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Between Scylla and Charybdis: The role of the human immune system in the pathogenesis of hepatitis C

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Abstract

Hepatitis C virus (HCV) frequently elicits only mild immune responses so that it can often establish chronic infection. In this case HCV antigens persist and continue to stimulate the immune system. Antigen persistence then leads to profound changes in the infected host's immune responsiveness, and eventually contributes to the pathology of chronic hepatitis. This topic highlight summarizes changes associated with chronic hepatitis C concerning innate immunity (interferons, natural killer cells), adaptive immune responses (immunoglobulins, T cells, and mechanisms of immune regulation (regulatory T cells). Our overview clarifies that a strong anti-HCV immune response is frequently associated with acute severe tissue damage. In chronic hepatitis C, however, the effector arms of the immune system either become refractory to activation or take over regulatory functions. Taken together these changes in immunity may lead to persistent liver damage and cirrhosis. Consequently, effector arms of the immune system will not only be considered with respect to antiviral defence but also as pivotal mechanisms of inflammation, necrosis and progression to cirrhosis. Thus, avoiding Scylla - a strong, sustained antiviral immune response with inital tissue damage - takes the infected host to virus-triggered immunopathology, which ultimately leads to cirrhosis and liver cancer - the realm of Charybdis.

Core tip: This topic highlight on the immunopathogenesis of chronic hepatitis C addresses changes in innate immunity (interferons and natural killer cells), adaptive immunity and immunoregulation (regulatory T cells). Our review provides a succinct but comprehensive overview and presents the concept, that effective antiviral immunity is associated with pronounced acute liver damage, while during chronic infection the arms of immunity will acquire new functions, which will cause and maintain tissue damage. Thus, the immune response becomes part of the mechanisms that eventually lead to progressive inflammation, liver cirrhosis and death in chronic hepatitis C.

INTRODUCTION

Scylla and Charybdis were two immortal and irresistible sea monsters in Greek mythology believed to live on either side of the Strait of Messina between Sicily and Italy. Scylla was a six-headed supernatural creature - probably

reflecting a shoal that devoured whatever came within her reach, and Charybdis was a whirlpool off the coast of Sicily. Avoiding Charybdis meant passing too close to Scylla and vice versa. According to Homer, the Greek hero Odysseus opted for Scylla when passing the strait, and had to sacrifice six of his companions rather than to risk the loss of his vessel in the whirlpool. Thus, being "between Scylla and Charybdis" means to be forced to make a choice between two equally unpleasant evils.

This allegory matches the challenge of the human immune system when defending against a viral infection, such as hepatitis C which has a high potential to establish chronic persistence. On one hand a strong and efficient immune response rapidly clears the virus; accepting the risk of severe tissue damage from immune-mediated destruction. On the other hand a less vigorous response allows for viral persistence and facilitates a low-level smoldering inflammation, which eventually results in progressive liver disease and ultimately death of the individual. In line with this analogy, acute self-limited hepatitis C is frequently associated with symptomatic disease and jaundice, while chronic hepatitis C often establishes in the absence of any characteristic symptoms[1,2]. Studies in various expression systems (cell culture or transgenic mice) indicate that hepatitis C virus (HCV) is not directly cytopathic, and viral replication may occur in the absence of any detectable inflammatory reaction[3,4]. On the other hand, chronic hepatitis C is associated with liver cell damage and intrahepatic inflammatory infiltrates. Of note, hepatocellular damage coincides with the onset of an immune response during acute infection but not with that of viral replication[5]. Thus, activation of the immune response is a pivotal factor for the pathogenic processes in hepatitis C leading to progressive tissue injury. Ultimately, hepatic inflammation and progressive fibrosis in chronic hepatitis C may result in cirrhosis and carry a high risk for hepatocellular carcinoma.

BASIC FACTS

HCV is a hepacivirus of the *Flaviviridae* family. Its genome consists of a single strand positive sense RNA. After cell entry the viral genome is translated into a single polyprotein which is co- and post-translationally cleaved into structural and non-structural proteins by host peptidases and two virus-encoded proteases. Replication involves generation of an antigenomic replication intermediate, and probably intermediate double-stranded RNA (ds-RNA) products, which can trigger intracellular pattern recognition receptors. The new viral genomes are packaged into viral particles by the viral non-structural proteins, which then are released from hepatocytes in association with host lipoproteins. Thus, HCV circulates in blood as a lipoprotein-coated virus[6]. During replication HCV is sensed by pattern recognition receptors (PRRs) in the host cell which detect pathogen-associated molecular patterns within viral products. This process then leads to coordinated activation of innate and adaptive immune responses. Both arms of the immune response work together in an integrated fashion to recognize and defend against HCV infection.

Innate responses to HCV comprise both cellular responses, such as recognition of non-self by various types of natural killer (NK) cells and humoral components, such as induction of a variety of cytokines, especially interferons. These various elements of innate immunity act in a highly integrated fashion as do innate and adaptive immune responses. Thus, development of adaptive B and T cell immunity is shaped by the initial innate responses, in particular interferons and other inflammatory and immunoregulatory cytokines that are induced by viral invasion[7]. However, despite these immune defences, hepatitis C becomes chronic in about 70%-80% of acute infections[8]. Failing immunity and continued viral persistence lead to sustained inflammatory host responses which then become the key mechanism for tissue injury in chronic hepatitis C.

INNATE IMMUNITY IN HEPATITIS C

Three types of PRRs are known to detect HCV: (1) the retinoic acid inducible gene- I (RIG- I)-like receptors, RIG- I and melanoma differentiation antigen 5, which sense viral RNA in the cytosol; (2) toll-like receptors (TLRs), such as TLR3, which detects ds-RNA fragments in the endosomal compartment; and (3) the non-traditional pattern recognition receptor protein kinase R (PKR), which binds ds-RNA binding and upon activation promotes interaction with mitochondrial antiviral signaling protein (MAVS) to trigger innate immunity[9].

RIG- I signaling is initiated by binding of the HCV PAMP RNA which consists of an exposed 5'triphosphate and the 3'poly-U/UC-rich untranslated region of the HCV RNA[10,11]. These regions are located at opposite ends of the viral genome but are brought together by intra-genomic interactions. In this configuration the viral RNA comes into close contact with RIG- I and induces conformational changes of RIG- I . RIG- I activation leads to the formation of a multi-component complex with MAVS (also termed interferon beta promoter stimulator protein 1 or card adaptor inducing interferon beta, cardiff). Finally, the interferon signaling cascade results in the activation of multiple transcription factors, such as interferon-regulatory factor-3 (IRF-3) and nuclear factor kappa B and production of multiple pro-inflammatory cytokines[12].

HCV dsRNA intermediates, which occur late in HCV replication, have been identified as ligands for TLR3[13]. TLR3 signals are transmitted by the adaptor molecule TIR-domain-containing-adaptor-inducing-interferon-β (TRIF) and also lead to production of interferons and pro-inflammatory cytokines[14]. TLR3 mediated interferon and cytokine responses are considered a secondary innate immune defense after initial RIG- I activation to establish an antiviral state and trigger T cell recruitment in HCV infection.

The ligand for PKR is the structured RNA at the internal ribosomal entry site (IRES) of HCV RNA[15,16]. Binding of HCV RNA induces phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2α). In addition, RNA binding also triggers a kinase-independent signal transduction cascade involving MAVS which finally activates interferon-β and interferon-stimulated genes (ISGs)[9,16].

Although HCV can be detected effectively by RIG-Ⅰ, TLR3 and PKR, it frequently establishes chronic persistence in up to 80% of patients, because it has evolved several mechanisms to counter-act innate immunity. The multi-functional HCV NS3/NS4A protease is a key component of the HCV evasion strategy from innate immunity. Studies in Huh-7 cells indicate that HCV initially activates the RIG-Ⅰ pathway which is shut down as infection progresses and NS3/NS4 abundance increases[17]. In addition to proteolytically processing the HCV polyprotein, NS3/NS4A can block RIG-Ⅰ signaling, because it cleaves MAVS from intracellular membranes[18-21]. This cleavage prevents signal transduction, abrogates interferon induction and facilitates progression to chronic infection. However, other hepatotropic viruses, such as hepatitis A virus also encode proteases that can cleave MAVS but in general do not become chronic[22]. Thus, MAVS cleavage alone is not sufficient for viral chronicity. Nevertheless, cleavage of MAVS has been demonstrated in the livers of patients with chronic hepatitis C, and patients with cleaved MAVS revealed reduced interferon pathway activation, although this inverse correlation was rather weak[23].

The NS3/NS4A protease can also cleave TRIF[24], the adaptor protein of the TLR3 pathway, and the relative abundance of this protein is reduced after HCV infection, probably as a result of degradation following its cleavage by NS3/NS4A[25]. Although details are insufficiently understood at present, blocking of the TLR3 pathway by HCV also seems to contribute to establishing chronic infection. TLR3-independent sensing of RNA which signals *via* TRIF has also been described, and is likewise blocked by NS3/NS4A targeting of TRIF[26]. Finally HCV proteins E2, NS3, NS4A and NS5 provide several strategies to interfere both with PKR signaling and PKR-regulated inhibition of translation[27-29]. However, these interactions are complex and the exact mechanisms how they support HCV persistence are still unclear.

Continued triggering of PRR pathways in chronic hepatitis C is likely to contribute to immunopathology, such as hepatic inflammation, fibrosis progression and HCV-associated malignancy. In this context it is interesting to note that HCV proteins core and NS3 also trigger TLR 1-2 and 2-6 dimers[30,31], and there is evidence from genetic epidemiology and functional *in vitro* studies that HCV-TLR interactions might contribute to hepatic fibrogenesis and cirrhosis[32,33], development of liver cancer[34], HCV-associated autoimmunity and B cell lymphoma[35].

INTERFERONS

HCV recognition by PRRs ultimately leads to induction of antiviral cytokines termed interferons (IFNs). Type I IFNs (several interferons-α and interferon-β) bind to the ubiquitously expressed type I interferon receptor, while type Ⅲ IFNs [IFN-λ1 alias interleukin (IL)-29, IFN-λ2 alias IL-28A, IFN-λ3 alias IL-28B] have their own receptor consisting of the IL10R2 chain (IL-10 receptor beta chain) and a unique IFN-λ receptor chain with a limited expression mainly on hepatocytes[36,37]. The type Ⅱ interferon IFN-γ has its own IFN-γ receptor. All IFN receptors transmit signals from the cell surface to the nucleus *via* the Jak-STAT pathway to activate interferon stimulated genes (ISGs). Specifically type I and Ⅲ IFNs induce IFN-stimulated gene factor 3 consisting of phosphorylated STAT1 and 2 proteins and IRF9 which activate the IFN-stimulated response elements (ISRE) of multiple genes contributing to antiviral activity[38-40].

IFN signaling is regulated by suppressors, such as suppressor of cytokine signaling and ubiquitin specific peptidase 18 (USP18) which provide important negative feed-back loops[41-43]. USP18 is a protease cleaving ISG15 from its target proteins, also including STAT1[44]. ISG15 is conjugated to STAT1 by the sequential action of several enzymes[45]. This so-called ISG-ylation and its de-conjugation by USP18 modify signal transduction pathways and immune responsiveness[46,47]. However, recently it has been recognized that USP18 suppresses IFN-signaling independently from its de-conjugating activity by interfering with the interaction between Jak1 and the type Ⅰ IFN receptor[48]. USP18 is a major mediator of unresponsiveness to type 1 IFNs in liver cells[49]. However, it does not inhibit signal transduction of type Ⅱ and Ⅲ IFNs[50].

Activation of the endogenous IFN system in the liver exerts little anti-HCV activity, and it has been well established that patients with high activation of the endogenous IFN system respond poorly to IFNα based therapies[51-55]. It has been proposed that expression of HCV proteins inhibits binding of activated STATs to ISRE[56], and Jak-STAT signaling was found to be inhibited both in HCV transgenic mice and liver biopsies from patients with hepatitis C[57,58]. Beyond that, phosphorylation and activation of STAT3 is involved in the antiviral IFN activity[59], and STAT3 expression was found to be reduced in HCV-infected livers[60]. Indeed, HCV core protein can prevent STAT3 phosphorylation[57,60,61], and this has been associated with HCV resistance to IFN-α[62]. Next, HCV-induced PKR activation inhibits cap-dependent translation of antiviral host proteins at the ribosomes owing to phosphorylation of eIF2α while production of HCV proteins is not impaired, because translation occurs *via* an IRES-dependent mechanism[63]. Of note, most studies on endogenous ISG induction in hepatitis C were based on steady state mRNA level measurements rather than determination of protein concentrations[51-55]. Finally, HCV proteins might directly inhibit ISG antiviral

effector functions apart from their inhibition of ISG translation. This concept is supported by experimental evidence from knock-out mice which demonstrated that expression of the USP18 leads to a long-term refractory state towards IFNα stimulation[49]. Likewise, strong USP18 expression was found in many hepatocytes of patients with chronic hepatitis C and high endogenous IFN activity, when histological specimens were studied[64]. At present the cellular sources and involved types of IFNs that maintain long-term ISG expression in chronic hepatitis C are still a matter of debate. IFN-λs are strong candidates as triggers of ISG induction in patients with chronic hepatitis C and endogenous activation of the IFN system, because, unlike all other IFN types, IFN-λ mRNA is readily detected in liver biopsies[53], and their action is not inhibited by USP18[50].

In patients with chronic hepatitis C endogenous ISG induction varies considerably between individuals, and this variability, as well as differential responsiveness to exogenous IFN-α is attributed to a combination of viral and host factors. For instance, difficult-to-treat HCV genotypes 1 and 4 induce high levels of endogenous IFN expression in hepatocytes resulting in an IFN-insensitive state that attenuates treatment responses[65]. Of note, endogenous ISG induction in Kupffer cells, the resident liver macrophages, is also a strong predictor of treatment responsiveness[66]. However, the relationship between baseline ISG induction and treatment outcome is opposite to that observed in hepatocytes: Virtually all non-responders lack baseline induction of ISGs whereas strongly induced ISG expression is found in responders[67]. This finding suggests that ISG induction in Kupffer cells may have a protective role for the host concerning both spontaneous HCV elimination and treatment outcomes.

Apart from viral factors genome-wide association studies have identified single nucleotide polymorphisms (SNPs) upstream of the *IFNL3* gene on chromosome 19q13, which are associated with outcomes of HCV infection both under IFN-based therapy of chronic hepatitis C[68-70] and disease evolution during acute HCV infection[71,72]. Although some initial studies failed to find a relationship between the SNPs and IFNL3 mRNA expression[71,73], it has meanwhile become clear that SNPs in this region alter IFN-λ expression levels[53,68,70,74-76], and the unfavorable minor alleles result in less *IFNL3* expression. Thus, it is quite unlikely that these SNPs simply reflect linkage disequilibrium with some other gene. However, the molecular and cellular mechanisms that underlie this association between outcomes of HCV infection and the *IFNL3* gene locus are not yet understood. It has been proposed that the unfavorable *IFNL3* variants may lead to compromised innate immune functions in particular with respect to natural killer cell activity[77,78]. However, given the fact that NK cells do not express type Ⅲ IFN receptors this hypothesis needs refining[79]. In addition, a dinucleotide polymorphism upstream of the *IFNL3* gene has been described, which

can create or disrupt an alternative open reading frame giving rise to a new gene, termed *IFNL4*[80,81]. Although it has been proposed that loss of *IFNL4* expression should be protective against HCV, it is as yet not clear if the putative *IFN-λ4* gene product plays any role for differential immune responses to HCV infection.
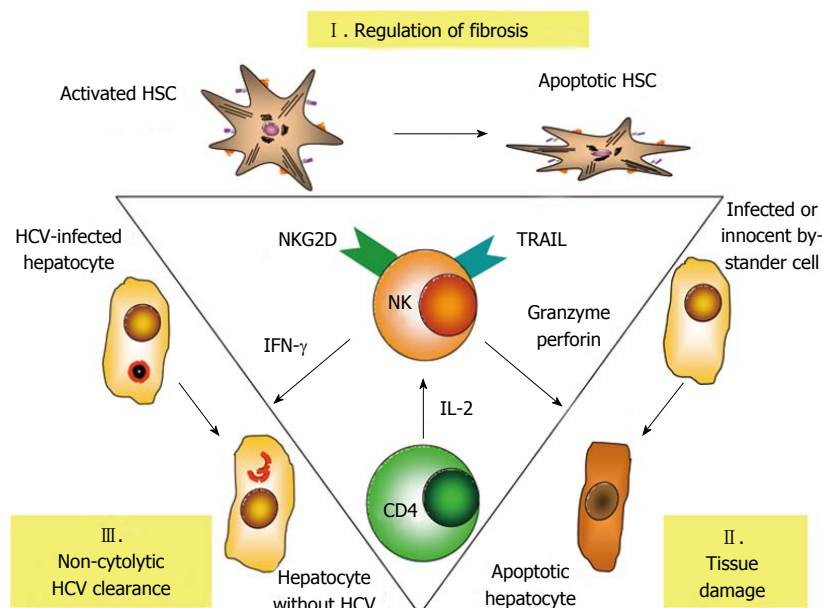
NATURAL KILLER CELLS

NK cells constitute a first line of defence against viral infections. They rapidly recognize and lyse virus-infected cells, inhibit viral replication but also exert immune-regulatory functions. NK cells constitute approximately 30% of resident lymphocytes in a normal liver, and may account for as many as 60% of lymphocytes in HCV infection[82].

Activation of natural killer cells results from the integration of multiple activating and inhibitory signals *via* specific receptors. The most important NK cell receptors (and their cognate ligands) comprise the killer immunoglobulin-like receptor (KIR) family (ligands: HLA-A, -B and -C), the CD94-NKG2A/C complex (ligand: HLA-E), NKG2D (ligands: MIC-A and MIC-B and others) and the natural cytotoxicity receptors NKp30, NKp44 and NKp46[83]. In addition, part of these receptors also exerts immune-regulatory functions in subsets of T lymphocytes. NK cells are activated, when there is a relative reduction of inhibitory signals, *e.g.*, down-regulated MHC class I expression on virus-infected cells, or a relative increase in signals from activating receptors, *e.g.*, binding of antibody-coated antigens[84]. However, conventional MHC class I expression is not substantially reduced in hepatitis C, and it has been proposed that NK cell functions might be altered by binding of HCV-derived peptides to non-polymorphic restriction molecules, such as HLA-E[85,86]. NK cells are recruited to inflammatory sites by a variety of chemokines and can also be stimulated by cytokines, such as IFN-α and ILs 8, 12, 15 and 18[87].

Activated NK cells with potent de-granulation and substantial cytokine production have been described in acute HCV infection[88,89], and there is accumulating evidence to suggest that NK cells play an important role in the antiviral immune response to hepatitis C and later on also in the immune-mediated pathogenesis of chronic hepatitis C. NK cells can inhibit HCV replication *in vitro* both by IFN-γ mediated non-cytolytic as well as granzyme/perforin and TRAIL-mediated cytotoxic mechanisms[90]. While HCV-infected hepatocytes up-regulate expression of TRAIL receptors[91], *in vivo* IFNγ-mediated clearance of HCV might be more important than direct cytolysis, because cytolytic elimination of all HCV-infected hepatocytes would lead to extensive liver damage[92]. Multi-functional NK cells are also detectable early after HCV exposure in health-care workers and iv drug users, who do not proceed to develop acute hepatitis; suggesting a potentially protective role of NK cells in early HCV infection[93,94]. Further support for a pro-

Figure 1 Central role of natural killer cells in the pathogenesis of hepatitis C. Natural killer (NK) cells regulate fibrosis by killing of activated hepatic stellate cells (HSC), which trigger NK cell activation *via* natural killer cell receptor with extracellular C-type lectin domains (NKG2D) signalling. The release of granzyme/perforin and cytotoxic cytokines, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce tissue damage. Interferon-γ (IFN-γ) released from NK cells can clear hepatitis C virus (HCV) infection from infected hepatocytes without cytolysis. On the other hand NK cell activity is critically dependent on sufficient supply with interleukin 2 (IL-2) from CD4$^+$ T cells.

tective role of NK cells in HCV infection comes from genetic studies, where genes encoding the inhibitory receptor KIR2DL3 and its ligand human leucocyte antigen group 1 (HLA-C1) seem to favour both spontaneous and treatment-induced elimination of HCV[95,96]. Since the affinity between inhibitory KIR2DL3 and HLA-C1 is weaker than all other combinations, it is reasonable to assume that a lower threshold of activation is needed to trigger KIR2DL3 NK cell responses in HLA-C1 homozygous individuals[88,97]. Finally, NK cells can become more activated upon IFN-based therapy and may contribute to HCV elimination by TRAIL-mediated cytotoxic mechanisms[98]. Interestingly, responsiveness in this setting again depends on the endogenous IFN-α activation state, since a rapid first phase HCV decline is associated with strong induction of STAT1 phosphorylation, whereas non-responders exhibit reduced STAT1 induction[99]. Chronic exposure of NK cells to IFN-α results in preferential STAT1 over STAT4 phosphorylation, which is associated with increased STAT1-dependent cytotoxicity but reduced STAT4-dependent IFN-γ production[99-101]. These findings correspond to NK cell phenotypes and functional differentiation seen at later stages in IFN-α responders and non-responders[100,102], although patients who achieve a sustained virological response also exhibit substantial NK cell cytotoxicity[103].

NK cells in chronic hepatitis C have been reported to also express altered patterns of NK receptors (Figure 1). Although reported patterns are somewhat inconsistent and may vary between peripheral blood and the liver, altered expression on NK cells has been reported for receptors NKp30, NKp44, NKp46, NKG2A, NKG2C, NKG2D and CD122[100,104-107]. In addition, NK cells ex-

press the tetraspanin CD81, a co-receptor of HCV, and *in vitro* binding of the HCV envelope 2 (E2) protein to CD81 has been shown to block antiviral functions of NK cells and to alter their migratory behaviour[108-111]. However, the experimental setting of these studies involved cross-linking of HCV E2 on plastic plates, whereas NK cells exposed to intact virions did not exhibit altered functionality[112]. Thus, it remains to be elucidated if cross-linking of CD81 by HCV E2 affects functions of NK cells to facilitate chronic infection. A particularly interesting NK cell receptor is NKp46, since it is considered a major activating receptor in hepatitis C, which also has a role in the regulation of adaptive immunity: High expression of NKp46 defines a NK cell subset with high cytotoxic activity and IFN-γ production that accumulates in the liver in chronic hepatitis C[113,114]. Of note, recently Pembroke *et al*[115] confirmed intrahepatic enrichment of NKp46+ NK cells in chronic hepatitis C and reported a high (> 80%) frequency of NKp46+ cells in the liver to be associated with pronounced inflammation in histology. Another important finding of this study was the observation, that expression of NKp46 could predict responses to IFN therapy. Patients with chronic hepatitis C, who successfully cleared their HCV infection, had lower mean frequencies of activated NKp46+ NK cells than patients who did not respond to therapy. The possible identification of NKp46 as a marker of both IFN-un-responsiveness and hepatic inflammation bears some similarity to the paradoxical relationship between IFN-un-responsiveness and high baseline ISG expression and may be linked to chronic endogenous interferon exposure. On the other hand, unlike Pembroke *et al*[115] the group of Golden-Mason[113] reported increased NKp46

expression in white female Americans as opposed to male African-Americans and proposed that a high proportion of functionally active NKp46+ NK cells could explain their higher response to IFN therapy. Thus, the precise role of NKp46+ NK still remains elusive.

Finally, NK cell-mediated cytotoxicity against hepatic stellate cells (HSC) may contribute to the regulation of intrahepatic fibrosis in hepatitis C. HSC store vitamin A, reside in the space of Dissé, and produce extracellular matrix proteins upon activation, *e.g.*, upon TLR stimulation, exposure to cytokines or reactive oxygen species[116]. HSC activation leads to trans-differentiation into myofibroblasts, which in the mouse also alters the balance in the expression between activating and inhibitory NK cell receptor ligands, so that they become target cells for NKG2D-, TRAIL- and granzyme-mediated killing by NK cells[117,118]. NKG2D- and TRAIL-mediated killing by NK cells has now also been reported for human HSC in chronic hepatitis C[119], and CXCR3 + CD56[Bright] as well as NKp46+ NK cells express particularly high cytotoxic capacity against HSC in chronic hepatitis C[114,120]. Importantly, when other processes, such as CD4 T cell depletion in HIV/HCV co-infection interfere with the regulation of hepatic fibrosis by NK cells, this may result in accelerated fibrosis progression[121].

ADAPTIVE IMMUNITY IN HEPATITIS C

A coordinated immune response involving both antibodies and T cell responses is normally required for efficient adaptive immunity. However, in hepatitis C the role of antibodies is complex: Circulating antibodies against structural and non-structural components are generated in virtually all patients irrespective from the outcome of HCV infection. A rapid induction of neutralizing antibodies early in the course of hepatitis C has been demonstrated to contribute to HCV clearance[122], but broad antibody responses usually occur at the stage of chronic infection and are not neutralizing[123,124]. Neutralizing antibodies frequently recognize the HCV envelope proteins[125-127]. However, these proteins have a high degree of mutational diversity, so that antibody responses are frequently directed against only a single strain or are easily evaded by viral mutations[124]. It is also quite likely that glycosylation of HCV proteins and the close association of the virus with lipoproteins further prevent antibody recognition. HCV antibodies are not required to clear HCV infection as has been demonstrated in patients with hypo-gammaglobulinemia[128]. HCV antibodies gradually disappear after successful HCV elimination[129]. Conversely, there is circumstantial evidence that HCV-specific cellular immune responses can protect individuals at high risk for hepatitis C without seroconversion[123,130,131]. Thus, adaptive cell-mediated immunity is considered a key mechanism for resolution of primary HCV infection[152]. Cell-mediated immunity involves CD8^{+} cytolytic T lymphocytes (CTL), which recognize linear HCV peptides of 8 to 11 amino acids in length

bound to self HLA class I molecules, and CD4^{+} T helper lymphocytes, which respond to longer viral peptides bound to class Ⅱ molecules. Single source outbreaks further support a clear relationship between distinct HLA types and the outcome of HCV infection: patients with HLA-A3, HLA-B27, and HLA-B57 exhibit greater chances to develop protective immunity, thus strengthening the importance of effective antigen presentation and the generation of efficient antigen-specific T cell responses for immune control of HCV infection[133-136].

T CELL RESPONSES

The most conclusive experiments to suggest an important role for T cells in protective immunity against HCV stem from chimpanzee experiments: Depletion of CD8^{+} T cells in animals, which had recovered from previous hepatitis C, resulted in prolonged viraemia, and viral clearance was correlated to recovery of HCV-specific CD8^{+} T cells[137]. Likewise, depletion of CD4^{+} T cells resulted in abrogation of a previously protective immune response[138]. In acute hepatitis C strong HCV-specific CTL[139,140] and TH1 type CD4^{+} T helper cell responses[141] have consistently been reported to be closely associated with a self-limited course of HCV infection. Moreover, several groups have reported an inverse relationship between the strength of the CTL response and HCV viral loads[142-144] further suggesting that in principle it is possible for cellular immunity to control HCV infection[145]. A substantial proportion of individuals who ultimately develop chronic hepatitis C also generate HCV-specific CD4(+) and CD8(+) T cell responses during the early acute phase of infection and may transiently gain some control over HCV[140,146-149]. However, early T cell responses decline to almost undetectable levels later on, and initial control over HCV replication is lost. If present, HCV-specific CD4^{+} and CD8^{+} T cells are detected at only low frequency in peripheral blood although they are somewhat enriched in the liver[150,151]. Thus, chronic hepatitis C is characterized by a progressive functional exhaustion and ultimately loss of HCV-specific CD4^{+} and CD8^{+} T cells[152,153].

Exhausted T cells exhibit a couple of characteristic abnormalities: They show increased expression of inhibitory receptors, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing molecule 3, corresponding to up-regulated expression of their cognate ligands in the liver[154-159]. Conversely, functional recovery of HCV-specific T cells can be achieved experimentally by the combined blockade of CTLA-4 and PD-1 signalling[157,160].

HCV replicates by an RNA-dependent RNA polymerase which has a high error rate and consequently generates considerable genomic diversity of HCV and T cell escape mutations. Mutations that affect CD8^{+} T cell epitopes and proteasomal processing have been observed in several HCV single source outbreaks[161-163].

Due to the exhausted state of T cells new epitope variants rarely elicit strong CD8^{+} T cell responses at this stage of infection, and consequently further escape mutation to secondary epitopes are selected infrequently in man and the chimpanzee[146,164,165]. Protective T cells seem to target epitopes that do not allow for escape mutations owing to the associated loss of viral replication fitness[133-135]. Conversely, T cells that are not stimulated any more after HCV viral escape, do not show features of exhaustion[166]. Thus, prolonged exposure appears to be the mechanism that leads to T cell dysfunction in chronic hepatitis C, and T cell exhaustion in hepatitis C seems to follow the same pattern as has been first described in mice for lymphocytic choriomeningitis virus (LCMV) infection[167,168]. In this model, persistent high level viremia can be established in susceptible mouse strains by pathogenic virus variants. Initially, mice develop a robust T cell response but fail to eliminate the virus and subsequently exhibit a gradual decline of CD8^{+} and CD4^{+} T cell responses. T cells undergo T cell exhaustion in this model, and first lose production of IL-2, a cytokine which supports T cell proliferation. Then, cytotoxicity and production of tumour necrosis factor alpha and IFN-γ are lost sequentially. Finally, intracellular expression of pro-apoptotic factors, such as Bcl2-interacting mediator (Bim), is up-regulated both in the LCMV model and hepatitis C[169]. In analogy to the LCMV model, virus-specific CD4^{+} and CD8^{+} T cell responses decline in chronic hepatitis C but full exhaustion with deletion of antigen-specific CD8 T cells does not occur, because at least *in vitro* T cell responses can be rescued.
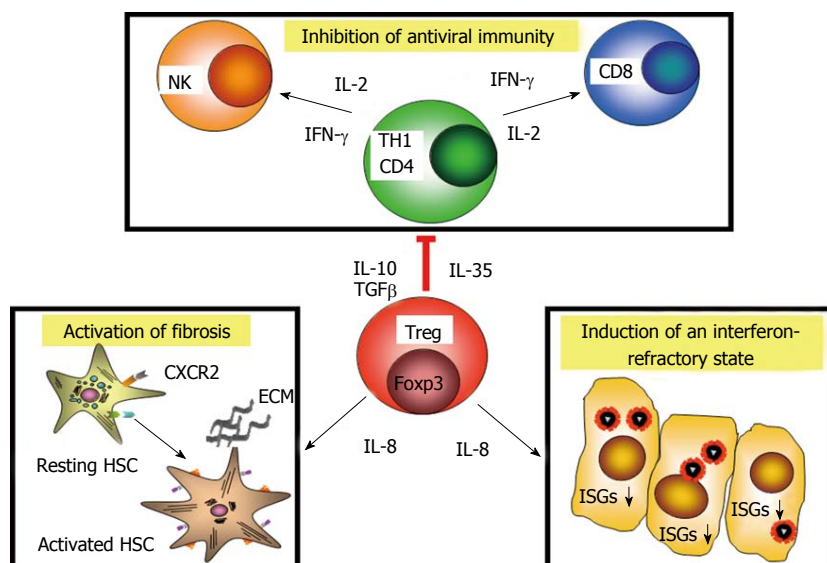
REGULATORY T CELLS

Recently CD8^{+} T cells have been reported in the livers of patients with chronic hepatitis C which were considered to represent CD8^{+} regulatory T cells, because they secrete IL-10 and suppress *in vitro* proliferation of liver-derived T cells[170]. In general, regulatory T cells (Tregs) actively control induction and activity of other immune cells by suppressing their functional activity *via* contact-dependent mechanisms and by release of immunosuppressive cytokines, such as IL-10 and transforming growth factor beta. The major cell type with these properties constitutes CD4^{+}CD25^{high}CD127^{-} T cells, which express the transcription factor Foxp3 (forkhead box P3). They can be divided into thymus-derived natural regulatory T cells, that prevent autoreactivity to self-antigens and induced regulatory T cells, that are generated in the peripheral immune system as a regulatory response to antigenic stimulation. Foxp3^{+} Tregs were rarely detected in acute hepatitis C[171] and they are also not found in patients who had managed to resolve HCV infection[172], suggesting that effector T cells in acute and self-limited hepatitis C are not under active suppression by Tregs. In chronic hepatitis C, however, numbers of CD4^{+} Tregs were increased in the peripheral blood of patients, and depletion of CD4^{+} CD25^{+} T cells was as-

sociated with increased numbers and function of CD8^{+} T cells in *in vitro* assays[173-176]. Such regulatory T cells may reduce inflammatory activity and are considered to contribute importantly to preventing immune-mediated pathology in chronic hepatitis C. Functional analysis of regulatory T cell clones generated from patients with chronic hepatitis C revealed that Tregs were directed against HCV antigens and showed the same pattern of HLA class Ⅱ restriction and epitope specificity as effector T cells[172]. Importantly, Treg clones from chronic hepatitis C inhibited *in vitro* proliferation and IFN-γ production of autologous reporter T cells *via* release of inhibitory cytokines, such as IL-10 and IL-35. Of note, intrahepatic regulatory T cells in chronic hepatitis C also produced substantial amounts of IL-8, and isolated Tregs as well as Treg clones activated fibrogenic genes of hepatic stellate cells *in vitro*[177]. High intrahepatic IL-8 mRNA levels in chronic hepatitis C have been linked with progression of fibrosis[178,179] and CD4^{+} Tregs are enriched in the liver[175,177,180-182]. Moreover, some but not all studies also reported a correlation between numbers of intrahepatic Tregs and the stage of fibrosis. Beyond that, IL-8 counter-acted the antiviral activity of IFN-α in the replicon model by down-regulation the expression of ISGs[183,184]. Moreover, *in vitro* studies suggest that part of the superior antiviral activity in IFN/ribavirin combination therapy may be due to preferential inhibition of Tregs by ribavirin[185] (Figure 2).

Thus, once the immune system has failed to clear HCV infection, regulatory T cells in chronic hepatitis C seem to exert multiple different effects: they dampen inflammatory responses associated with reduced antiviral activity of the immune system, facilitate HCV persistence, and also contribute to the regulation of fibrosis in the liver.

CONCLUSION

When an individual becomes infected with HCV, the immune system has to make a choice between Scylla and Charybdis. If it takes a course close to Scylla, it generates strong antiviral immune responses, which eliminates virus infected liver cells by the combined action of its several innate and adaptive defense mechanisms. This may cause extended liver damage and eventually liver failure. To avoid this risk, immune responses may be softer. Then, the virus has a chance to escape from control by immunity, and functions of innate and adaptive immune mechanisms become diverted owing to continued antigenic stimulation. An inflammatory state is induced, which, however, is refractory to stimulation by antiviral cytokines, and NK cells as well as cells in the adaptive immune system take over regulatory functions. Necro-inflammatory and pro-fibrotic activities maintained by diverted immune responses inevitably take a course towards Charybdis, and may ultimately result in liver cirrhosis, liver cancer and death of the individual. Thus, the immune system holds the steer to find the way between

Figure 2 Multiple activities of hepatitis C virus-specific regulatory T cells in chronic hepatitis C. Regulatory T cell (Tregs) inhibit antiviral immunity *via* release of immunosuppressive factors, such as interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and interleukin 35 (IL-35). Tregs in hepatitis C are also differentiated towards interleukin 8 (IL-8) production. Release of IL-8 binds to its receptors, such as CXCR2 on hepatic stellate cells (HSC), which become activated and produce extracellular matrix (ECM) components. IL-8 down-regulates interferon-stimulated genes in infected cells and induces an interferon (IFN)-refractory state, which also counter-acts antiviral immunity. ISGs: Interferon-stimulated genes.

Scylla and Charybdis.

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