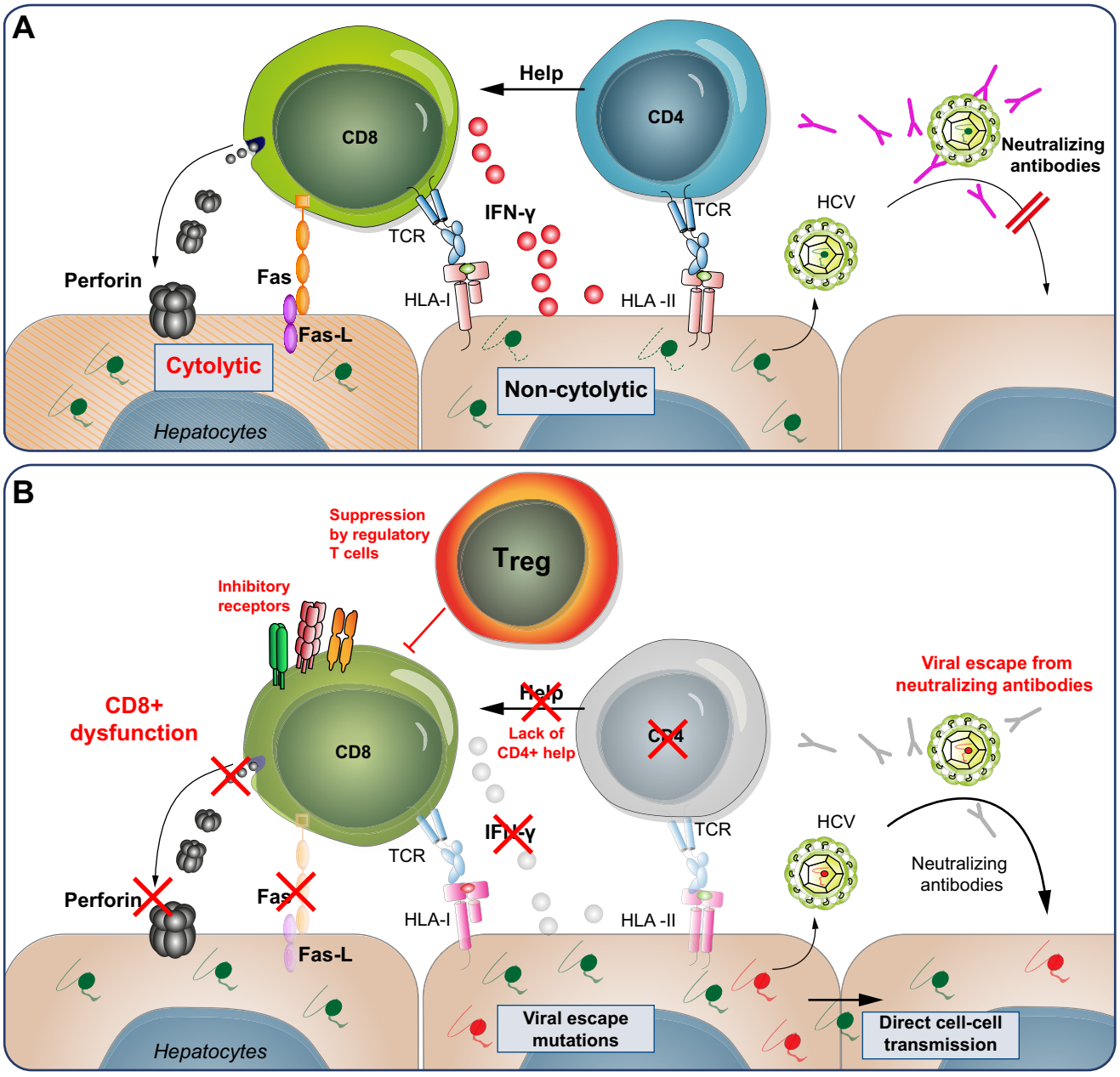

Fig. 3. Success and failure of virus-specific CD8+ T cell responses. (A) CD8+ T cells can inhibit viral replication by cytolytic (perforin) and non-cytolytic (IFN-γ) effector mechanisms. Help by CD4+ T cells is required. Neutralizing antibodies may block the virus and thus infection of further hepatocytes. (B) Several different mechanisms contribute to the failure of adaptive immune responses in HCV infection, such as viral escape, T cell exhaustion, as indicated by the expression of inhibitory receptors, lack of CD4+ T cell help or the action of regulatory T cells.

[70]. Apart from a strong association of allelic variants near the IFN-λ4 gene with ISG induction [71–75], little is known about the factors that determine the activation level of the IFN system. Inter-individual 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 [76].

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 [14,77,78]. Furthermore, there is no significant correlation between serum or intrahepatic viral loads with ISG expression levels [70,76]. The mechanisms of viral interference with the hepatic IFN system remain to be elucidated. There are numerous reports of viral interference with Jak-STAT signalling and ISG induction in cell culture systems. However, this has not been confirmed in human liver biopsies. On the contrary, using a recently developed *in situ* hybridization method that allows the detection of HCV RNA in human liver biopsies, HCV RNA and mRNA of ISGs were found to be co-expressed in hepatocytes [70]. Alternatively, HCV could inhibit cap-dependent protein translation at the ribosomes [14,77,78]. In cell culture experiments, HCV infection triggers

phosphorylation and activation of the RNA-dependent protein kinase PKR, which phosphorylates eukaryotic translation initiation factor eIF2a [79]. 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 [79]. If HCV indeed inhibits ISG protein translation in hepatocytes of infected patients remains to be clarified. A third possible level of viral interference with the IFN system could also be downstream of ISG protein production. HCV replication could occur in subcellular compartments that are not accessible to antiviral proteins, induced by IFNs, or HCV proteins could bind to and antagonize antiviral ISG proteins [13]. 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 [14,77,78,80]. 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 [14]. In such biopsies, staining for the phosphorylated (activated) form of STAT1 revealed a faint staining in nuclei of hepatocytes in pre-treatment biopsies, and no further increase of phospho-STAT1 signals 4 h after PegIFN-α injections [14]. In contrast, no phospho-STAT1 signals were detected in pre-treatment 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 [14]. The reason for the apparent refractoriness of IFN-α induced Jak-STAT signalling 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 [13]. Moreover, there is convincing genetic evidence from knockout mice experiments that USP18 is responsible for the long-term refractoriness of IFN-α signalling in the liver [31]. These observations generate an apparent paradox: Since USP18 is not constitutively expressed in cells, but is only expressed after IFN stimulation, how can its expression level be maintained at high levels despite complete refractoriness of IFN-α signalling? Or in more general terms: How can an IFN-α induced negative regulator of IFN-α signalling 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. mRNA expression of *IFN-αs*, *IFN-β*, *IFN-γ* has not been consistently detected in liver biopsies from 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 [13,14,81]. IFN-αs can be tentatively excluded because IFN-α signalling is subject to strong negative feedback inhibition, specifically by USP18, that would prevent long-lasting activation of ISGs [13,30,31]. One could only argue that the refractory state, caused by USP18, is leaky and allows low-level STAT1 activation below the detection limit of phospho-STAT1 Western blots or immunostaining techniques. However, there are more appealing alternative explanations. Interestingly, USP18 does not inhibit IFN-λ signalling [32]. Contrary to all other IFN subtypes, *IFN-λ1*, *-λ2*, *-λ3*, and the recently discovered *IFN-λ4* mRNA can be detected in liver biopsies [71,82]. 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 signalling is not refractory, IFN-λs remain strong candidates for being the drivers of constitutive ISG induction in CHC patients with an activated endogenous IFN system.

IFNλ3/4 genotype and innate immune responses to hepatitis C virus

Key Points 3

- Genetic variants of the IFN-λ3 and IFN-λ4 locus are strongly associated with spontaneous clearance of HCV and with response to therapy with pegylated IFN-α and ribavirin
- The ancestral IFN-λ4 ΔG allele (associated with poor response to therapy) is positively associated with IFN-stimulated gene expression in CHC
- The molecular mechanisms that link genetic variants near the IFN-λ4 gene with constitutive activation of the endogenous IFN system in the liver are not entirely known, but might involve an ongoing stimulation of the Jak-STAT pathway by INF-λ4 through the IFN-λ receptors on hepatocytes

The discovery of a strong association of genetic variants near the *IFN-λ3* 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 factors that control natural history, host-virus interactions and IFN responsiveness in individual patients [83–87]. More recently, an additional variant in this gene region has been described, and contrary to the other single nucleotide polymorphisms (SNPs) that have no obvious functional consequences in terms of gene expression or amino acid changes, the newly discovery TT/ΔG SNP directly controls the expression of IFN-λ4 (Fig. 4) [74]. The ancestral allele with the sequence gccGctg at position rs368234815 can give rise to a transcript with an open reading frame of 179 AA, coding for IFN-λ4. The insertion of a T and the change of the G to a T (resulting in the sequence gccTTctg at rs368234815) disrupt the open reading frame [74]. The TT allele is more frequent in Caucasians, but not in Africans. Paradoxically, the IFN-λ4 producing allele is associated with reduced spontaneous clearance rates of HCV infections and also

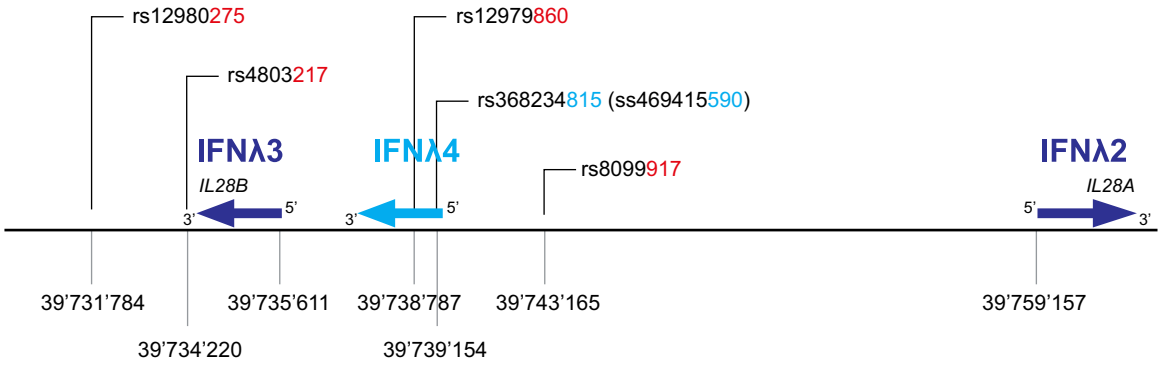

Fig. 4. *IFN-λ3/4* gene locus on human chromosome 19. The originally described SNPs strongly associated with spontaneous and treatment induced HCV clearance are located in the flanking regions of the *IFN-λ3* (*IL28B*) gene (highlighted in red) [83–87]. The more recently described SNP rs368234815 (highlighted in blue) is linked to two alleles, the ΔG and the TT alleles. The ΔG allele codes for IFN-λ4 (light blue). The insertion of a T in the TT allele introduces a frame shift and premature stop codon. The TT allele cannot code for a functional IFN-λ4 transcript. Nucleotide numbers on chromosome 19 are indicated for the SNPs and for the start of the IFN-λ3 and IFN-λ2 genes.

a dramatically reduced rate of sustained virological response to treatment with pegylated IFN-α and ribavirin [74,75,88]. The molecular link between genotype and phenotype remains to be elucidated. Importantly, the IFN-λ4 producing ΔG allele is associated with high ISG expression in pretreatment biopsies [82,89,90], and given the strong association of hepatic ISG expression with non-response to treatment with pegylated IFN-α and ribavirin, it is reasonable to speculate that IFN-λ4 induced ISG expression could be the molecular link between genotype and phenotype [83,84,86,87,91].

NK cell responses in chronic infection

In chronic HCV infection, NK cells are activated but may display alterations in phenotype and function [92]. For example, NK cells from chronically HCV infected patients express higher levels of several activating receptors, such as NKp30 and NKp46 [46,93]. Chronic exposure of NK cells to endogenous INF-α can result in increased STAT expression, and preferentially STAT1 over STAT4 phosphorylation [94,95]. Interestingly, however, similar NK cell phenotypic and functional alterations can equally be observed in chronic HBV and HDV infection, suggesting that these alterations may not be mediated by the virus but rather by disease-specific factors [96]. On the other hand, specific downregulation of, e.g., NKp30 in HCV infected cells and subsequent inhibition of NK cell function argues for a virus-specific effect [97]. Whatever the explanation, several groups have shown that NK cells, obtained from chronically infected patients, are impaired in antiviral effector function [98,99]. Interestingly, NK cells seem to be impaired especially in their ability to secrete IFN-γ, as has been reported by several but not all groups. However, cytokine-stimulated NK cell lines and primary NK cells, isolated from healthy donors, can lyse HCV-replicating cells, particularly at high effector-to-target ratios [100] and also secrete IFN-γ that mediates the inhibition of HCV replication [101]. Importantly, IFN-γ production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells, such as monocytes and plasmacytoid dendritic cells [102]. HCV may directly interfere with the action of NK cells. For example, a recent report suggests that NS5A-containing apoptotic bodies can trigger monocytes to produce increased amounts of IL-10 and decreased levels of IL-12 that leads to a significant downregulation of NKG2D on NK cells via TGF-β [103]. Another study has suggested that cell-to-cell contact with HCV-infected cells reduces functional capacity of natural killer cells [104] although NK cell function remains intact after exposure to infectious virus [105]. HCV mediated inhibition of NK cell mediated augmentation of complement synthesis has also recently been reported [106].

Of note, similar to the predictive value of high pretreatment ISG levels, NK cell responses can be used as an indicator of a patients' IFN responsiveness. Indeed, higher pretreatment levels of inhibitory receptors, such as NKG2A or activating receptors, such as NKp46 on NK cells, predict treatment failure [107–109]. Also, dynamic changes of NK cells are observed during therapy with an association between higher NK perforin content, lower CD16 expression, and higher natural and antibody-dependent NK cell cytotoxicity with a virological response [110]. Patients with a rapid first phase HCV RNA decline after initiation of IFN-based therapy show a maximal phospho-STAT1 induction *in vivo* and are refractory to further IFN-α stimulation *in vitro*. In contrast, patients with a slow first phase HCV RNA control show lower phospho-STAT levels in their NK cells and the IFN-α responsiveness is retained. Also, treatment responders show greater levels of NK cell degranulation than non-responders, specifically in the first 12 weeks of therapy [94,111].

Adaptive immune responses in chronic hepatitis C

HCV can persist in the majority of chronically HCV infected patients despite the presence of HCV specific neutralizing antibodies and T cell responses. The latter contribute most likely to the progression of liver disease. Multiple mechanisms for the failure of the adaptive immune responses have been suggested (Fig. 3B). For example, evolution of viral quasi-species within targeted epitopes may lead to escape from neutralizing antibodies and T cells [112]. Interactions of HCV glycoproteins with high-density lipoprotein (HDL) and the scavenger receptor B1 (SCARB1) may protect from neutralizing antibodies (100), and specific glycans on E2 may modulate cell entry and confer protection from neutralizing antibodies [113,114]. Interestingly, it has also been suggested that HCV may evade neutralization by direct cell to cell transfer of the virus [115,116].

HCV specific T cell failure is primarily caused by T cell exhaustion and the emergence of viral escape mutations. However, results obtained from the early phase of acute HCV infection in chimpanzees [38] and in health care workers infected via

needlestick exposure [33] also support the hypothesis that at least in some patients virus-specific T cells are not or only weakly primed during acute HCV infection. Impaired priming of HCV-specific CD8+ T cells might be mediated by low numbers or functional impairments of antigen-presenting cells such as macrophages or dendritic cells [47].

In most chronically HCV infected patients, however, virus-specific T cells are present and even enriched in the liver [65]. Viral escape in HCV infection has first been reported in chronically infected patients [117] and experimentally infected chimpanzees [118,119], and subsequently in acutely infected humans [120,121]. Of note, the emergence of viral escape mutations seems to be associated with the development of chronic infection, and the absence of escape mutations is associated with viral clearance [120]. Viral escape, however, is not universal. Indeed, the occurrence of viral escape may be limited by insufficient CD4+ T cell help, by a limited TCR diversity, by functional alterations of CD8+ T cells, or by viral fitness cost, e.g., the inability of the virus to tolerate mutations in certain viral regions [65]. Indeed, fitness cost might not only explain the occurrence of reversion after removal of T cell pressure or the absence of viral escape in specific CD8+ T cell epitopes [122,123], but may also directly contribute to the protective effect of specific CD8+ T cell responses [122]. Importantly, this has been suggested to be the case for the protective HLA-B27 and HLA-A3 alleles [124,125] where epitopes are targeted that do not easily allow viral escape mutations because of high costs to viral replicative fitness. It is important to note that viral escape is not limited to CD8+ T cell epitopes. Indeed, escape mutations can also occur in MHC class II-restricted epitopes although they are rarely found in chronically infected patients and chimpanzees [126].

A hallmark of chronic HCV infection is the presence of functionally impaired virus-specific CD8+ T cells that are characterized by their inability to secrete antiviral cytokines, such as IFN-γ, or to proliferate [57,127]. This state of T cell exhaustion is characterized by an upregulation of inhibitory receptors, such as PD-1 [128–131] and a low expression of CD127 [128,131]. Intrahepatic HCV-specific CD8+ T cells with a high PD-1 expression are prone to apoptosis [132]. Importantly, the impaired proliferative response of CD127-PD-1+ HCV-specific CD8+ T cells to antigenic stimulation can be increased by blocking antibodies targeting PD-1 [128,129,131]. However, the dysfunction of CD127- cells is not solely caused by inhibitory signals via PD-1, since PD-1 blockade alone was unable to restore the function of strongly inhibited HCV-specific CD8+ T cells in the liver, and targeting of additional inhibitory receptors, e.g., CTLA-4 or TIM-3 may be required for restoration of T cell function [133,134]. Noteworthy, the expression of TIM-3 may specifically identify exhausted HCV specific CD8+ T cells in the liver [135]. Indeed, the liver environment itself has been recently shown to affect the expression pattern of inhibitory receptors on virus-specific CD8+ T cells [135]. A recent study has also suggested a role for 2B4 in HCV-specific CD8+ T cell dysfunction [136]. Thus, it appears that T cell exhaustion is not mediated by a single but rather by the co-expression of several different inhibitory receptors. Indeed, CD127low HCV-specific CD8+ T cells were shown to co-express the inhibitory receptors 2B4, KLRG1, and CD160 in addition to PD-1 in chronic HCV infection [137]. These observations may also explain the limited clinical efficacy of PD-1 treatment in chronically infected humans and chimpanzees [138,139].

It is also important to note that PD-1 expression during acute HCV infection does not predict the outcome of infection, suggesting that PD-1 may rather be a marker of activation than exhaustion, at least during acute infection [140,141].

Next to the expression of inhibitory receptors, the lack of CD4+ T cell help or the action of regulatory T cells or cytokines may also contribute to virus-specific CD8+ T cell exhaustion. Indeed, weak and dysfunctional HCV-specific CD4+ T cell responses have been reported in chronic infection [142]. Also, a higher frequency of suppressive CD4+CD25+ T cells has been found in in chronically HCV-infected patients [143–145]. HCV specificity *in vivo* might be mediated by the enrichment of CD4+CD25+ T cells in the liver [146] where they might limit immunopathology in the chronic phase of HCV infection by inhibiting virus-specific CD8+ T cells by direct cell to cell contact [147]. Another type of regulatory T cells in HCV infection are virus-specific regulatory CD8+ T cells that express high levels of IL-10. These regulatory T cells have been detected in the liver of HCV-infected individuals and their suppression of virus-specific CD8+ effector T cells could be blocked by neutralizing IL-10 antibodies [148,149].

Collectively, these results suggest that several different mechanisms contribute to HCV-specific T cell dysfunction, however, the relative contribution of each of these different pathways needs to be clarified in future studies. The contribution of ongoing antiviral therapy is also not entirely clear. Indeed, in contrast to early successful treatment with pegylated type I IFN during acute infection that has been shown to lead to restoration of virus-specific CD8+ T cell function, IFN therapy in chronic infection has not been reported to lead to HCV specific CD8+ T cell restoration [150–153]. Interestingly, however, direct antiviral IFN free therapies can restore HCV-specific CD8+ T cell function [154]. Although this needs to be confirmed in further studies using different antiviral regiments, these results may indicate that ongoing replication may directly contribute to HCV-specific CD8+ T cell failure.

Conclusion and perspectives

In recent years, we have seen a tremendous development of potent new direct acting antiviral IFN free therapy regiments that lead to sustained virological response rates of almost up to 100%. This is amazing, considering that HCV has been identified only a quarter of a century ago. Also, in these years, important novel insights into innate and adaptive immune responses and their role in determining the outcome of natural infection and treatment response have been made. Indeed, the study of host virus interactions in HCV infection 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 and adaptive immunity of chronic viral infections in general. For example, we have learned about several general but also about unique escape strategies, utilized by HCV to avoid recognition by innate and adaptive immune responses. It is striking that HCV can persist, despite a rapid, strong and sustained IFN response. Also, when chronically infected patients with induced ISGs are treated with PegIFN-α and ribavirin, virological response is very rare. Thus, the endogenous IFN system does not only fail to eliminate 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. In these patients, the IFN system can be activated therapeutically by treatment with PegIFN-α and ribavirin with a high chance of a sustained virological response. The underlying molecular mechanisms are not known. It is interesting to note, however, that ISG induction is positively correlated with viral load and that inhibition of viral replication, e.g., by anti-miR122 resulted in a simultaneous decline of ISG induction in the liver [155]. Adaptive immune responses fail, due to the emergence of viral escape mutations, and the development of functional alterations. Although we have learned a great deal about the possible mechanisms contributing to the failure of innate and adaptive immune responses, there are still several important questions that yet have to be solved. For example, very little is currently known about the interaction between innate and adaptive immune responses. This important question is difficult to address in the absence of infectious small animal models. Recent studies in the LCMV mouse model, however, have suggested that early IFN induction may interfere with the induction of virus-specific T cell responses [156]. Thus, it is tempting to speculate that early IFN induction may also be associated with the late priming of T cells or may even contribute to viral persistence by inhibiting HCV replication to a degree that it is not recognized by the adaptive immune response.

In addition, a thorough understanding of host-virus interactions is a prerequisite for the rational design of a vaccine. Furthermore, the strong association of the *IL28B* (*IFN-λ4*) 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 the *IFN-λ4* genotype with basic host reactions, such as spontaneous virus control and with the response to antiviral therapies remains an important challenge in the field. Taken together, there is clearly no reason for a declining interest of the hepatology research community in host-virus interactions in HCV infections as several important biological and clinical relevant questions still need to be addressed. This is of utmost importance not only for a better understanding of HCV but also of liver disease and viral hepatitis immunobiology in general.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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