Animal Models of Hepatitis C Virus Infection

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Hepatitis C virus (HCV) is an important and underreported infectious disease, causing chronic infection in ~71 million people worldwide. The limited host range of HCV, which robustly infects only humans and chimpanzees, has made studying this virus in vivo challenging and hampered the development of a desperately needed vaccine. The restrictions and ethical concerns surrounding biomedical research in chimpanzees has made the search for an animal model all the more important. In this review, we discuss different approaches that are being pursued toward creating small animal models for HCV infection. Although efforts to use a nonhuman primate species besides chimpanzees have proven challenging, important advances have been achieved in a variety of humanized mouse models. However, such models still fall short of the overarching goal to have an immunocompetent, inheritably susceptible in vivo platform in which the immunopathology of HCV could be studied and putative vaccines development. Alternatives to overcome this include virus adaptation, such as murine-tropic HCV strains, or the use of related hepaciviruses, of which many have been recently identified. Of the latter, the rodent/rat hepacivirus from *Rattus norvegicus* species-1 (RHV-rn1) holds promise as a surrogate virus in fully immunocompetent rats that can inform our understanding of the interaction between the immune response and viral outcomes (i.e., clearance vs. persistence). However, further characterization of these animal models is necessary before their use for gaining new insights into the immunopathogenesis of HCV and for conceptualizing HCV vaccines.

CONTINUED NEED FOR ANIMAL MODELS FOR HCV INFECTION AND IMMUNITY

Worldwide, an estimated 71 million people are infected with hepatitis C virus (HCV) (WHO 2017) and are consequently at greater risk for developing severe liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Perz et al. 2006; Mohd Hanafiah et al. 2013). The mechanism underlying such HCV-induced pathogenesis remains incompletely understood.

The Centers for Disease Control and Prevention (CDC) estimates that in the United States alone, 3.5 million people are infected with HCV and 40%–85% of these individuals are unaware (Smith et al. 2012). These numbers may even be underestimated because of bias in the populations included in surveys (Edlin 2011). In addition, there has been an increase

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in acute HCV infections in younger people. Data collected from 2006 to 2012 found a growing number of injection drug users (IDUs) presenting with acute HCV infection on admission to substance abuse treatment facilities (Zibbell et al. 2015). Among IDUs around the world, HCV is considered hyperendemic, with midpoint prevalence estimates of HCV antibodies present in 60%–80% of IDUs in 25 different countries, including Japan (65%), China (67%), the United Kingdom (51%), and the United States (73%) (Nelson et al. 2011).

Although highly effective treatments for HCV now exist, their pricing in the United States is prohibitive for many of the most at-risk individuals, such as IDUs, who suffer disproportionately from underinsurance or complete lack of insurance (McGowan and Fried 2012). Therefore, developing preventive measures, such as vaccines, remains crucial (Callaway 2014).

The increasing incidence and prevalence of HCV, inaccessibility of the most effective treatments, and lack of a vaccine altogether underscore the importance of continued HCV research. Because HCV robustly infects only humans and chimpanzees, in vivo modeling of chronic infection and immunopathogenesis has been extremely difficult as a result of increased restrictions on chimpanzee use in biomedical research and the official inclusion of captive chimpanzees on the U.S. Fish and Wildlife Services endangered species list. Most importantly, the immune correlates of protection against HCV persistence are not fully defined and this knowledge is crucial for conceptualizing an effective HCV vaccination approach. Additionally, an in-depth understanding of intricate virus–host interactions culminating in delayed virus clearance (3–12 months postinfection [p.i.]) and more common lifelong viral persistence is necessary but is impeded by lack of a fully immunocompetent animal model that recapitulates these unique and dual HCV infection outcomes.

PRIMATE MODELS FOR HCV

Chimpanzees played a pivotal role in early HCV research, including the discovery of HCV as the etiologic agent of hepatitis C, validation of the first infectious cDNA clones, characterization of the natural course of infection, and as proof-of-concept models for antiviral therapies and vaccination attempts (for review, see Bukh 2004). However, high costs, genetic heterogeneity, small cohort sizes, limited access to relevant tissue compartments, the inability to genetically manipulate large apes, and growing ethical concerns have all impeded the continued use of chimpanzees.

To identify alternative experimental models, a variety of other nonhuman primates (NHP) species have been tested for their susceptibility to HCV. However, signs of infection were not observed in cynomolgus, rhesus, Japanese, and green monkeys; Doguera (Abe et al. 1993) and Chacma baboons (Sithebe et al. 2002); cottontop tamarins (Garson et al. 1997); and marmosets. This apparent resistance could be attributed to multiple reasons: (1) simian orthologs of host factors essential for HCV infection may be incompatible; (2) restriction factors may exist that antagonize part(s) of the HCV life cycle; and/or (3) NHP immune responses may be faster and/or more vigorous, preventing (persistent) HCV infection.

However, more recent work has questioned the role of some of these possibilities. Stem cell–derived hepatocyte-like cells from pig-tailed macaques (*Macaca nemestrina*) (Sourisseau et al. 2013) and primary rhesus macaque (*Macaca mulatta*) hepatocytes (Scull et al. 2015) both support the entire HCV life cycle in vitro. Furthermore, inoculation of simian liver chimeric mice, that is, immunocompromised xenorecipients transplanted with rhesus macaque hepatocytes, support persistent HCV infection, albeit at lower levels than humanized mice (Scull et al. 2015). Experimental conditions still need to be defined, under which HCV persistence could be achieved in rhesus or pig-tailed macaques.

Tree shrews (*Tupaia belangeri*), which were previously designated as small, squirrel-like primates but are now classified in the separate order Scandentia, are the only other species shown to support HCV infection. Following inoculation with HCV, tree shrews can become intermittently viremic but only if immunosup-

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pressed (Xie et al. 1998). Subsequent work has suggested that acute infection can progress to chronicity (Xu et al. 2007; Amako et al. 2010), resulting in clinically symptomatic liver disease, including steatosis, fibrosis, and cirrhosis after 3 years (Amako et al. 2010). These data are certainly intriguing but require further validation. Additionally, tree shrews are a challenging model not easy to maintain in captivity, genetically highly diverse as an outbred species and having fewer available species-specific tools and reagents for investigating the immune response to HCV.

RODENT MODELS FOR HCV

HCV Transgenic Mice

Mice are one of the most commonly used species in biomedical research but are resistant to HCV infection. To study HCV-induced liver disease, several mouse lines have been created that transgenically express individual or combinations of HCV gene products (for review, see Kremsdorf and Brezillon 2007). HCV transgenic mice have found some use for studying intrahepatic, virus-specific adaptive immune responses against HCV or the impact of specific HCV-derived gene products on the development of liver disease. However, it should be noted that disease severity is highly strain dependent. Although HCV transgenic mouse models may have improved some of our understanding of the mechanisms involved in HCV-induced pathogenesis, numerous caveats remain. First and foremost, transgenic expression of HCV protein(s) does not adequately mimic the inflammatory milieu that is established in the liver during acute and chronic viral infection. Consequently, the mechanisms leading to any histopathologic manifestations in HCV transgenic mice—even if they are similar to those observed in chronic HCV carriers—may differ significantly from those driving pathogenesis in patients. Furthermore, expression of HCV gene products in transgenic mice is often under strong viral or cellular promoters that yield viral protein expression levels far exceeding those during normal infection.

Xenotransplantation Models: HCV Infection in Human Liver Chimeric Mice

To establish a murine environment conducive to HCV infection, different host adaptation approaches have also been pursued. Liver chimeric mice harboring human tissue have proven very useful for HCV infection. To achieve engraftment, murine xenorecipients must be immunodeficient to avoid rejection of the transplanted human cells and also suffer from an endogenous liver injury to selectively ablate mouse hepatocytes while promoting expansion of human cells. Numerous liver injury mouse strains have been created including lines that express hepatotoxic transgenes (tg) such as the urokinase-type plasminogen activator (uPA) (Sandgren et al. 1991), a mutant form of α-1 antitrypsin (AAT-Z, referred to as PiZ) (Ding et al. 2011), or the herpes simplex virus thymidine kinase (HSV-TK) (Hasegawa et al. 2011). A different mouse liver injury model is based on the deficiency of fumarylacetoacetate hydrolase (FAH), an enzyme in the tyrosine catabolic pathway. Knockout of FAH leads to accumulation of hepatotoxic metabolites, and $FAH^{-/-}$ mice die from liver failure (Grompe et al. 1993). When crossed to immunodeficient backgrounds, uPA tg (Mercer et al. 2001; Meuleman et al. 2005; Tesfaye et al. 2013; Carpentier et al. 2014), HSV-tg (Hasegawa et al. 2011), $FAH^{-/-}$ (Azuma et al. 2007; Bissig et al. 2007, 2010), and PiZ mice (Borel et al. 2017) support robust engraftment with human hepatocytes. Highly engrafted human liver chimeric mice readily become viremic and can sustain persistent HCV infection for several months (Mercer et al. 2001; Meuleman et al. 2005; Lindenbach et al. 2006; Bissig et al. 2010; de Jong et al. 2014) when injected with cell culture- or patient-derived HCV. Such humanized mice have helped to analyze many aspects of HCV biology, including viral entry (Lacek et al. 2012; Meuleman et al. 2012), the role of anti-HCV antibodies (Vanwolleghem et al. 2008; de Jong et al. 2014), and infectious particle composition (Lindenbach et al. 2006; Steenbergen et al. 2010). Several anti-HCV therapeutics (Vanwolleghem et al. 2008; Kneteman et al. 2009; Kamiya et al. 2010; Ohara et al. 2011)

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have also been evaluated preclinically in human liver chimeric mice.

Challenges and Opportunities in Creating Dually Engrafted Human Immune System and Liver Mice

A major drawback of humanized mice is their highly immunocompromised status, thereby limiting their use for studying HCV pathogenesis, human adaptive immune responses, or vaccine development. Consequently, efforts have focused on establishing humanized mice coengrafted with both human liver cells and a functional human immune system (HIS).

Double humanization of both the liver and immune system has been achieved in mice using human hematopoietic stem cells (HSCs) and either adult (Gutti et al. 2014; Wilson et al. 2014; Strick-Marchand et al. 2015; Dusséaux et al. 2017) or fetal (Washburn et al. 2011; Bility et al. 2016; Billerbeck et al. 2016) hepatocytes. Dually engrafted mice can support HCV infection and have shown T-cell activation and HCV-associated liver disease (Washburn et al. 2011).

Although it is well documented that the engrafted HIS does become activated on challenging such mice with different viral pathogens (for review, see Douam and Ploss 2018), the quality of the immune response in these humanized models is rather weak (Baenziger et al. 2006; Yu et al. 2008; Brainard et al. 2009; Jaiswal et al. 2009, 2012; Strowig et al. 2009; Dudek et al. 2012; Billerbeck et al. 2013; Lüdtke et al. 2015; Bird et al. 2016). For example, whereas the engrafted components of the HIS become activated following microbial challenge, the HIS in conventional humanized mice—in contrast to humans—does not control very efficiently replication of viruses such as Epstein–Barr virus (EBV) (Strowig et al. 2009). Following vaccination with very potent vaccines such as the yellow fever virus vaccine, the immunogenicity profiles in peripheral blood mononuclear cells derived from conventional humanized mice resemble only poorly those of human vaccinees (Douam et al. 2018). Furthermore, titers of pathogen-specific antibodies are lower than those reported in humans and switching from immunoglobulin M (IgM) to other immunoglobulin subclasses is limited in conventional models. These and other caveats can be at least partially attributed to the inadequate cellular complexity of the engrafted HIS and aberrant architecture of secondary lymphoid organs. Engineering xenorecipients and refining humanization protocols to support development of human lymph nodes (Li et al. 2018) and the selective expansion of underrepresented immune population cells (Rongvaux et al. 2014; Li et al. 2016b; Douam et al. 2018) have translated into significantly improved functionality (Douam et al. 2018). Conceivably, combining these features with liver injury strains may yield dually engrafted mice much better suited to study HCV infection and immunity.

Genetically Humanized Mice

Despite their proven use as a versatile animal model for studying HCV, work with human liver chimeric mice can be limited by their fairly high cost, low throughput, and technical difficulties in generating them. An inbred mouse model with inheritable susceptibility to HCV would overcome these downsides. The challenge is to systematically identify and overcome any restrictions to HCV growth in murine cells.

It remains incompletely understood why mouse cells are largely resistant to HCV infection. Prior work established that CD81 and occludin (OCLN) represent the minimal set of human factors required to facilitate HCV entry into mouse cells in cell culture (Ploss et al. 2009). Scavenger receptor class B type 1 (SCARB1) and claudin-1 (CLDN1) are still needed for HCV uptake but do need to be of human origin (Ploss et al. 2009). Subsequent studies showed that transgenic expression of human CD81 and OCLN are sufficient to overcome the species barrier at the level of entry in mice (Dorner et al. 2011, 2013). Minimally humanizing residues that contribute to species restriction in the second extracellular loops (ELs) of murine CD81 and OCLN sufficiently enabled HCV uptake in mCD81/hEL2 mOCLN/hEL2 knockin mice (Ding et al. 2017).

Besides a genetic host adaptation approach, species barriers at the level of entry can also be

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overcome via viral adaptation. Mutating the HCV envelope proteins E1 and E2 can also permit HCV entry in cell lines expressing only mouse CD81, SCARB1, CLDN1, and OCLN (Bitzegeio et al. 2010). Such a murine-tropic HCV strain was subsequently shown to enter murine primary hepatocytes in vitro and in vivo (von Schaewen et al. 2016). However, such mouse-adapted HCV did not cause chronicity, indicating that further viral adaptations to murine environment are needed.

These genetically humanized mice have proven useful for a variety of applications, including the genetic dissection of HCV entry in vivo as well as preclinical assessment of entry inhibitors and vaccine candidates. Combining genetically humanized mouse models with mouse knockout technology can be used to genetically dissect molecular pathways involved in HCV entry. A proof-of-concept experiment showed that SCARB1 (Dorner et al. 2011) are essential for HCV uptake in vivo. Genetically humanized mice were further used to assess the protective efficacy of immunity induced via vaccination with recombinant vaccinia virus expressing the HCV polyprotein against challenge with HCV strains carrying heterologous structural proteins (Dorner et al. 2011). Similarly, it was shown that altered glycosylation patterns can increase the immunogenicity of a subunit HCV vaccine, yielding neutralizing antibodies that confer protection in genetically humanized mice (Li et al. 2016a). It was further demonstrated that passive infusion (Dorner et al. 2011; Giang et al. 2012) or vectored expression (de Jong et al. 2014) of broadly neutralizing anti-E2 antibodies or anti-CD81 blocking antibodies protects hCD81/hOCLN-expressing mice against viral challenge. Genetically humanized mice also aided in the identification of flunarizine, a diphenylmethylpiperazine traditionally used to treat migraines, as genotyped-dependent inhibitor of HCV entry both in vitro and in vivo (Anggakusuma et al. 2013).

Despite these exciting advances to facilitate viral entry through genetic adaptation of the host, a major shortcoming is the limited ability of HCV to replicate in mouse hepatocytes. Mouse hepatoma cell lines and embryonic fibroblasts can sustain HCV RNA replication at very low levels (Zhu et al. 2003; Uprichard et al. 2006; Vogt et al. 2013), suggesting that all host factors necessary for HCV replication are present. However, HCV relies on a large number of host factors to establish replication, and conceivably the murine orthologs of such factors may cooperate less efficiently with the virally encoded components of the HCV RNA replication machinery. One of such factors may be cyclophilin A (CypA), which is essential for HCV replication (Yang et al. 2008; Kaul et al. 2009). It was recently shown that orthologs from different primate and rodent lineages differ in the ability to support HCV replication (Gaska et al. 2019). In particular, mouse CypA could only poorly substitute for human CypA's function in the HCV life cycle because of differences in four amino acid residues in positions 11, 12, 14, and 52. However, (over)expression of human CypA in mouse cell lines augmented only slightly HCV's ability to replicate, suggesting that additional (human-specific) components of the HCV replication complex are missing or are incompatible. Undoubtedly, identifying any additional human-specific factors capable of augmenting HCV RNA replication in mouse cells will be critical for the development of additional mouse models for HCV infection.

The low levels of HCV RNA replication could also be indicative of the virus' inability to robustly evade host antiviral defenses. Indeed, blunting the innate immune response in mouse cells (Chang et al. 2006; Lin et al. 2010; Dorner et al. 2013; Nandakumar et al. 2013; Vogt et al. 2013) and mice (Dorner et al. 2013) boosted viral replication. It should be noted that HCV entry factor transgenic mice on a different immunocompetent background (ICR) were reported to sustain chronic HCV infection (Chen et al. 2014). However, these data await independent confirmation. In-depth analysis of differences in host-specific immunity will be very informative to determine how the murine environment could be adapted to be more conducive to viral replication.

Conceivably, species-specific dominant restriction factors such as those identified for human immunodeficiency virus (HIV) (for re-

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view, see Bieniasz 2004) may pose additional hurdles for interspecies transmission of HCV. However, heterokaryons of mouse and human cells support the entire HCV life cycle (Frentzen et al. 2011), suggesting these restriction factors may not exert a dominant-negative effect.

Future efforts must focus on identifying additional factors behind the limited host tropism of HCV. Results from these studies could inform iterative improvements to existing models, ultimately yielding a mouse model with inheritable susceptibility to HCV infection.

SURROGATE MODELS FOR HCV

In the absence of a fully immunocompetent animal model for HCV, researchers started looking for HCV-like animal viruses or hepaciviruses to develop surrogate models (Bukh 2011; Hartlage et al. 2016). Animal homologs of human viruses have been widely recognized as an important, and often the only, alternative for studying the pathogenesis of, and immune responses to, related human viruses. Studies of murine norovirus (MNoV), simian immunodeficiency viruses (SIVs), and several different rodent herpesviruses yielded data that significantly improved our understanding of how their human counterparts establish viral persistence and evade host immunity (Wobus et al. 2006). Notably, although HIV can infect chimpanzees, the immune response to SIV infection and resultant pathogenesis in NHPs more closely mirrors that of HIV in humans, including the development of acquired immunodeficiency syndrome (AIDS) (Hatziioannou and Evans 2012).

HCV is the prototypic member of the genus *Hepacivirus* of the family *Flaviviridae* (Smith et al. 2016). Until 2011, the only known hepacivirus besides HCV was the distantly related GBV-B (George–Barker virus B) (Stapleton et al. 2011). Although initially discovered in tamarins experimentally infected with a human serum sample, no evidence of natural GBV-B infection in humans or tamarins exists, leaving the origins of this virus unknown. In 2011, a metaviromics study of shelter dogs suffering with respiratory diseases led to the identification of the first hepacivirus directly from an animal sample (Kapoor et al. 2011). Later, horses were identified as the natural host of this virus and therefore the canine hepacivirus was renamed as nonprimate hepacivirus (NPHV) or equine hepacivirus (EqHV) (Burbelo et al. 2012). Subsequently, several species of new hepaciviruses were identified from different animals, including wild and feral rodents (Drexler et al. 2013; Kapoor et al. 2013; Lauck et al. 2013; Firth et al. 2014; Hartlage et al. 2016). Multiple recent review articles (Pybus and Gray 2013; Pfaender et al. 2014a; Scheel et al. 2015b; Thézé et al. 2015; Hartlage et al. 2016; Pybus and Thézé 2016) have elaborated on the discovery of these animal hepaciviruses, their genetic relatedness, and evolutionary origins. Here, we will focus our discussion on the potential use of these animal hepaciviruses for developing meaningful surrogate models for HCV. Until 2012, GBV-B was the only animal hepacivirus used as an HCV surrogate, but from 2012 to 2017, researchers changed their focus to NPHV. However, more recent efforts to develop a useful (Tanaka et al. 2014; Scheel et al. 2015a; Pfaender et al. 2017) HCV surrogate model have looked to a rodent hepacivirus (RHV) species found in rats (Billerbeck et al. 2017; Trivedi et al. 2018).

George–Baker Virus B

GBV-B is the oldest known genetic relative of HCV. The origin and natural host of GBV-B remains a mystery after its initial identification in 1967 from captive tamarins (*Saguinus labiatus*) inoculated with the blood sample of a surgeon suffering from acute hepatitis (Deinhardt et al. 1967). To date, no evidence of GBV-B infection has been found in humans or wild primates (Smith et al. 2016). Experimental infection of GBV-B in laboratory tamarins, a New World monkey species, induces hepatitis and mostly an acute self-resolving infection (Takikawa et al. 2010; Bukh 2012; Manickam et al. 2016). Thus, GBV-B was frequently used as a surrogate model for HCV to characterize the immune response associated with acute virus clearance (Woollard et al. 2008; Manickam et al. 2016). Like HCV, the acute phase of GBV-B infection in tamarins is associated with

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appearance of virus-specific T cells that primarily target the nonstructural proteins (Woollard et al. 2008). More interestingly, the natural clearance of primary GBV-B infection in tamarins confers protective immunity against reinfection by a homologous virus. Similar to HCV, the GBV-B NS3/4A protein can also disrupt the host innate immune response by cleaving the adapter protein MAVS (mitochondrial antiviral signaling protein) and subsequently disrupting RIG-I signaling (Chen et al. 2007). In a recent study, Manickam and colleagues characterized the nature of innate immunity during GBV-B infection in tamarins and showed association of acute hepatitis with significant lymphocytic infiltration of the liver marked by an increase in natural killer (NK) and dendritic cells (Manickam et al. 2016). Although a lack of species-specific biological and immunological reagents for tamarins has hindered an in-depth analysis of immunity during GBV-B infection, these studies clearly show the usefulness of GBV-B as a surrogate of HCV. A major limitation of the GBV-B model, however, is the inability of this virus to establish lifelong or even long-term viral persistence in most tamarins, a hallmark of HCV infection in humans. This precludes the use of GBV-B model to characterize the nature of the immune response in developing persistent hepacivirus infection and, more importantly, the ability to test and optimize vaccines or other approaches for preventing HCV-like viral persistence.

Nonprimate or Equine Hepacivirus

NPHV was first identified as a common infection in horses using a serological survey (Burbelo et al. 2012). Phylogenetic analysis indicates that NPHV is the closest genetic relative of HCV. In 35% of screened horses, high-titer anti-NS3 antibodies were observed, whereas only a fraction (8/36 horses) of these seropositive horses had ongoing viremia. These results suggested that a majority of horses clear the NPHV infection. A few years later, Pfaender and colleagues reported evidence of NPHV infection in 31.4% of 433 surveyed horses from Germany, but only ~8% of these seropositive horses had viremia, again indicating the rarity of chronic NPHV infection (Pfaender et al. 2014, 2015). Similarly, Ramsay and colleagues (2015) recently reported early virus clearance of experimental NPHV infection in all immunocompetent adult horses and viral persistence in foals and immunodeficient horses. Although horses allow the serial analysis of immune responses in the blood and liver during NPHV infection, the low incidence of viral persistence and scarcity of horse-specific molecular and biological reagents restricts the usefulness of NPHV as a surrogate model.

Rodent Hepacivirus Models

Since 2013, a plethora of RHV species have been identified from feral and wild rodents worldwide (Table 1; Drexler et al. 2013; Kapoor et al. 2013; Van Nguyen et al. 2018). However, considering the species specificity of known hepaciviruses, the two RHV species identified in feral rats from New York City (Firth et al. 2014) offered the best opportunity to develop animal models using laboratory rats and mice. Later, one of these two RHV species was successfully isolated in outbred Sprague Dawley (SD) rats and used to infect laboratory mice (Billerbeck et al. 2017; Trivedi et al. 2018). Despite their shared origin, two different names were used for this virus, NrHV (Norway rat hepacivirus) or RHV-rn1 (rodent/rat hepacivirus from *Rattus norvegicus* species-1). Because the first reverse genetic system-derived virus was named RHV-rn1 (Trivedi et al. 2018) and this clone is now used by several laboratories worldwide, we will use this name for consistency. Although all RHV species are genetically distinct and share only limited sequence identity with HCV genotypes (Table 1), the well characterized genome of RHV-rn1 shares high-sequence homology with HCV in the conserved miR-122 seed sites in viral 5′UTR, RNA secondary structures, and polyprotein cleavage sites (Table 1; Hartlage et al. 2016; Trivedi et al. 2018).

RHV Mouse Model

Considering the close genetic relatedness of laboratory mice and rats, successful isolation and

Table 1. Comparison of hepatitis C virus (HCV) surrogate models

Model	GBV-B	NPHV	RHV mouse	RHV rat
Protein similarity HCV (NS5B)[a]	37%	59%	40%	40%
Genotypes	1	1	1	>2
Reverse genetic system	Yes	Yes	Yes	Yes
Cell culture system	No	No	Yes	Yes
Cell tropism	Hepatocytes	Hepatocytes	Hepatocytes	Hepatocytes
Acute infection	Yes	Yes	Yes	Yes
Chronic infection	No	Rare	No	Yes
Acute liver disease	Yes	Mild	Mild	Mild
Chronic liver disease	No	No	No	Yes
HCV antiviral sofosbuvir	Not known	Not known	Not known	Yes
Use for vaccination studies	No	No	No	Yes

(GBV-B) George–Barker virus B, (NPHV) nonprimate hepacivirus, (RHV) rodent hepacivirus.

[a]HCV isolate H77 (GenBank AAB67063, NS5B region amino acid residues 2423–2934) was used as reference. Most diverse HCV genotype (YP_001469631) shows only 75% similarity in this region.

infection of RHV-rn1 in rats was a promising step toward developing a mouse model for hepacivirus. A RHV-rn1 stock generated in SD rats was used for infecting immunocompetent laboratory mice (Billerbeck et al. 2017). As expected, both C57BL/6J and Balb/c mice showed complete susceptibility to virus infection but universally cleared the virus in 2–3 weeks, which is markedly different from the course of infection observed in humans and even wild rats (please see below). Notably, RHV-rn1-infected immunodeficient mice developed persistent long-term viremia. Furthermore, although mice lacking functional B, T, and NK cells (NOD-Rag1$^{-/-}$ IL2R$\gamma^{-/-}$), the type I (A129, IFNRα/$\beta^{-/-}$) or the type I and III (AG129, IFNRα/$\beta^{-/-}$, and IFNR$\gamma^{-/-}$) interferon (IFN) receptors failed to clear the virus, whereas MAVS knockout mice cleared the infection in 3 weeks. These results suggest that the failure of RHV-rn1 to establish persistence in mice is not primarily because of its inability to cleave mouse MAVS (Parera et al. 2012; Anggakusuma et al. 2016). Most interestingly, RHV-rn1 inoculum passaged in NRG mice did produce a higher titer and prolonged viremia in normal mice. Although most immunocompetent mice cleared this adapted virus within ∼4–5 weeks of infection, these results suggested that RHV-rn1 can be adapted to establish persistent infection in normal laboratory mouse strains. Comparative sequence analysis of virus present in the rat inoculum, NRG and C57BL/6J mice not only revealed adventitious mutations but also acquisition of adaptive mutations during the passaging of this rat virus in mice (Billerbeck et al. 2017).

RHV-rn1 infection in mice resulted in expansion of Ly6C^{+} monocytes and NKp46^{+} NK cells in the liver during the early acute phase of infection. The number of these cells declined in correlation with viral clearance at week 3 p.i. T-cell expansion (Ki67^{+}) in the livers of infected mice started at day 6 p.i. and were characterized by a CD44^{+} effector phenotype and Th1 differentiation as evident by expression of T-bet and IFN-γ. These changes in T-cell function and phenotype were more profound in the liver compared with peripheral blood mononuclear cells. Kinetics of T-cell responses correlated with an increase in ALT, suggesting T-cell-mediated clearance of infected hepatocytes. The role of T cells in RHV-rn1 clearance was further studied by selectively depleting T-cell subsets before infection. Although depletion of CD8^{+} T cells led to prolonged viremia in mice, depletion of CD4^{+} T cells culminated in long-term viral persistence. These studies not only confirmed the importance of T-cell immunity in RHV-rn1 clearance in mice but also the indispensability of helper T-cell responses. Clearance of primary RHV-rn1 infection generated T-cell memory because the duration and titer of viremia were lower on reinfection and accompanied by the rapid expansion of IFN-γ-producing, virus-

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specific $CD8^+$ T cells. Finally, analysis of T cells in mice depleted of $CD4^+$ T cells and with persistent viremia showed a significant increase in $Foxp3^+$ $CD4^+$ T_{regs} and PD-1^{High} $CD44^{Low}$ $CD8^+$ T cells, indicating an exhausted T-cell phenotype. Notably, NK-cell depletion in mice before RHV-rn1 infection had no major effect on virus clearance. Although all infected mice showed evidence of seroconversion (anti-NS3 IgG) at day 21 p.i., the serum of these cleared mice was able to neutralize only very low titers of the virus.

Further work with RHV-rn1 could also provide new insights into hepacivirus entry, receptor usage, and the role of host factors during infection and replication. However, many important aspects of the intricate interactions with the host conceivably may be virus-specific and not conserved between HCV and RHV-rn1. Studying chronic RHV-rn1 infection seems to be a possibility as passaging in mice leads to adaptation for delayed clearance in normal mice. In the future, such an RHV variant adapted for establishing long-term persistence in normal mice will be an enormously useful model to study the nature of host responses culminating in lifelong viral persistence and development of virus-induced liver diseases (Klenerman and Barnes 2017; Grakoui and Walker 2018). Testing and optimization of vaccination concepts for HCV requires an immunocompetent animal model susceptible to viral persistence and, therefore, adapting RHV to establish spontaneous persistence in mice is also necessary before this model can be used for HCV vaccine research.

RHV Rat Model

Metaviromics-based screening of feral rats for RHV-rn1 and related variants indicated active viral infection (viremia) in 10%–30% of animals, making rats likely the natural host of these viruses (Firth et al. 2014). The complete genome of the first RHV-rn1 variant isolated from laboratory SD rats infected with serum from a feral brown rat was used to construct the reverse genetic system for RHV-rn1 (Trivedi et al. 2018). Clone-derived transcripts produced infectious virus following intrahepatic injection in laboratory rats and assays for negative-strand RNA confirmed the virus is hepatotropic. The RHV-rn1 genome contains 9656 nucleotides and encodes a single, 2991 amino acid polyprotein flanked by a 485-nucleotide-long 5′UTR and 297-nucleotide-long 3′UTR. In addition to resembling HCV in genome organization and polyprotein cleavage pattern, the RHV-rn1 5′UTR also contains two miR-122 seed sites, which are known to play an important role in viral RNA replication and accumulation in hepatocytes (Jopling et al. 2005). miR-$122^{-/-}$ mice could not be infected with RHV-rn1 and mutagenesis of these miR-122 sites revealed that one of the two seed sites is indispensable for virus replication in rats (Billerbeck et al. 2017; Trivedi et al. 2018). More recently, the miR-122 seed sites in the HCV 5′UTR were shown to sequester miR-122 as a means to de-repress miR-122 target genes, suggesting the biological and evolutionary significance of miR-122 sites in hepacivirus 5′UTR are not fully understood (Jopling et al. 2005; Luna et al. 2015; Yu et al. 2017). Availability of the RHV-rn1 reverse genetic system, presence of miR-122 seed sites in the 5′UTR and a tractable model of hepatotropic viral infection together bode well for defining the interaction of miR-122 with the hepacivirus 5′UTR and its biological and pathological significance.

RHV-rn1 infection in a wide range of outbred and inbred rats highlights its remarkable ability to establish lifelong persistence in a majority of immunocompetent rats (Trivedi et al. 2018), which is a distinct advantage over the RHV-rn1 mouse model. Notably, spontaneous resolution of primary infection has been observed in outbred rats and, therefore, comparative analysis of host responses culminating in different infection outcomes can be studied. Histopathologically, the livers of RHV-rn1-infected rats showed lymphocytic infiltration during acute viral infection and the presence of micro- and macrovesicular steatosis during the chronic phase (Trivedi et al. 2018). In situ hybridization assay showed disseminated viral infection in the liver and wide variability in the amount of viral RNA in infected hepatocytes. Analysis of liver transcriptome changes

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in RHV-rn1-infected rats showed overexpression of IFN-stimulated genes (ISGs) during both the acute and chronic phases of infection (Trivedi et al. 2018). However, to better understand liver ISG responses during RHV-rn1 infection, the liver transcriptomes of animals that clear the virus must be compared with those that develop chronic infection.

The full susceptibility of immunocompetent rats to RHV-rn1 persistence can be exploited to test and develop vaccines for preventing viral persistence, the main goal of a desired HCV vaccine (Walker 2017; Grakoui and Walker 2018). As the first step forward, Hartlage and colleagues (2019) used a rat model to determine whether T-cell vaccination can be used to prevent hepacivirus persistence. Although the immune correlates of protection against HCV persistence are not fully defined, several lines of evidence from chimpanzees and humans indicate an important role of T-cell immunity. Importantly, vaccination of chimpanzees to prime functional HCV-specific T cells led to suppression of viremia and accelerated virus control following experimental HCV challenge (Folgori et al. 2006). Analysis of T-cell immunity in RHV-rn1-infected rats showed spontaneous failure of T cells as characterized by an early and transient expansion of virus-specific T cells incapable of producing Th1 cytokines, IFN-γ, TNF-α, and IL-2. Additionally, serial analysis of RHV-infected rats revealed persistent failure of T-cell immunity during chronic viral infection. Although these results indicated that spontaneous T-cell failure leads to RHV persistence, immunization by a T-cell vaccine protected 40%–60% of rats against RHV persistence and thus confirmed the seminal role of T-cell immunity in determining the infection outcome. Interestingly, vaccine-primed T cells in vaccinated rats showed 10- to 100-fold higher expansion of $CD4^+$ T cells compared with $CD8^+$ T cells during RHV clearance, indicating the importance of helper T cell responses in the initial control of RHV. In vivo cell depletion of T-cell subsets in vaccinated rats showed that although transient depletion of $CD8^+$ T cells prolongs the viremia that ultimately clears, $CD4^+$ T-cell depletion results in lifelong RHV persistence. Overall, this study provided direct in vivo evidence that cooperation between $CD4^+$ and $CD8^+$ T cells is necessary for resolution of a primary hepacivirus infection and that hepacivirus persistence caused by subversion of T-cell immunity can be prevented by T-cell vaccination. Notably, a T-cell vaccination approach failed to protect against HCV persistence in a recently concluded human clinical trial (NCT01436357). Although the reasons for vaccine failure remains unknown and will be challenging to study in human clinical cohorts, a better and more thorough evaluation of vaccine failure and success in the RHV-rat model can provide data for optimizing more efficacious HCV vaccination approaches. Notably, the role of innate and humoral immunity during RHV infection in rats remains unstudied and should also be prioritized. Furthermore, the rat model is unique in that the mechanisms of T-cell subversion leading to delayed virus clearance and lifelong viral persistence during hepacivirus infection can be delineated. Such studies of in-depth T-cell characterization require the construction of novel MHC class I and II tetramers. However direct in vivo analysis of virus-specific T cells from the liver, a site of viral replication rarely accessible in humans, will likely yield unprecedented insights into HCV persistence and in general can also identify the common mechanisms of immune failure culminating in the persistence of other hepatotropic viruses.

Despite their phylogenetic relatedness, RHVs share very low DNA and protein sequence identity with HCV. One potential solution could be the construction of RHV/HCV chimeras. However, at this point, it is unclear whether viral chimeras between RHV and HCV could be constructed that do not lose viral fitness, as previously seen for almost all GBV-B/HCV chimeras (Rijnbrand et al. 2005; Haqshenas et al. 2007; Li et al. 2014; Suzuki et al. 2016; Zhu et al. 2016).

CONCLUDING REMARKS

Despite the availability of highly effective, direct-acting antiviral (DAA) therapies for HCV, incidences of new infections are increasing in the United States and worldwide. Development

of a prophylactic vaccine is necessary to prevent new infections and reduce the size of the HCV epidemic. Considering the availability of surrogate models, humanized mice models, and effective treatment, we propose that finally the time has come to conceptualize and test prophylactic vaccines for HCV. Recent restrictions on the use of human fetal tissue in the United States (grants.nih.gov/grants/guide/notice-files/NOT-OD-19-128.html) jeopardize access to existing humanized mouse models and their further refinement. Given the scarcity of alternative models for these and many other human-tropic pathogens and the fact that human fetal tissue is currently absolutely irreplaceable, such shortsighted attempts to curtail biomedical advancement will hurt those who are at risk of contracting HCV or are already suffering from other, currently untreatable diseases. We believe that the testing of potential vaccination regimens should start on surrogate models, followed by validation using HCV infection studies in humanized mice models, and lastly, vaccination in high-risk populations or uninfected, healthy volunteers with full and free access to DAA therapy should the vaccination fail.

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