Experimental models to study the immunobiology of hepatitis C virus

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Effective host immune responses are essential for the control of hepatitis C virus (HCV) infection and persistence of HCV has indeed been attributed to their failure. In recent years, several in vitro and in vivo experimental models have allowed studies of host immune responses against HCV. Numerous observations derived from these models have improved our understanding of the mechanisms responsible for the host’s ability to clear the virus as well as of the mechanisms responsible for the host’s failure to control HCV replication. Importantly, several findings obtained with these model systems have been confirmed in studies of acutely or chronically HCV-infected individuals. Collectively, several mechanisms are used by HCV to escape host immune responses, such as poor induction of the innate immune response and escaping/impairing adaptive immunity. In this review, we summarize current findings from experimental models available for studies of the immune response targeting HCV and discuss the relevance of these findings for the in vivo situation in HCV-infected humans.

Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus that belongs to the genus Hepacivirus within the family Flaviviridae. The positive-strand genome has a length of ~9.6 kb and it encodes a polyprotein that is cleaved by viral and cellular proteases into 10 different proteins (reviewed by Moradpour et al., 2007). The structural proteins core, envelope protein 1 (E1) and E2 reside in the amino-terminal region of the polyprotein and they are the main constituents of infectious virus particles. The hydrophobic p7 protein together with non-structural protein 2 (NS2) are required for virion assembly. The serine-type protease residing in NS3 forms a stable complex with the NS4A cofactor and contributes to polyprotein cleavage. NS4B induces alterations of intracellular membranes, designated the membranous web, which is thought to be the site of viral RNA replication. NS5A is required for RNA replication and assembly and NS5B is the RNA-dependent RNA polymerase.

HCV has a narrow host range and infects only humans and chimpanzees. It is estimated that ~130 million people are persistently infected by HCV worldwide (reviewed by Shepard et al., 2005). Limited therapy options and the lack of a preventive vaccine aggravate this medical issue (reviewed by Lauer & Walker, 2001). Of note, the virus is able to establish persistence in approximately two-thirds of infected subjects and has thus become a major cause of chronic liver inflammation that can culminate in liver cirrhosis or even hepatocellular carcinoma (reviewed by Lauer & Walker, 2001). It is still not completely clear why successful immune responses allow only one-third of infected subjects to clear HCV. A major limitation when addressing this issue is the lack of an immunocompetent small animal model. Importantly, however, by using novel cell culture systems, some of these hurdles have now been overcome and first important insights into the intimate interaction between HCV and its host cell have been gained. In this review, we will summarize our current state of knowledge about how HCV may evade innate and adaptive immune responses. We will first compile data gained with in vitro culture systems, then describe results obtained with in vivo models and finally raise the question as to how these results compare to prospective and retrospective analyses conducted with samples from infected individuals. Advantages, disadvantages and important findings from various experimental models are summarized in Table 1.

In vitro experimental models

It took a decade after the molecular identification of HCV as the cause of transfusion-associated non-A, non-B
Table 1. Experimental models of HCV immunobiology

DCs, Dendritic cells; pDCs, plasmacytoid dendritic cells; HCVcc, cell culture-grown HCV; HVR1, hypervariable region 1; OKT3, CD3-specific mAb.

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<thead>
<tr>
<th>Models</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Important findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture</td>
<td>Stable HCV-replicating human cell lines.</td>
<td>Primarily based in human hepatoma Huh7 cell lines.</td>
<td>IFN-α production by pDCs due to direct contact with HCV-replicating cells [Takahashi et al., (2010)]. HCVcc does not productively replicate in DCs and B cells [Decalf et al. (2007); Marukian et al. (2008); Stamatakis et al. (2009); Takahashi et al. (2010)]. Non-cytolytic effector functions (via IFN-γ) of HCV-specific CD8⁺ T cells to inhibit HCV replication [Jo et al. (2009)]. Immune escape and viral fitness cost [Dazert et al. (2009); Salloum et al. (2008); Soderholm et al. (2006); Uebelhoer et al. (2008)].</td>
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<td>Chimpanzees</td>
<td>Can be infected by HCV.</td>
<td>Have a milder hepatitis.</td>
<td>Induction of hepatic ISG mRNAs in an early phase of HCV infection [Bigger et al. (2001, 2004); Major et al. (2004); Su et al. (2002); Thimme et al. (2002)]. Association of viral clearance with intrahepatic IFN-γ-producing CD8⁺ T cells [Thimme et al. (2002)]. Crucial roles of HCV-specific CD4⁺ and CD8⁺ T cells for the protection [Cooper et al. (1999); Erickson et al. (1993); Thimme et al. (2002)]. Viral escape mutations in MHC class I-restricted epitopes [Erickson et al. (2001); Weiner et al. (1995)]. HVR1 of E2 as an antibody-neutralized domain [Farci et al. (1996)]. Induction of hepatic ISG mRNAs, lipid peroxidation and oxidative stress-inducible genes in the human liver chimeric mice after infection [Walters et al. (2006)]. Protection effect of IL-2/OKT3-activated liver allograft-derived lymphocytes (via IFN-γ) [Ohira et al. (2009)]. Immune-mediated liver injury in the Cre/loxP-controlled HCV transgenic mice [Wakita et al. (1998, 2000)].</td>
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<tr>
<td>Small animals</td>
<td>Genomic information is available.</td>
<td>Not a natural host for HCV infection.</td>
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hepatitis (Choo et al., 1989) to establish the first effective cell culture model supporting autonomous replication of HCV (Lohmann et al., 1999). Additional subgenomic HCV replicons were established later on for other genotype 1b isolates as well as for genotype 1a (isolate H77) and 2a (isolate JFH1) (reviewed by Bartensschläger & Sparacio, 2007). Due to the lack of structural proteins, however, subgenomic replicons cannot be used to study immune responses against these proteins.

A hallmark of all genotype 1 replicons is their dependence on replication-enhancing mutations (reviewed by Bartensschläger & Sparacio, 2007). These mutations increase RNA replication up to several orders of magnitude, but at the same time interfere with virion assembly (Pietschmann et al., 2009). A fully permissive cell culture system supporting the production of infectious HCV only became possible with the identification of the genotype 2a isolate JFH1 that was cloned from the serum of a Japanese patient with fulminant hepatitis (Kato et al., 2003). In fact, upon transfection of JFH1 genomes into Huh7 cells, HCV particles that are infectious for naive Huh7 cells were obtained for mice with human liver xenografts and for a chimpanzee (Lindenbach et al., 2006; Wakita et al., 2005; Zhong et al., 2005). Both the replicon and the HCVcc model became important tools, because they allow – amongst others – studies of HCV-specific immune responses in cells containing persistently replicating viral RNA. Nevertheless, there are several limitations such as the strong dependence on human hepatoma cells of the Huh7 line and in case of the HCVcc system, the dependence on the JFH1 isolate (reviewed by Bartensschläger & Sparacio, 2007), restricting the interpretation of immunological findings. In this respect, persistent HCV replication in primary human hepatocytes may overcome these limitations, but even when using HCVcc this system is limited by low infection rates and low virus titres (Ploss et al., 2010; Podevin et al., 2010).

Apart from these culture systems, models to be used primarily for HCV entry have been developed such as HCV pseudoparticles (HCVpp), recombinant HCV envelope glycoproteins or the HCV-like particles. These models have been instrumental in identifying host cell receptors for HCV infection and the study of neutralizing antibodies (reviewed by Burlone & Budkowska, 2009; Stamataki et al., 2008). However, they are not of use for studies of innate and adaptive immune responses and therefore are not discussed in this review.

**In vitro experimental models: insight into innate immune responses**

For many viruses it has been shown that innate immune responses, most notably type 1 interferons (IFNs) are the first line of defence limiting viral replication and spread, and presumably contributing to the outcome of an infection such as acute lytic or chronic persistent (reviewed by Haller & Weber, 2007). Given the importance of this defence, viruses have evolved numerous strategies to counteract it, including the block of induction of IFNs, the interference with signals triggered by IFNs or the inhibition of the action of one or several IFN-stimulated genes (ISGs) (reviewed by Haller & Weber, 2007). As illustrated in Fig. 1, the same applies to appear to HCV as it was demonstrated that the viral NS3/4A protease proteolytically cleaves two key molecules required for the induction of ISG transcription: MAVS, an adaptor protein relaying the signal from the RNA sensor RIG-I to the transcription factor IRF-3; and TRIF, an adaptor required for signalling from the RNA sensor toll-like receptor (TLR)-3 to IRF-3 (Li et al., 2005; Meylan et al., 2005). For this reason, cells infected with HCV are impaired in their production of type I IFNs. This block might be relevant to counteract the antiviral response triggered by the HCV RNA genome that was reported to be a potent activator of RIG-I (Saito et al., 2008b). However, others have found that the HCV genome is a rather weak inducer of IFNs arguing that this poor induction augments the block imposed by the viral protease (Binder et al., 2007).

While the block of the RIG-I and TLR-3 pathway by the NS3/4A protease has been observed consistently, contradicting reports exist as to whether HCV interferes with the antiviral effect exerted by IFNs. Several studies described a partial block of Jak/Stat signalling in transgenic mice expressing HCV proteins or cells harbouring a genomic HCV replicon (Blidenbacher et al., 2003; Luquin et al., 2007), whereas HCV replication in the replicon system or in HCVcc-infected cells still is highly sensitive to treatment with type I, II or III IFNs (Frese et al., 2001, 2002; Marcello et al., 2006). In this respect, HCV appears to control the early stages of IFN induction, but might remain susceptible to the antiviral state triggered by IFNs.

An additional mechanism by which HCV may attenuate the very early antiviral response is the block of RNA translation via the dsRNA activated protein kinase PKR (Garaigorta & Chisari, 2009). It was found that in comparison to type I IFN-treated uninfected Huh7 cells or Huh7 cells containing genomic replicons, HCVcc-infected Huh7 cells treated with type I IFNs display reduced levels of MxA and ISG15, although no reduction in transcription or nucleo-cytoplasmic transport of the corresponding mRNAs has been observed (Garaigorta & Chisari, 2009). This protein reduction that is only seen in ‘acute’ infection (HCVcc), but not in a persistent state (replicons) was linked to the phosphorylation of PKR, resulting in phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α). Non-phosphorylated eIF2α is essential for translation of capped RNA, including the ISGs, whereas non-capped RNAs such as the HCV genome are translated independent from this factor (Robert et al., 2006; Shimoike et al., 2009; Terenin et al., 2008). In this respect, HCV would block translation of ISG mRNAs without affecting expression of its polyprotein. The activation of PKR is, however, very transient and observed only 8–12 h after infection (Arnaud et al., 2010). Thus, impairing production of antiviral effectors at a very early
stage after infection may give the virus a ‘head-start’ until sufficient amounts of the protease have accumulated to block IRF-3-mediated ISG expression. We note that per viral RNA ~1000 polyprotein molecules are translated, resulting in high abundance of NS3/4A even when low copy numbers of the viral RNA genome are present.

Although these observations provide an interesting explanation as to how HCV manipulates PKR at an early stage of infection, contradictory reports have been published as to the role of this kinase in controlling viral replication. For instance, two reports described an inhibition rather than enhancement of HCV replication by phosphorylated PKR (Erickson et al., 2008; Kang et al., 2009). Moreover, several groups have reported that HCV resistance to type I IFNs is mediated through suppression of PKR kinase activity (Gale et al., 1997, 1998; Taylor et al., 1999). Apart from technical reasons such as the use of different cell lines or HCV-replicating RNAs the time point when type I IFNs is added might be crucial. Indeed, early addition may block HCV replication to reach a minimum level that induces phosphorylation of PKR and subsequently suppression of other ISGs. In contrast, if type I IFN has been added at the peak of HCV replication, maximal suppression of ISG protein has been achieved (Garaigorta & Chisari, 2009). Moreover, besides phosphorylating eIF2α, activated PKR assists in the production of autocrine IFN, thus accelerating the establishment of an antiviral state (Balachandran et al., 2000). The balance between these various functions of PKR may determine its pro- or antiviral activity.

Several ISGs have been reported to block HCV replication. These include, apart from PKR, the OAS/RNaseL system (Han & Barton, 2002), adenosine deaminase acting on RNA (Taylor et al., 2005) and the lipid droplet-binding protein viperin (Helbig et al., 2005; Hinson & Cresswell, 2009). Moreover, the induction of non-coding microRNAs (miRNAs) upon treatment of cells with IFNs has been reported (Pedersen et al., 2007). Interestingly, some of these miRNAs may bind to and block the HCV RNA genome, whereas the proviral miR-122 is reduced in abundance arguing for a dual mode of antiviral action of IFNs at the level of miRNAs. While these are interesting
observations, others did not find an effect of IFNs on expression levels of miRNAs (Sarasin-Filipowicz et al., 2009). Thus, the jury is still out if and to what extent miRNAs contribute to the antiviral status triggered by type I IFNs.

**In vitro experimental models: role of NK cells**

HCVcc has been used to study the impact of hepatitis C virions on NK-cell functionality. Briefly, HCVcc do not stimulate NK-cell activation and IFN-γ production and they do not inhibit activation, cytotoxicity or IFN-γ production of anti-CD16-stimulated NK cells isolated from healthy donors (Yoon et al., 2009).

The replichon model has also been used to analyse the functions of activated NK cells. Of note, most tumour cell lines, including Huh7 cells express only very low amounts of HLA molecules, in the case of HuH7 cells HLA-A11, -B54 and -B55 (Windisch et al., 2007). The downregulation of MHC class I should allow the activation of NK cells by HCV-replicating Huh7 cell lines (i.e. the ‘missing-self’ hypothesis) (Ljunggren & Karre, 1985) and thus facilitate the *in vitro* study of innate cellular immune responses. In partial agreement with this hypothesis, 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 (Larkin et al., 2006; Stegmann et al., 2010) and also secrete IFN-γ that mediates the inhibition of HCV replication (Li et al., 2004). However, a major caveat underlying these studies is that NK cells require stimulation by cytokines [e.g. interleukin (IL)-2, IL-15 or IFN-α], questioning whether *in vitro* findings of cytokine-stimulated NK cells can be applied to the *in vivo* situation. This also implies that current HCV-replicating cells may not activate NK cells. Collectively, these results suggest that the HCV-replicating Huh7 cells may not be a suitable *in vitro* model for functional studies of NK cells.

**In vitro experimental models: role of dendritic cells (DCs)**

DCs initiate and regulate the pathogen-specific adaptive immune responses and thus are central to the development of adaptive immune responses as well as immune tolerance (reviewed by Geissmann et al., 2010). Of note, there are two major subsets of human DCs, i.e. myeloid and plasmacytoid DCs (mDCs and pDCs, respectively) (reviewed by Liu et al., 2001). For the sake of simplicity, in this review the term ‘mDCs’ includes *in vivo* generated mDCs and *in vitro* monocyte-derived DCs, although differences exist between these subsets of mDCs, e.g. in their ability to stimulate T cells (Osugi et al., 2002). After receiving proper signals from the innate immune system (through type I IFN and/or NK cells), mDCs and pDCs mature (and upregulate the expression of MHC class II and co-stimulatory molecules, e.g. CD80 and CD86) and perform their respective functions to activate antigen-specific immune responses, mDCs mainly present antigen to T cells and pDCs mainly produce high levels of IFN-α upon viral infection (reviewed by Banchereau et al., 2000; Ueno et al., 2007; Villadangos & Young, 2008).

HCVcc and the replicon models have been used to address two important issues regarding DCs during HCV infection, i.e. whether DCs can be infected by HCV and whether maturation and functionality of DCs are impaired during HCV infection. It is clear that HCVcc do not infect and replicate in DCs (Decalf et al., 2007; Ebihara et al., 2008; Eksioglu et al., 2010; Marukian et al., 2008; Shiina & Rehermann, 2008). This non-permissiveness of DCs can be attributed to several factors. First, pDCs and mDCs isolated from healthy subjects express some of the HCV entry molecules, the tight-junction protein claudin-1 and the scavenger receptor class B type I, at low levels or not at all, thus precluding entry of HCVcc (Decalf et al., 2007; Lambotin et al., 2010). However, HCVcc acquisition by mDCs and pDCs (which does not necessarily mean infection and productive HCV replication) can occur in the presence of blocking antibody to each of these co-receptors (Lambotin et al., 2010). Second, there is a possibility that the JFH1 isolate does not have a tropism for haematopoietic cells. It has been reported that patients infected with HCV genotype 1 have a higher detection rate of positive- or negative-strands of HCV RNA in haematopoietic cells compared with patients infected with other genotypes (Lerat et al., 1998). However, it is still an unresolved issue as to whether HCV indeed infects and replicates in blood cells or whether the detectable viral RNA is just bound to or taken up by blood cells without productive replication (Boisvert et al., 2001; Muratori et al., 1996; Pal et al., 2006; Rodrigue-Gervais et al., 2007).

With respect to maturation and functionality of DCs during HCV infection, studies using HCVcc have demonstrated that HCV does not induce maturation of mDCs, based on a lack of upregulation of co-stimulatory molecules, including CD80, CD86 and HLA-DR (Ebihara et al., 2008; Eksioglu et al., 2010; Shiina & Rehermann, 2008). However, findings from studies regarding possible inhibitory effects of HCV on the induced maturation of mDCs are conflicting (Eksioglu et al., 2010; Saito et al., 2008; Shiina & Rehermann, 2008). Similarly, studies regarding the functionality of mDCs, based on the induction of the proliferation of CD4+ and CD8+ T cells, have also led to conflicting results (Saito et al., 2008a; Shiina & Rehermann, 2008). Thus, it is still not clear whether hepatitis C virions can inhibit the maturation and/or the functionality of mDCs. IFN-α production by pDCs cannot be induced by Huh7 cells containing replication-defective subgenomic HCV RNA. However, replication-competent subgenomic HCV replicons are able to induce IFN-α production (Takahashi et al., 2010). This suggests that pDCs recognize HCV-replicating cells and that the induction of IFN-α production can occur in the absence of HCV structural proteins and virion production (Takahashi et al., 2010). In line with these findings, direct incubation
with HCV-infected cells, but not with HCVcc, triggers IFN-α production by pDCs (Decalf et al., 2007; Takahashi et al., 2010), which requires active HCV replication in infected Huh7 cells, direct cell–cell contact and TLR7 signalling. Although the exact mechanism for the HCV RNA uptake from living cells by pDCs is still elusive, a supporting finding has been derived from a study with a mouse model showing that DCs can internalize cytosolic and membrane material of living cells in vivo (Matheoud et al., 2010).

**In vitro experimental models: insight into adaptive immune responses**

Obviously, the original replicon model cannot be used to analyse the adaptive cellular immune responses, particularly HCV-specific CD8+ T-cell responses. Indeed, the very low expression of MHC class I on Huh7 cells impairs antigen recognition by virus-specific CD8+ T cells (Liu et al., 2003). To overcome this limitation, we have developed a novel immunological model based on a subgenomic JFH1-replicating Huh7-lunet cell line (Jo et al., 2009). Specifically, we have stably transduced these cells with the common MHC class I allele HLA-A2 gene, matched the epitope sequence in NS5B of the JFH1 isolate to the sequence targeted by an HLA-A2-restricted HCV genotype 1-specific CD8+ T-cell clone established in our laboratory, and used luciferase activity to quantify HCV RNA replication (termed Huh7A2/HCVEM cells, as EM stands for epitope matched), as illustrated in Fig. 2. This model allows the analysis of the mechanisms of cytolytic and non-cytolytic effector functions of HCV-specific CD8+ T cells and their relative contribution to antiviral efficacy. The latter is achieved by parallel measurements of the luciferase activity of HCV subgenomic replicons (for quantifying viral replication) and aspartate aminotransferase activity in the supernatant (for quantifying cytolytic of subgenomic HCV-replicating Huh7 cells). Of note, we found that HCV-specific CD8+ T cells exert strong antiviral effects primarily through IFN-γ-mediated non-cytolytic effector functions (Jo et al., 2009), suggesting that IFN-γ secreted by CD8+ T cells is important to control HCV replication.

The replicon model has also been used to determine the impact of viral mutations in MHC class I-restricted epitopes observed in chronically infected humans and chimpanzees on viral T-cell escape as well as on viral fitness. For example, it has been shown that in order to escape from CD8+ T-cell recognition, HCV may be forced to adopt relatively unfavourable sequences, as these mutations reduce viral replication in the *in vitro* model (Salloum et al., 2008; Soderholm et al., 2006). Importantly, the role of CD8+ T-cell pressure in the emergence of viral escape mutations is supported by findings of an *in vitro* study comparing different variants that were originally observed in a persistently infected chimpanzee over a period of 7 years (Uebelhoer et al., 2008). Briefly, by comparing the stability of two different mutations (transient L1637P and stable L1637S variants) introduced at the MHC-binding anchor residue of a NS3-specific CD8+ T-cell epitope in replicon cells in the absence of HCV-specific CD8+ T cells, it was found that five of 15 clones containing the L1637P mutation, in contrast to clones containing the L1637S mutation, reverted to the parental sequence *in vitro*. However, HCV can accumulate escape mutations within a particular epitope that have a less pronounced impact on viral fitness. For example, within an immunodominant HLA-B27-restricted epitope, clustered mutations occur that only had a modest impact on viral fitness. Importantly, the clustering of mutations is required for efficient viral escape from the HLA-B27 restricted T-cell response (Dazert et al., 2009).

The ineffectiveness of the humoral immunity to protect hepatocytes from HCV infection has also been studied by using HCVcc. Of note, HCV can be transmitted *in vitro* by
at least two routes, i.e. cell-free virus infection and direct transfer between cells (Jones et al., 2010; Timpe et al., 2008; Witteveldt et al., 2009). While neutralizing antibodies may prevent the former transmission route, the latter route is not protected by antibodies (Timpe et al., 2008), partially explaining the ineffectiveness of the humoral immunity to confer protection. In addition, the ineffectiveness of the humoral immunity can be caused by HCV infection of B cells. However, HCVcc do not infect primary and immortalized B cells, even though it may bind to B cells (Marukian et al., 2008; Stamatakis et al., 2009). Interestingly, this B cell-associated virus shows an enhanced infectivity in Huh7 cells and becomes resistant to neutralizing antibodies, as compared with the cell-free virus (Stamatakis et al., 2009). In addition, HCV promotes the adhesion of primary B cells to Huh7 cells, thus providing a possible mechanism for B-cell retention in the infected liver that can help viral spreading (Stamatakis et al., 2009). In summary, these findings suggest a possible mechanism underlying the failure of neutralizing antibodies to prevent HCV infection and a possible pathogenic role of B cells.

**In vivo experimental models**

Currently, the chimpanzee is the only immunocompetent animal model available to study HCV. In addition, small animal models including immunodeficient mice with human liver xenografts and tree shrews have been developed, but they are of limited use for studying HCV-specific immune responses.

**In vivo experimental models: lesson learned from the chimpanzee model**

Chimpanzees have been used to define the strain and the titre of HCV used in primary and rechallenge infections, to collect appropriate samples (e.g. liver biopsies) before and during the course of infection, to manipulate the immune system (e.g. by performing T-cell depletion), and to use the same reagents and tests that have been developed for HCV-infected humans (reviewed by Bukh, 2004). In addition, during HCV infection, MHC class I-restricted epitopes are generated that are recognized by both humans and chimpanzees with their HLA and Patr orthologues, respectively, indicating a close functional homology between individual HLA and Patr alleles (Mizukoshi et al., 2002). However, there are two major limitations of the chimpanzee model. First, chimpanzees and humans may respond differently to HCV infection. For example, it is known that HCV-infected chimpanzees have a milder clinical course and also have a higher rate of viral clearance compared with infected humans (reviewed by Bukh, 2004). In addition, although infected chimpanzees can suffer from persistent infections (reviewed by Bukh, 2004) and even develop hepatocellular carcinoma (Muchmore et al., 1988), the incidence of HCV-related liver fibrosis or cirrhosis has not been reported thus far (reviewed by Boonstra et al., 2009). Second, the chimpanzee studies are limited by ethical concerns, restricted availability and prohibitively high costs (reviewed by Bukh, 2004). Nevertheless, despite these limitations, studies performed in chimpanzees have indeed provided key insights into the role of innate and adaptive immune responses during HCV infection, as discussed below.

**Chimpanzee model: innate immune responses and role of DCs**

Transcription of hepatic ISGs of chimpanzees is upregulated in the early phase of HCV infection (Bigger et al., 2001, 2004; Major et al., 2004; Su et al., 2002; Thimme et al., 2002), suggesting that infected hepatocytes are the primary target cells. Whether IFNs are produced from the infected cells or from other sources (e.g. pDCs migrating into the liver) (Bigger et al., 2001) remains to be determined. However, these responses do not eliminate the virus, suggesting that type I IFNs are insufficient to control viral replication in infected hepatocytes (Bigger et al., 2001; Major et al., 2004; Su et al., 2002). The underlying reasons are not clear, but the discussed observations in vitro may also operate in these animals. This includes the early block of PKR, the cleavage of MAVS (and eventually also TRIF) and the inhibition of NK cells.

Limited studies have been performed to analyse the role of DCs derived from infected chimpanzees. In chronically infected animals, no impairment either in the maturation or in the stimulatory capacity of DCs has been observed, although DCs isolated from infected animals with very high viral loads were reduced in their stimulatory capacity. In addition, no evidence of active HCV replication in DCs was found (Larsson et al., 2004; Röllert et al., 2003).

**Chimpanzee model: adaptive immune responses**

Importantly, multispecific intrahepatic HCV-specific CD4+ and CD8+ T-cell responses have been found to correlate with viral clearance (Cooper et al., 1999; Erickson et al., 1993; Thimme et al., 2002). Indeed, the entry and accumulation of HCV-specific IFN-γ-producing T cells in the liver coincides with viral clearance (Thimme et al., 2002), suggesting a crucial role of functional virus-specific T cells in the control of viral replication. Interestingly, viral clearance may not require the destruction of infected cells, as indicated by the absence of elevated serum alanine aminotransferase (ALT) levels and minimal histological evidence of liver-cell injury in some of the resolved animals (Thimme et al., 2002), thus supporting the in vitro findings of a dominant role of non-cytolytic CD8+ T-cell-mediated effector functions.

In contrast, persistent infection is associated with weak and narrowly focused intrahepatic T-cell responses (Cooper et al., 1999; Erickson et al., 1993; Thimme et al., 2002) and the emergence of viral escape mutations in MHC class I-restricted epitopes (Erickson et al., 2001; Weiner et al., 1995). Most viral escape mutations already appear in
infected chimpanzees during the acute phase of infection in animals that become persistently infected. In contrast, most epitopes remain stable in animals that are able to clear the virus (Erickson et al., 2001). Importantly, escape mutations are temporally associated with a narrowly focused intrahepatic HCV-specific CD8\(^+\) T-cell response (Erickson et al., 2001), suggesting that restricted clonotypic CD8\(^+\) T-cell response may contribute to the emergence of escape mutations.

Interestingly, humoral immunity does not seem to be associated with HCV clearance in chimpanzees (Bartosch et al., 2003; Bukh et al., 2008; Cooper et al., 1999; Major et al., 1999, 2004; Thimme et al., 2002). One possible explanation is that titres of anti-hypervariable region 1 (HVR1) antibody, an antibody to the critical neutralization domain of E2 protein (Farci et al., 1996), do not correlate well with titres of HCV-specific neutralizing antibodies in vivo (Bartosch et al., 2003; Major et al., 1999). This suggests that the titre of the anti-HVR1 antibody is either not high enough to confer protection (Bartosch et al., 2003) or that anti-HVR1 antibody cannot neutralize emerged variant mutants (Farci et al., 1996).

One important issue regarding adaptive immunity is whether it can protect the resolved host against HCV reinfection. Studies in reinfected chimpanzees that previously cleared the infection have revealed the existence of a protective immunity, which may, however, not be able to consistently prevent reinfection (Bukh et al., 2008; Major et al., 2002). The protection seems to be T-cell-mediated (reviewed by Bowen & Walker, 2005). Indeed, two key studies in immune chimpanzees that were reinfected with the same HCV strain have shown that HCV-specific memory CD4\(^+\) and CD8\(^+\) T cells are essential for protection from persistent HCV infection (Grakoui et al., 2003; Shoukry et al., 2003). The importance of HCV-specific memory CD8\(^+\) T cells is confirmed by a prolonged viraemia after antibody-mediated depletion of CD8\(^+\) T cells and by a strong correlation between recovery of intrahepatic HCV-specific CD8\(^+\) T cells and viral clearance (Shoukry et al., 2003). In parallel, antibody-mediated depletion of CD4\(^+\) T cells results in viral persistence, despite the presence of HCV-specific memory CD8\(^+\) T cells (Grakoui et al., 2003), indicating that an effective viral control by virus-specific CD8\(^+\) T cells requires help from virus-specific CD4\(^+\) T cells. Indeed, the inability of HCV-specific CD8\(^+\) T cells to control viral replication in the absence of HCV-specific memory CD4\(^+\) T cells has been shown to be associated with the emergence of escape mutations in MHC class I-restricted epitopes (Grakoui et al., 2003). Collectively, these findings suggest that virus-specific CD4\(^+\) T cells behave as the master regulator, while virus-specific CD8\(^+\) T cells function as the key effector.

The mechanisms responsible for the failure of HCV-specific CD4\(^+\) T-cell responses are still unclear. Although escape mutations can also occur in MHC class II-restricted epitopes, they are rarely found in chronically infected chimpanzees, suggesting that other mechanisms, e.g. CD4\(^+\) T-cell dysfunction, may be more dominant (Fuller et al., 2010).

**In vivo experimental models: small animal models**

Due to the limitations of the chimpanzee model, numerous attempts have been performed to establish immunocompetent small animal models that can be infected by HCV. Interestingly, the tree shrew *Tupaia belangeri* can be infected by HCV, although the viral titres remain low (Amako et al., 2010; Xie et al., 1998; Xu et al., 2007; Zhao et al., 2002). HCV-infected tree shrews produce infectious viral particles and, interestingly, can develop chronic hepatitis leading to fibrosis and cirrhosis (Amako et al., 2010). However, the main limitation of using this species is the lack of its genetic information because it is an outbred stock. In addition, the tree shrew's genome has not been completely sequenced, further limiting their potential for studying host immune responses (Bissig et al., 2010).

The development of inbred HCV-permissive mice could solve the limitations of the tree shrew model, since a lot of information is available about the genetic characteristics as an inbred stock, the genome and the immune systems. The use of mice has also technical advantages, e.g. high fecundity and ease of genetic manipulation (reviewed by Mullins & Mullins, 2004). However, the main limitation is that HCV does not infect mouse hepatocytes. Nevertheless, several models have been developed to address this issue (reviewed by Barth et al., 2008; Kremsdorf & Brezillon, 2007). In the following, two mouse models for HCV immunobiology will be briefly summarized.

The first model is the creation of a human liver chimera in immunodeficient mice (the SCID/Alb-uPA or the *Fah* / *Rag2* / *Il2rg* / mice). Despite its technical difficulties (e.g. transplantation of human hepatocytes), this model is permissive for HCV infection (Bissig et al., 2010; Mercer et al., 2001). Since it is immunodeficient, the host adaptive immune responses cannot be studied. However, innate immune responses can be analysed in the infected mice. In addition, liver injury after HCV infection can also be studied (Walters et al., 2006). For example, similar to findings in HCV-infected chimpanzees, microarray analysis of liver tissue from the HCV-infected mice revealed the transcriptional upregulation of many antiviral ISGs (Walters et al., 2006), further supporting the rapid activation of innate immunity by HCV. In addition, an upregulation of β-oxidation enzymes and oxidative stress-inducible genes and an increased apoptotic rate have also been observed in infected livers (Walters et al., 2006), suggesting that HCV infection by itself may induce lipid peroxidation, thus liver injury. However, the increased liver injury in this model might also be caused by the activity of accumulated inflammatory cells (mainly mononuclear and Kupffer cells) in the infected livers (Walters et al., 2006). Importantly, this model has also been used to demonstrate the role of human allograft-derived lymphocytes to
prevent HCV infection (Ohira et al., 2009). Briefly, adoptive transfer of IL-2/OKT3-activated liver lymphocytes into the mice at 2 weeks after infection indeed can prevent HCV replication, most likely via IFN-γ. However, the preventive effect is no longer observed if the adoptive transfer is performed at 4 weeks after infection, the time point when high titres of virus are already detectable.

Another model is HCV transgenic mice created by inserting a viral cDNA into mouse embryos. The major limitation of this model is that the transgenic mice are immunotolerant to HCV proteins due to their constitutive expression, thus host immune responses are not induced (reviewed by Barth et al., 2008). Nonetheless, by introducing the Cre/loxP system that allows temporal (inducible) control of the expression of HCV proteins, host immune responses against HCV proteins can be triggered (Wakita et al., 1998). Indeed, after transgene activation, HCV structural proteins are strongly expressed in the mouse liver with a concurrent elevation of serum ALT levels (Wakita et al., 1998). Anti-HCV core antibodies also become detectable subsequently, suggesting that immune responses are indeed induced (Wakita et al., 1998). Interestingly, depletion of CD4+ and CD8+ T cells before transgene activation leads to a normalization of both serum ALT levels and histopathological changes in the liver biopsies, supporting the role of immune-mediated liver injury (Wakita et al., 1998, 2000).

In summary, the utility of current murine models is very limited for HCV immunological studies. Hence, the creation of an immunocompetent murine model that can be infected by HCV, either through xenotransplantation of human liver and human haematopoietic cells into the same animal, genetic host humanization by expressing humanspecific co-receptors for HCV in mice, or viral adaptation to murine hepatocytes (reviewed by Ploss & Rice, 2009), is necessary for a better in vivo study of HCV immunobiology. Of note, HCV adaptation to mouse CD81, one among HCV entry co-receptors, has been reported to permit viral entry into NIH3T3 cells, a mouse embryonic fibroblast cell line, in the absence of human factors (Bitzegeio et al., 2010), suggesting that this strategy can contribute to the development of an immunocompetent murine model fully permissive to HCV.

**HCV immunobiology in HCV-infected humans**

Since HCV naturally infects only humans, immunological findings obtained with experimental models are only relevant if they simulate or can be confirmed by findings in HCV-infected humans. Hence, studies in both acutely and chronically infected patients are important. In recent years, several important insights into the interaction between HCV and host immune responses have been derived from these human studies. In the following, we will discuss findings from human studies that support or contradict the results from the experimental models.

**Innate immune responses in HCV-infected humans**

Importantly, the observation that in HCV-infected Huh7 cells, MAVS is cleaved by the viral protease has been confirmed in humans (Bellecave et al., 2010). By using Western blot analyses of cell lysates prepared from liver biopsies a convincing correlation was found between the degree of MAVS cleavage and HCV infection. Moreover, the amount of uncleaved MAVS was clearly reduced as compared with liver biopsies from patients with non-HCV liver diseases (Bellecave et al., 2010). Whether TRIF is also cleaved in vivo has not been determined thus far. Next, genome-wide association studies on HCV genotype 1-infected humans have supported the antiviral role of type III IFN (IFN-λ). Indeed, these studies have demonstrated that certain single-nucleotide polymorphisms located near or at the locus of IL-28B gene, the gene that encodes IFN-λ3, are strongly associated with HCV clearance (Ge et al., 2009; Rauch et al., 2010; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009). Further studies are obviously required to elucidate the protective mechanism of certain IL-28B polymorphisms against HCV infection.

In parallel, two lines of evidence from human studies support the notion that HCV infection triggers innate immunity: first, transcription of ISGs is upregulated in chronically HCV-infected livers (Chen et al., 2005; Patzwahl et al., 2001; Sarasin-Filipowicz et al., 2008); second, NK cells are activated in acutely infected subjects, as determined by an increased expression of the activating receptor NKG2D on both CD56dim and CD56bright subsets of NK cells. This is accompanied by an increased production of IFN-γ and cytotoxicity (Amadei et al., 2010). While the overall killing capacity of NK cells was not impaired or even enhanced in chronically infected patients (Ahlenstiel et al., 2010; Amadei et al., 2010; Golden-Mason et al., 2008; Oliviero et al., 2009), it is unclear whether the ability of NK cells to produce antiviral cytokines during chronic infection is altered. Some groups reported an impairment in IFN-γ production (Ahlenstiel et al., 2010; Oliviero et al., 2009), while others came to the opposite conclusion (Amadei et al., 2010; Golden-Mason et al., 2008). However, irrespective of these conflicting results, there seems to be a strong correlation between the degree of HCV infection and the state of activation of the innate immunity.

**Role of DCs in HCV-infected humans**

In contrast to the inability of HCVcc to actively replicate within DCs, several human studies suggested that HCV may infect and replicate within DCs in vivo, as partly supported by the detection of HCV RNA within DCs (Bain et al., 2001; Goutagny et al., 2003; Lerat et al., 1998; Rodrigue-Gervais et al., 2007). However, it remains inconclusive whether HCV can alter the maturation and functionality of DCs in vivo. Of note, studies on the impairment of the maturation capacity of DCs during
chronic HCV infection yielded conflicting results (Auffermann-Gretzinger et al., 2001; Lai et al., 2007; Liang et al., 2009; Mengshol et al., 2009; Yonkers et al., 2007). Similarly, while some studies suggested that the functionality of DCs was impaired during chronic infection, e.g. mDCs being poor activators of CD4+ T cells (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Dolganiuc et al., 2008; Kanto et al., 1999) and pDCs producing less IFN-α (Anthony et al., 2004; Yonkers et al., 2007), other studies reported that mDCs and pDCs from chronically infected patients remained functional (Decalf et al., 2007; Lai et al., 2007; Longman et al., 2004). It is interesting to note that, however, several groups have reported that the frequency of circulating mDCs and pDCs during chronic HCV infection is reduced, probably due to an intrahepatic enrichment (Dolganiuc et al., 2006; Lai et al., 2007; Mengshol et al., 2009; Nattermann et al., 2006; Wertheimer et al., 2004). Hence, it is possible that HCV retains DCs in the liver, causing a delay or even a reduction in the migration of DCs to the draining lymph nodes, thereby affecting T-cell activation and ultimately resulting in an impairment of HCV-specific immune responses.

Adaptive immune responses in HCV-infected humans

Similar to the related findings from the chimpanzee model, strong and sustained multispecific HCV-specific CD4+ and CD8+ T-cell responses are indeed present during acute and after resolved infections (Diepolder et al., 1995; Gerlach et al., 1999; Lechner et al., 2000; Missale et al., 1996; Thimme et al., 2001). It is important to note, however, that these findings were obtained only from analyses of T cells derived from the peripheral compartment, since liver biopsies are not performed in acutely and spontaneously resolved HCV-infected subjects. Of note, during acute HCV infection, the emergence of a strong CD4+ T-cell response coincides with the first time CD8+ T cells start to produce IFN-γ in response to HCV and a rapid 100 000-fold decrease in viraemia that occurs without a corresponding surge of disease activity (Thimme et al., 2001). This indeed supports findings from the experimental models regarding the important role of non-cytolytic IFN-γ-mediated effector functions of virus-specific CD8+ T cells to control HCV replication.

In chronically infected HCV patients, virus-specific CD4+ and CD8+ T-cell responses are weak and/or narrowly focused, similar to findings in chronically infected chimpanzees, for reasons that are poorly understood (Diepolder et al., 1995; Gerlach et al., 1999; Lechner et al., 2000; Thimme et al., 2001). However, the two main mechanisms seem to be primarily associated with viral persistence, as illustrated in Fig. 3. The first mechanism is an HCV-specific T-cell dysfunction (e.g. inability to proliferate, to produce cytokine and to exert cytotoxicity) despite enrichment at the site of disease, namely the liver (Gerlach et al., 1999; Mueller et al., 2010; Nakamoto et al., 2008; Neumann-Haefelin et al., 2008; Semmo et al., 2005; Spengenberg et al., 2005; Ulsenheimer et al., 2003). The T-cell dysfunction can be caused by several factors, including high viral load, altered antigen-presenting cells (non-professional antigen-presenting cells or impairment of DCs), upregulation of multiple inhibitory receptors (e.g. PD-1, 2B4, or CD160) or suppression by viral factors, by immunoregulatory cytokines (e.g. IL-10 or TGF-β), or by regulatory T cells (reviewed by Crawford & Wherry, 2009; Neumann-Haefelin et al., 2005; Virgin et al., 2009). The second mechanism is the emergence of viral mutations in certain MHC class I-restricted epitopes (more often associated with HLA-B alleles), in which the virus can thereby escape CD8+ T-cell recognition and establish persistence (Cox et al., 2005; Neumann-Haefelin et al., 2008; Ray et al., 2005; Tester et al., 2005; Timm et al., 2004). Similar to the chimpanzee model, the emergence of viral escape mutations within CD8+ T-cell epitopes is associated with loss of CD4+ T-cell responses (Tester et al., 2005).

In contrast to the elusive role of humoral immunity in the chimpanzee model, an early induction of neutralizing antibodies in humans who have spontaneously cleared the infection suggests a correlation for neutralizing antibodies in HCV control (Dowd et al., 2009; Logvinoff et al., 2004; Pestka et al., 2007). This notion is further supported by the finding that the sequence of HVR1 is less variable in the absence of neutralizing antibodies. This rapidly changes, however, when neutralizing antibodies emerge, indicating evolution of viral variants in response to pressure from neutralizing antibodies (Dowd et al., 2009; Farci et al., 2000; Liu et al., 2010). Importantly, it has been shown that amino acid substitutions in HVR1 are responsible for a dramatic decrease in neutralization sensitivity over time (Dowd et al., 2009). In an agreement with these findings, mutations in defined neutralizing epitopes of E1 and E2 proteins are associated with continuous escape of HCV from recognition by neutralizing antibodies in chronically infected individuals, suggesting that HCV subjects to selection pressure from humoral immunity that results in the continuous generation of escape variants during chronic infection (von Hahn et al., 2007). However, neutralizing antibodies from acutely and chronically infected patients have been shown to be restricted to IgG1 isotype and to be produced at low titre (Chen et al., 1999), and thus may not be sufficient to confer protection.

Finally, with respect to the protective immunity against HCV infection, a few studies have been conducted to analyse HCV reinfection rate on intravenous drug users who had spontaneously resolved HCV infection. Importantly, the reinfection rate is approximately 50% lower in individuals who had spontaneously resolved the infection compared with individuals who had no previous HCV infection (Grebely et al., 2006; Mehta et al., 2002). Thus, similar to the findings in reinfected immune chimpanzees, humans seem also to acquire protective immunity against HCV infection.
**Future perspectives**

In summary, the experimental models have provided important and new insights into HCV immunobiology, such as the activation of innate immunity at the early phase of infection and the essential role of HCV-specific T cells for viral control. However, several key issues of HCV immunobiology remain unresolved, including central questions such as:

(i) whether TRIF cleavage also occurs in vivo, and if so, in which cells, and how does it contribute to establishment of persistence, (ii) why HCV appears to counteract the induction of type I IFNs, but otherwise remains rather sensitive to the antiviral state triggered by this cytokine, (iii) whether the protective effect of the IL-28B polymorphism is due to antiviral actions of IFN-λ, (iv) how HCV can persist in the face of an IFN-induced antiviral state, (v) whether there are reservoirs of HCV replication and if so, whether they are accessible to innate and adaptive antiviral control, (vi) how innate effector cells, e.g. NK cells, NK T cells and γδ T cells interact with HCV-infected cells, DCs, and adaptive effector cells, (vii) whether presentation of viral antigens by different hepatic antigen-presenting cells (e.g. hepatocyte and liver sinusoidal endothelial cells) lead to an activation or inhibition of HCV-specific adaptive immune responses, (viii) whether functions of DCs are impaired during HCV replication, (ix) why there is a delay in intrahepatic T-cell responses in the early phase of HCV infection [is it due to lack of priming, place of priming (lymph nodes versus liver) or immunosuppressive cytokines, such as IL-10?], (x) which mechanism is primary responsible for the failure of HCV-specific CD4+ T-cell responses in two-third of infected subjects who progress to chronicity, (xi) how CD161+ Th17 cells and other T-cell lineages contribute to HCV immunobiology, and (xii) whether humoral immunity is ineffective to confer protection.

Key to address these questions is the availability of an easy to manipulate and immunocompetent small animal model. Given our rapidly increasing knowledge about the interaction between HCV and its host cell and the identification of critical host factors that may confer species specificity or restrict HCV replication, establishment of such an animal model has become a realistic goal. In combination with the power of transgene technologies, immunological studies in such an animal model will...
undoubtedly provide important insights that are ultimately required to develop more efficient preventive and therapeutic regimens to combat this insidious disease.

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