Innate Immunity to Enteric Hepatitis Viruses

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Although hepatitis A virus (HAV) and hepatitis E virus (HEV) are both positive-strand RNA viruses that replicate in the cytoplasm of hepatocytes, there are important differences in the ways they induce and counteract host innate immune responses. HAV is remarkably stealthy because of its ability to evade and disrupt innate signaling pathways that lead to interferon production. In contrast, HEV does not block interferon production. Instead, it persists in the presence of an interferon response. These differences may provide insight into HEV persistence in immunocompromised patients, an emerging health problem in developed countries.

Textbooks often consider hepatitis A virus (HAV) and hepatitis E virus (HEV) together because their modes of transmission and clinical presentations are relatively similar. Historically, both viruses have been thought to cause only acute disease, diminishing the interest in studying them, in contrast to hepatitis B virus (HBV) and hepatitis C virus (HCV) that persist and cause chronic hepatitis. As a result, the immune responses generated by HAV and HEV and the pathogenesis of both infections remain relatively poorly understood. However, recent advances in molecular techniques and animal models have provided new insight into these viruses and show that the host responses to these two agents are quite different. Many unexpected findings have been made in recent years that challenge old concepts about these two human pathogens.

The intestinal epithelium stands as the first physical barrier that HAV and HEV must cross

before either can enter the bloodstream and gain access to the liver (Peterson and Artis 2014). Despite epidemiologic evidence of efficient natural enteric transmission, the mechanisms by which these viruses penetrate the tight gut epithelial barrier are not known. There is no compelling evidence that HAV actually replicates in the gastrointestinal epithelium. HAV-related antigen was putatively detected by immunofluorescence microscopy in crypt cells of the small intestine shortly after per-oral challenge of New World owl monkeys (*Aotus trivirgatus*) (Asher et al. 1995), but a similar study of marmosets failed to show viral antigens in gut tissue (Mathiesen et al. 1980). Similar detailed studies have not been performed with HEV. Because there is a dearth of information on interactions of HAV and HEV with the gut, only events occurring in the liver, the main target for both viruses, will be considered here.

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INNATE IMMUNITY IN THE LIVER

The liver is constantly exposed to food and microbial products coming from the intestine via the portal venous blood, and therefore hepatic immune responses are generally geared toward tolerance (Crispe 2009). However, the liver can mount robust immune responses on microbial infection. Liver resident cells, including hepatocytes, resident macrophages (Kupffer cells), sinusoidal endothelial cells, and stellate cells, express a variety of pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs). These include Toll-like receptors (TLRs) and retinoic acid activated gene I (RIG-I)-like RNA helicases (RLRs) (Pandey et al. 2014), as well as nucleotide-binding oligomerization domain (NOD)-like protein receptors (NLRs) that have both proinflammatory and regulatory activities (Guo et al. 2015; Coutermarsh-Ott et al. 2016). On ligand binding, PRRs assemble multiprotein complexes culminating in the production of antiviral cytokines and chemokines.

Hepatocytes, the primary cell type targeted by HAV and HEV for infection, express both RLRs and TLRs (Li et al. 2005). RIG-I and the melanoma differentiation-associated protein-5 (MDA5), two well-characterized RLRs, detect cytoplasmic single-stranded RNA (ssRNA) with a 5'-di- or triphosphate moiety, and lengthy double-stranded RNA (dsRNA), respectively (Fig. 1) (Pandey et al. 2014). On RNA binding, both RIG-I and MDA5 recruit the mitochondria-associated antiviral protein (MAVS) to assemble a "signalosome," which results in the activation of transcription factors (e.g., interferon [IFN] regulatory factor [IRF3], nuclear factor κ B [NF- κ B]) leading to type I and type III IFN expression (Horner and Gale 2013). Hepatocytes also express TLR3, which senses dsRNA in an endosomal compartment and activates IRF3 and NF- κ B via the adaptor protein TIR-domain-containing adapter-inducing IFN- β (TRIF) (Fig. 1) (Pandey et al. 2014). IFNs are secreted from the cell, subsequently binding to specific receptors on the cell surface and inducing hundreds of IFN-stimulated genes (ISGs). ISGs thus induced through both autocrine and

paracrine signaling inhibit the replication and spread of viruses (Schoggins and Rice 2011). These cell-protective responses are augmented by the IFN-inducible protein kinase R (PKR), which is activated by viral dsRNA replication intermediates and which, by phosphorylating the translational initiation factor eIF2 α , globally suppresses cellular protein synthesis (Garcia et al. 2006).

In addition to hepatocytes, other cell types that either reside in or are recruited to the liver during infection participate in the innate response to virus infection. For example, in addition to their role in antigen presentation, Kupffer cells and dendritic cells (DCs) express a variety of PRRs and produce antiviral cytokines and chemokines. Kupffer cells and monocytes express high levels of NLRs, which play important roles in mediating inflammatory responses and modulating liver injury (Dixon et al. 2013). Plasmacytoid DCs (pDCs), also known as professional IFN-producing cells, express high levels of TLR7 and TLR9 and rapidly produce high quantities of both type I and type III IFNs (within hours) on activation (Swiecki and Colonna 2015). The liver also contains highly abundant natural killer (NK) cells and NK T cells that produce IFN- γ on activation and exhibit perforin-dependent cytotoxicity against virus-infected cells (Golden-Mason and Rosen 2013).

INNATE IMMUNE RESPONSES TO HAV INFECTION

Innate Immune Responses in Hepatitis A

With only very rare exceptions, HAV infection causes only short-term, acute liver injury. Fecal shedding of virus and viremia (both detected by reverse transcription polymerase chain reaction [RT-PCR]) may be prolonged for months in infected neonates and severely immunocompromised liver transplant recipients (Rosenblum et al. 1991; Foster et al. 2017), but such reports are very rare in the literature. The replication of wild-type HAV does not cause cytopathic effects in vitro or in vivo (Daemer et al. 1981; Binn et al. 1984; Hirai-Yuki et al. 2016a), and the disease is thought to be mediated by the

Figure 1. Innate immune signaling pathways leading to interferon (IFN) production in hepatocytes. The cytosolic retinoic acid activated gene I (RIG-I)-like helicases, melanoma differentiation-associated protein 5 (MDA5) and RIG-I, and endosomal Toll-like receptor (TLR)3 are important pathogen recognition sensors within hepatocytes, signaling downstream through the adaptors mitochondria-associated antiviral protein (MAVS) and TIR-domain-containing adapter-inducing IFN-β (TRIF), respectively, inducing phosphorylation, dimerization, and, ultimately, nuclear translocation of the critical transcription factor IRF3 along with activation of nuclear factor (NF)-κB, resulting in transcription of type I (IFN-α/β) and type III (IFN-λ) IFN genes. IL, Interleukin. (From Lemon 2010; adapted, with permission, from The American Society for Biochemistry and Molecular Biology © 2010.)

host immune response to the virus (see Shin and Jeong 2018).

Although a number of nonhuman primate species are susceptible to HAV infection and have been used for many years to study pathogenesis (see Lanford et al. 2018), the innate immune response to acute HAV infection has not been carefully examined until recently. Lanford and colleagues reported the results of microarray assays documenting changes in the intrahepatic transcriptome during acute HAV infection in experimentally infected chimpanzees (Lanford et al. 2011). This study revealed

HAV to be remarkably stealthy. Only a very limited type I IFN response was detected in the liver of these animals, and only during the early stages of the infection, before peak replication of virus in the liver (Fig. 2A) (Lanford et al. 2011). These results are in sharp contrast to HCV, which elicits robust innate responses within days after virus inoculation of chimpanzees (Fig. 3) (Bigger et al. 2001; Su et al. 2002), and confirm very early suggestions in the literature that hepatitis A is accompanied by a minimal IFN response (Taylor and Zuckerman 1968; Wheelock et al. 1968).

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Figure 2. Intrahepatic interferon (IFN)-stimulated gene expression in a chimpanzee with experimental hepatitis A virus (HAV) infection. (A) Intrahepatic IFN-stimulated gene (ISG) messenger RNA (mRNA) expression, shown as fold change from baseline in Affymetrix GeneChip assays, in a chimpanzee (4x0395) infected experimentally with HAV. With the exception of *CXCL10* (IP10) and *ISG20*, both of which are dually regulated by IFN- α/β and IFN- γ , low-level ISG induction is restricted to the first weeks of infection, and subsides before peak HAV RNA abundance in the liver. (From Lanford et al. 2011; adapted, with permission, from the National Academy of Sciences © 2011.) (B) Early course of HAV infection in chimpanzee 4x0395, showing alanine aminotransferase (ALT) elevation and IFN and cytokine detection in serum samples.

The early but limited expression of ISGs is associated with a brief period during which minimal amounts of IFN- α can be detected in serum from infected chimpanzees (Fig. 2B). This correlates temporally with the transient presence of pDCs in hepatic sinusoids (Feng et al. 2015). In vitro studies using human pDCs freshly isolated from buffy coats indicate that pDCs respond in a robust fashion, secreting type I IFN when exposed to quasi-enveloped virus (eHAV) (see McKnight and Lemon 2018), but not naked, nonenveloped HAV (Feng et al. 2015). eHAV is endocytosed rapidly and efficiently by pDCs. This is likely a result, at least in part, of recognition of phosphatidylserine

(PtdSer) residues on the surface of the quasi-envelope by PtdSer receptors on the pDC membrane as it is partially blocked by annexin V (Feng et al. 2015). The early type I IFN response diminishes before peak replication of HAV within the liver and the onset of liver injury, when a type II IFN (IFN- γ) response becomes evident (Lanford et al. 2011; Zhou et al. 2012; Feng et al. 2015). Type III IFN (IFN- λ) responses induced by HAV infection have not been critically examined in the chimpanzee model.

Several attributes of HAV likely to contribute to its stealthy nature. The 5' end of newly synthesized HAV RNAs are covalently linked to a small protein, VPg, which serves to prime

Figure 3. Comparative analysis of intrahepatic interferon (IFN)-stimulated genes (ISGs) expression resulting from infection with hepatitis C virus (HCV) versus hepatitis A virus (HAV) or hepatitis E virus (HEV) in chimpanzees. Columns reflect the average maximum-fold change for each of the indicated ISGs from baseline during the course of acute infection, as determined in two independent studies, one of HAV and the other of HEV. Data from HAV-infected animals are from Lanford et al. (2011), whereas HEV-infected animal data are from Yu et al. (2010). Contemporaneous data from chimpanzees with experimental HCV infection are shown for comparison from each of these two studies, both of which used Affymetrix GeneChips.

RNA synthesis (Weitz et al. 1986; McKnight and Lemon 2018). Although studies with other picornaviruses indicate that this is likely to be removed from incoming genomic RNA shortly after viral entry and uncoating, VPg may shield the 5' end of newly synthesized RNAs from cytoplasmic RNA sensors such as RIG-I. In addition, the HAV genome terminates at its 3' end in a poly(A) tract similar to cellular messenger RNAs (mRNAs). Thus, HAV RNA may be distinguished from host mRNAs only by the secondary structure within the 5' untranslated (5'UTR) RNA (see McKnight and Lemon 2018), or by its duplex structure with minus-strand RNA (Myong et al. 2009; Schlee et al. 2009). The replication of HAV is very slow compared to other picornaviruses, possibly because of the low guanine-cytosine (GC) content of viral RNA and the fact that viral protein synthesis is dependent on codons that are relatively rarely used by host cells (see Pintó et al. 2018). Newly synthesized HAV genomes appear to be rapidly packaged into the viral capsid, leaving little free HAV RNA in the cytoplasm (Anderson et al. 1988). Both of these attributes could also limit the abundance of

viral RNA available for detection by PAMP receptors.

HAV Actively Suppresses IFN Responses by Proteolytically Degrading MAVS and TRIF

In addition to these features that likely limit innate immune recognition, HAV actively disrupts signaling pathways leading to IFN production in infected cells (Fig. 4A) (Fensterl et al. 2005). Both cytoplasmic and endosomal RNA-sensing pathways are checked by proteinases expressed by the virus. A virally encoded, catalytically active polyprotein processing intermediate, 3ABC, degrades MAVS, a critical adaptor protein for signaling downstream of cytoplasmic RNA sensors, and thereby ablates RLR-mediated induction of IFNs (Figs. 4B, top, and 5) (Yang et al. 2007). The mature, fully processed HAV 3C^{pro} protease (see McKnight and Lemon 2018) is normally distributed throughout the cytoplasm. However, when fused with 3AB in the processing intermediate, 3ABC, it assumes a mitochondrial localization because of a mitochondrial membrane localization signal in the 3A transmembrane domain (Yang

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Figure 4. Hepatitis A virus (HAV) infection actively blocks virus-induced interferon (IFN) responses and degrades mitochondria-associated antiviral protein (MAVS). (A) Confocal fluorescence microscopic images of IFN-regulatory factor (IRF)3 expression in a Huh7 cell line containing a replicating subgenomic HAV RNA (HAV-Bla cells) or its cured derivative lacking HAV RNA (Bla-C cells), following infection with Sendai virus (SenV), a potent retinoic acid activated gene I (RIG-I) agonist, or mock infection (Mock). IRF3 is predominantly nuclear in localization in Bla-C cells, indicating its activation, whereas it remains cytoplasmic in HAV-Bla cells because of disruption of RIG-I/MAVS signaling. (Panel A from Yang et al. 2007; adapted, with permission, from the National Academy of Sciences © 2007.) (B) Confocal microscopic images showing MAVS and viral antigens in human liver-derived HepG2 cells infected with either HAV (*top* panels) or HEV (*lower* panels). MAVS expression is lost because of 3C^{pro} cleavage in HAV-infected cells, whereas MAVS abundance is not altered in HEV-infected cells. Nuclei are counterstained with 4', 6-diamidino-2-phenylindole (DAPI). (Panel B from Yin et al. 2017; adapted under the terms of the Creative Commons Attribution License, which permits unrestricted use.)

et al. 2007). In vitro, the cleavage of MAVS in HAV-infected cells is complete, and cells no longer respond to exogenous RLR ligands.

A second catalytically active 3C^{pro} processing intermediate, 3CD (see McKnight and Lemon 2018), cleaves the TLR3 adaptor protein TRIF (Fig. 5) (Qu et al. 2011). TRIF does not contain any canonical 3C^{pro} dipeptide cleavage sites. However, when fused to the polymerase 3D^{pol} in 3CD, the 3C substrate specificity is altered such that it cleaves TRIF at two noncanonical sites (Qu et al. 2011). It is interesting that the targeting of MAVS and TRIF for proteolytic degradation by HAV proteins mirrors the capacity of the phylogenetically unrelated HCV serine protease, NS3/4A, to degrade these same adaptor proteins (Lemon 2010). However, 3C^{pro} also cleaves the regulatory subunit of the IκB kinase complex, NF-κB essential modulator (NEMO) (Fig. 5) (Wang et al. 2014), disrupting NF-κB-mediated responses and further crippling the host's ability to produce IFN in HAV-infected cells.

Despite efficient cleavage of MAVS and TRIF, cultured cells infected with HAV may still develop at least a transient IFN response initi-

ated by RIG-I or TLR3 (Yang et al. 2007; Qu et al. 2011; Sung et al. 2017). This may occur before a significant fraction of MAVS or TRIF are degraded by the viral proteases. ISG responses could also result from TLR3 sensing of dsRNA in uninfected hepatocytes following endocytosis of replication intermediates released by neighboring infected cells (Dansako et al. 2013). Class A scavenger receptor proteins such as MSR1 play key roles in this process, facilitating the endocytosis of viral RNA that is then detected by endosomal TLR3 (DeWitte-Orr et al. 2010; Dansako et al. 2013). Macrophages could similarly contribute to the IFN response via TLR3 within the liver, as there is little evidence that they are actively infected and express HAV proteases. Plasmacytoid dendritic cells also sense eHAV via TLR7, as mentioned above, and respond with production of type I IFN in a MAVS- and TRIF-independent fashion (Feng et al. 2015).

Early studies showed that HAV can be eradicated from infected fibroblasts by treatment with either IFN-α or IFN-β (1000 IU/mL) (Vallbracht et al. 1984), whereas more recent data indicate that HAV RNA replication is inhibited by recombinant IFN-α2B at an EC₅₀ of

Figure 5. Hepatitis A virus (HAV) proteases degrade key innate immune signaling proteins: mitochondria-associated antiviral protein (MAVS), TIR-domain-containing adapter-inducing interferon (IFN) (TRIF), and nuclear factor (NF)- κ B essential modulator (NEMO) (IKK γ). Schematic showing how HAV polyprotein processing intermediates with 3C^{pro} catalytic activity cleave MAVS (3ABC), TRIF (3CD), and NEMO also known as IKK γ (mature 3C^{pro}), thereby disrupting signaling leading from pathogen-associated molecular pattern (PAMP) sensors to the critical transcription factors IFN-regulatory factor (IRF)3 and NF- κ B that drive type I and III IFN expression.

~100 U/mL (Yang et al. 2007). Thus, the capacity of HAV to infect cells is likely to be very dependent on its ability to block IFN responses.

Inflammasome Activation and NLR Proteins in Hepatitis A

In addition to RLRs and TLRs, NLRs can play an important role in the host response to infections by RNA viruses, both promoting and suppressing innate immunity and inflammation (Wen et al. 2013; Jha and Ting 2015). The NLRs are a large protein family containing evolutionarily

conserved domains involved in sensing and responding to cellular stress and pathogen invasion. NLRP3 (also known as cryopyrin, NALP3, or CIAS1) is the best-studied NLR protein, and it is pivotal for elaboration of interleukin (IL)-1 β and IL-18 by macrophages in response to a broad spectrum of RNA viruses, including picornaviruses (Rajan et al. 2011). NLRP3 acts as a component of a multimolecular “inflammasome” complex that mediates cleavage of procaspase-1 to caspase-1 (Dowling and O’Neill 2012). Inflammasome assembly leads to maturation of caspase-1, which processes

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pro-IL-1 β and pro-IL-18 to their mature, biologically active forms.

Relatively little is known about the role of NLR proteins in viral hepatitis, including hepatitis A and E. However, HCV has been reported to induce inflammasome assembly and IL-1 β secretion mediated by NLRP3 in a differentiated human macrophage (THP-1) cell line (Negash et al. 2013). Such a response, mediated by Kupffer cells, could contribute to the inflammatory response in hepatitis C. In contrast, unpublished data from our laboratory indicate that exposure to either eHAV or HAV neither initiates nor blocks NLRP3 inflammasome assembly or IL-1 β secretion by THP-1 cells (Z Feng and SM Lemon, unpubl.). Despite an IFN- γ response and (likely) activation of NF- κ B during the inflammatory phase of the infection (which can drive pro-IL-1 β transcription), serum IL-1 β levels are not prominently elevated either in experimentally infected chimpanzees or in infected *Ifnar1*^{-/-} mice (Lanford et al. 2011; Hirai-Yuki et al. 2016a). In contrast, other cytokines, such as IP10 (CXCL10) and MCP-1, are prominently elevated in these animal models of hepatitis A, as well as in infected humans (Lanford et al. 2011; Hirai-Yuki et al. 2016a; Sung et al. 2017; Shin and Jeong 2018).

Nonetheless, other NLR proteins contribute to cytokine responses evoked by HAV infection. Recent data from our laboratory indicate that NLRX1 positively regulates very early (3 h), RLR-induced cytokine responses to HAV in T antigen-transformed adult human hepatocytes (Feng et al. 2017). NLRX1 is usually considered a negative regulator of innate immunity (Allen et al. 2011), but we found it to promote IL-6 and other early cytokine responses by inhibiting activation of the dsRNA-induced PKR. Suppression of PKR activation allows for early, virus-induced increases in synthesis of IRF1 protein, which plays a key role in regulating these cytokine responses in hepatocytes (Feng et al. 2017). In the absence of NLRX1, translation of *IRF1* mRNAs is blocked by PKR-mediated global shutdown of cellular protein synthesis. NLRX1 appears to compete with PKR for binding to dsRNA, thereby reducing PKR activation. NLRX1 is likely to similarly regulate the synthe-

sis of ISG proteins in hepatocytes infected with HAV, as well as HCV (Garaigorta and Chisari 2009; Feng et al. 2017). These recent findings point to the extreme complexity with which virus-activated intrahepatic innate immune pathways activated are regulated.

The Role of Innate Immunity in Host Range Restriction and HAV Pathogenesis

The importance of type I IFNs in host defenses against HAV infection was recently confirmed in a novel murine model of human hepatitis A (Hirai-Yuki et al. 2016a). Normal C57Bl6 mice are not susceptible to intravenous challenge with wild-type HAV, but *Ifnar1*^{-/-} mice that lack expression of the receptor for type I IFNs are susceptible to infection and develop acute hepatitis with histopathologic features typical of the disease in humans, including numerous apoptotic hepatocytes (see Hirai-Yuki et al. 2018). HAV failed to infect *Ifngr1*^{-/-} mice lacking the type II IFN receptor, indicating that type I IFNs, but not type II IFNs, are a major barrier for cross-species infection by HAV. Mice lacking MAVS, but not TRIF, were also susceptible to HAV (Hirai-Yuki et al. 2016a). This suggests that MAVS-dependent, RLR-induced IFN responses play a much more important role in restricting HAV replication than TLR3 in vivo, at least in mice, despite the fact that HAV targets adaptors in both signaling pathways for degradation (Fig. 5). Mice lacking expression of both IRF3 and the redundant transcription factor, IRF7, were also susceptible to HAV infection (Hirai-Yuki et al. 2016a).

Interestingly, unlike *Ifnar1*^{-/-} mice that developed histologic evidence of hepatitis with high serum ALT elevations, HAV-infected *Mavs*^{-/-} and *Irf3*^{-/-}*Irf7*^{-/-} mice showed little or no evidence of disease despite replicating virus to higher titers in the liver than infected *Ifnar1*^{-/-} mice (Hirai-Yuki et al. 2016a). These results show that liver injury in HAV-infected mice is not dependent on IFN-mediated responses, but rather results from the activation of MAVS and IRF3/7 leading to hepatocellular apoptosis. This could result from expression of proapoptotic ISGs in the infected liver, some of which can be directly transcriptionally activated

by IRF3 without the need for IFNs or Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) signaling (Grandvaux et al. 2002). One such ISG is IFN-induced protein with tetratricopeptide repeats 2 (IFIT2), which was highly induced in HAV-infected *Ifnar1*^{-/-} mice (Hirai-Yuki et al. 2016a). Human IFIT2 has been shown to promote mitochondrial apoptosis when expressed in murine cells (Stawowczyk et al. 2011; Reich 2013).

Alternatively, HAV-induced hepatocellular apoptosis could result from a transcription-independent function of IRF3 in infected *Ifnar1*^{-/-} mice. When activated by RLR-MAVS signaling, IRF3 is ubiquitinated by the linear polyubiquitylating enzyme complex, LUBAC, resulting in a direct interaction between the BH3 domain of IRF3 and the mitochondrial protein, Bax, thereby inducing apoptosis (Chattopadhyay et al. 2013, 2016). This proapoptotic activity of IRF3 is termed RLR-induced IRF3-mediated pathway of apoptosis (RIPA). Additional studies are needed to determine the exact mechanism of IRF3-induced liver injury in this murine model, but it is interesting that acute ethanol-induced liver injury has also been linked to IRF3 activation (Petrasek et al. 2013).

INNATE IMMUNE RESPONSES TO HEV INFECTION

Like HAV, HEV causes acute, self-limited infection in humans. However, HEV can persist in persons with a weakened T-cell immunity, particularly those who have received solid organ transplantation or those infected with human immunodeficiency virus (HIV) (see Kamar and Pischke 2018). This is indicative of a key difference in host control of HEV and HAV infections. Innate immune responses during acute and chronic HEV infections are particularly poorly understood and have not been studied intensively.

Innate Immunity in Animal Models of HEV Infection

There are multiple HEV genotypes, at least four of which cause disease in humans. These differ

in their geographic distribution, epidemiology, and pathogenesis (Emerson and Purcell 2003; Dalton and Izopet 2018; Nelson et al. 2018). Genotype 1 (gt1) and gt2 HEV cause endemic and epidemic waterborne infections in developing countries, whereas gt3 and gt4 HEV are zoonotic and mostly found in developed countries. HEV gt3 is responsible for the majority of chronic HEV cases. Studies in nonhuman primates have shown that gt1 HEV and gt2 have greater infectivity and virulence than gt3 and gt4 (Purcell et al. 2013). In mice with chimeric human livers, gt1 HEV also replicates much more efficiently than gt3 HEV (Allweiss et al. 2016; van de Garde et al. 2017). Currently, it is not clear whether the lower replication efficiency of gt3 is because of a restriction mediated by a stronger host innate response, or simply an intrinsic property of the virus.

Using Affymetrix microarrays, Yu et al. (2010) examined the intrahepatic transcriptional response to gt1 HEV in experimentally infected chimpanzees. A rough comparison of the results of this study with the similar study of HAV-infected chimpanzees conducted by Lanford et al. (2011) described above suggests that the IFN response to HEV may be somewhat stronger than in HAV infection and closer to that induced by acute HCV infection (Fig. 3). Elevated ISG expression is detected in the sera of patients with chronic HEV infection (Moal et al. 2013), as well as in the liver of HEV-infected chimeric mice engrafted with human hepatocytes, where adaptive immunity is absent (Sayed et al. 2016). Whereas a very recent study reported no induction of intrahepatic ISGs in humanized uPA/NOG mice infected with either gt1 or gt3 HEV (van de Garde et al. 2017), it is important to note that the earliest time point examined was 2 weeks after HEV inoculation. An early, transient IFN response could have been missed.

Innate Immune Responses to HEV in Cultured Cells

During its replication, HEV generates a sub-genomic RNA for translation of the structural proteins (see Kenney and Meng 2018). Both the

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HEV RNA genome and the subgenome are capped at their 5' end with a 7-methylguanosine cap (similar to cellular mRNAs), and the 5'UTR RNA segment is much shorter and less structured than in the HAV genome. The 3'UTR of both HEV RNAs terminates in a lengthy poly (A) tract. In essence, the HEV genome and sub-genomic RNA are very similar to host mRNAs in their structure, more so than in HAV, which would be expected to help the virus evade detection by cytoplasmic RNA sensors.

Nonetheless, HEV infection induces ISGs in a number of different types of cultured cells, including human lung epithelial cells (A549) (Devhare et al. 2013), human hepatoma cells (PLC/PRF/5 and HepG2) (Zhang et al. 2014; Yin et al. 2017), primary human hepatocytes (Yin et al. 2017), and human embryonic stem cells/induced pluripotent stem cell-derived hepatocyte-like cells (Helsen et al. 2016). HEV has not been shown to have the capacity to proteolytically degrade host proteins engaged in signaling downstream of PAMP receptors, as HAV and HCV do. Consistent with this, MAVS abundance is not altered in cells transfected with replication-competent HEV RNAs (Fig. 4B, bottom), although the response to RIG-I and MDA5 agonists is reduced in such cells (Nan et al. 2014b; Yin et al. 2016). The underlying mechanism is uncertain, but overexpression of the papain-like cysteine protease (PCP) domain of the HEV open reading frame (ORF)1 protein has been shown to result in deubiquitination of RIG-I and TBK1, thereby blocking RIG-I signaling (Nan et al. 2014b). The X domain of the ORF1 polyprotein also appears to act on an independent step in the induction of IFN, inhibiting RIG-I-induced phosphorylation of IRF3 (Nan et al. 2014b). These findings suggest that HEV replication impedes IFN induced via activation of RIG-I-like helicases. The deubiquitinase activity of the PCP domain may extend also to host proteins conjugated to the ubiquitin-like ISG15 (Karpe and Lole 2011).

Proteins expressed from the other two HEV ORFs have also been suggested to modulate IFN responses. Overexpression of the ORF2 protein in hepatoma cells stabilized I κ B α , thereby reducing NF- κ B activity (Surjit et al. 2012).

ORF3 has similarly been reported to reduce TLR3-induced NF- κ B activity (He et al. 2016). On the other hand, ORF3 was reported to interact with RIG-I and enhance its ubiquitination, and also boost poly-(I:C)-induced IFN- β expression (Nan et al. 2014a). The contradictory findings reported in these studies likely reflect the hazards of relying on protein overexpression and highlight the need for a robust cell culture system for HEV that accurately recapitulates events in the liver.

Interferon Resistance of HEV in Persistently Infected Cells

Importantly, HepG2 cells that were persistently infected with gt3 HEV or that harbored an HEV replicon RNA, were found to continuously produce type III IFNs (Yin et al. 2016). The cellular response to this IFN and resulting ISG expression was insufficient to eliminate the HEV RNA, despite continuous stimulation of the JAK/STAT pathway. Under these conditions, HEV infection was refractory to even high concentrations of IFNs, persisting despite treatment with 100 ng/mL (2×10^5 U/mL) of recombinant IFN- α 2a (Yin et al. 2016). HEV itself does not appear to interfere with JAK/STAT signaling, as blocking IFN production by RNAi-mediated depletion of MAVS largely restored the responsiveness of HEV-infected cells to both type I and type III IFNs. Nor does HEV block ISG protein synthesis, as the abundance of several ISGs (e.g., RIG-I, ISG56) was substantially increased in cells supporting HEV replication. How HEV maintains its replication capacity in the presence of ongoing IFN responses is unclear, but it is possible that the resistance to exogenous IFN stems at least in part from continuous stimulation of the IFN receptor (Sarasin-Filipowicz et al. 2009).

The ability of gt3 HEV to persist in cell culture in the presence of an IFN response and to resist treatment with exogenous IFNs may be important to the pathogenesis of HEV infection and virus persistence in patients with a weakened immune system. Commonly used immunosuppressive drugs suppress adaptive T-cell immunity but not innate IFN responses. Thus the hepatocytes infected with HEV in such pa-

tients are likely to produce IFNs, as are different types of immune cells (e.g., pDCs, NK cells) in the chronically HEV-infected liver (Yu et al. 2010). The ability of HEV to continue to replicate in the presence of IFNs may facilitate its persistence as long as T-cell function is impaired.

tence in vivo. Recent success in developing a mouse model for HAV offers new opportunities to investigate the pathogenesis of hepatitis A, and may conceptually aid our understanding of HEV pathogenesis. Numerous questions await future investigation.

CONCLUDING REMARKS

Although both HAV and HEV are transmitted enterically and cause similar clinical presentations, they use different strategies to counteract host IFN responses. HAV actively blocks IFN production in infected cells, whereas HEV appears capable of persisting despite the expression of multiple antiviral ISGs (Fig. 6). This unique ability of HEV may facilitate its persis-

How Important Is It for Hepatitis Viruses to Block IFN Production in Hepatocytes?

Efficient cleavage of MAVS and TRIF is not unique to HAV, because MAVS and TRIF are also degraded in HCV-infected cells by the viral NS3/4B protease (Lemon 2010). The fact that both viruses target both adaptors suggests that the disruption of IFN production via these pathways may be critical to enable hepatotropic

Figure 6. Contrasting strategies for disrupting interferon (IFN)-mediated responses in hepatitis A virus (HAV) versus hepatitis E virus (HEV) infections. (A) In HAV-infected cells, viral double-stranded RNA (dsRNA) replication intermediates are sensed by cytosolic retinoic acid activated gene I (RIG-I)-like RNA (RLRs) (RIG-I and melanoma differentiation-associated protein 5 [MDA5]) as well as endosomal Toll-like receptor (TLR)3. However, the adaptor proteins mitochondria-associated antiviral protein (MAVS) and TIR-domain-containing adapter-inducing IFN-β (TRIF), and the regulatory subunit of the IκB kinase complex, NF-κB essential modulator (NEMO), are degraded by viral proteinases (Fig. 5), disrupting signals extending from RLRs and TLR3 such that little or no activated IFN-regulatory factor (IRF)3 and nuclear factor (NF)-κB cells reach responsive promoters in the nucleus, and very little or no IFNs are produced. (B) In contrast, in HEV-infected cells, HEV RNA is recognized by RIG-I and MDA5, and possibly by TLR3 as well. Signaling pathways may be impeded by open reading frame (ORF)1 papain-like cysteine protease (PCP) domain antagonism of RIG-I activation and X domain inhibition of IRF3 phosphorylation, but they are not disrupted and substantial amounts of IFNs (predominantly IFN-λ) are produced. Secreted IFNs bind to their receptors on the surface of infected cells and induce IFN-stimulated gene (ISG) expression via the Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathway in both an autocrine and paracrine fashion. Despite this activation of JAK/STAT signaling, HEV replication persists.

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viruses to establish infection (Qu and Lemon 2010). HEV also, as described above, may impede RLR-induced MAVS signaling via the PCP and X domains of ORF1 (Nan et al. 2014b). In addition, although it is a DNA virus, HBV replicates its genome via an RNA intermediate and has been reported recently to block MAVS function to suppress IFN production (Khan et al. 2016). Whereas the latter finding remains to be confirmed, these observations suggest that disruption of RIG-I/MAVS signaling may be especially important for RNA viruses infecting hepatocytes. Is this to reduce pathogenicity, given the role of MAVS in liver inflammation in the murine model of hepatitis A (Hirai-Yuki et al. 2016a), or to enhance viral replication, or both? A particularly interesting question is whether distantly related hepatoviruses identified recently in bats, which are separated from humans by over 80 million years of evolution (Drexler et al. 2015; Sander et al. 2018), similarly target the orthologs of these innate immune adaptor proteins for degradation in their host species.

How Does HEV Persist in the Face of Active IFN Responses?

While HEV may be less capable of blocking MAVS signaling than HAV, the virus persists in HEV-infected cell cultures despite the continuous production of type III IFN (Fig. 6) (Yin et al. 2016). How does the virus do this? HEV RNA replication is relatively resistant to both IFN- α and IFN- λ compared to HCV, with an IFN- α 2a EC₅₀ more than 100-fold higher in comparative studies of subgenomic replicon RNAs (Todt et al. 2016; Wang et al. 2016). Good comparative studies of HAV and HEV would be of interest, especially studies looking at the complete viral life cycle. The mechanisms by which HEV resists IFN-mediated antiviral responses are unknown, and need to be explored.

What Are the Roles of Type I IFNs versus Type III IFNs in Innate Immune Responses to HAV and HEV?

Although type I IFNs clearly play a pivotal role in restricting HAV infection in mice (Hirai-Yuki

et al. 2016a), type III IFNs are predominantly detected in cultured human hepatocytes infected with HAV, HBV, HCV, or HEV (Sheahan et al. 2014; Sato et al. 2015; Sung et al. 2017; Yin et al. 2017). This apparent discrepancy is likely because of a species difference. Human hepatocytes produce and respond robustly to IFN- λ as noted above, but mouse hepatocytes express negligible amounts of the receptor for type III IFN and respond poorly to IFN- λ (Pott et al. 2011; Hermant et al. 2014). Thus, while *Ifnar1*^{-/-} mice provide a useful model for studying the pathogenesis of hepatitis A, this species-specific difference indicates a need for caution when considering the roles of the various types of IFNs. Studies are needed in *Ifnlr1*^{-/-} mice lacking functional expression of the type III IFN receptor, and in mice with dual knockouts of both the type I and type III receptors.

Do Polymorphisms in Type III IFNs Influence HAV or HEV Infection Outcome?

Polymorphisms in type III IFN genes have been associated with spontaneous and treatment-induced HCV clearance in humans (Ge et al. 2009; Thomas et al. 2009; Prokunina-Olsson et al. 2013). Whether such type III IFN polymorphisms also play a role in modulating HAV and HEV infection is not known. Some patients with chronic HEV infection clear the virus after withdrawal of immunosuppressive therapy, whereas others do not. It would be of interest to determine whether type III IFN polymorphisms are associated with clearance versus persistence of HEV, or fulminant disease in patients with either HAV or HEV infection.

Does Gut Innate Immunity Play a Role in HAV or HEV Pathogenesis?

We were unable to infect *Ifnar1*^{-/-} mice with HAV administered by gavage, even using 1000 $\times$ the minimal intravenous (i.v.) infectious dose (Hirai-Yuki et al. 2016a). This seems surprising, because HAV is typically transmitted fecal-orally, yet other studies have shown that the oral infectious dose of virus in chimpanzees is 10^{4.5}-fold higher than the i.v. infectious dose

(Purcell et al. 2002). We also found no evidence of replication in the ileum or colon of productively infected *Ifnar1*^{-/-} or *Mavs*^{-/-} mice (Hirai-Yuki et al. 2016a). Bile from infected mice contains abundant virus, indicating that fecally shed virus is produced in the liver, as in primates (Schulman et al. 1976; Hirai-Yuki et al. 2016b). Whereas the apparent lack of permissiveness of the gastrointestinal epithelium for HAV infection could reflect the absence of a key virus receptor, a plausible alternative hypothesis is that type III IFN responses might restrict HAV replication in the gut, as is the case for murine rotavirus and norovirus (Pott et al. 2011; Nice et al. 2015). It would not be surprising to find that type III IFNs restrict HAV replication in the murine gut, whereas type I IFN does so in the liver. *Ifnar1*^{-/-} and *Ifnlr1*^{-/-} mice should prove useful in investigating these possibilities.

Do Either HAV or HEV Replicate in the Gut?

Determining whether either HAV or HEV replicates in the gut will be central to understanding the pathogenesis of these enteric hepatitis viruses. As described above, data addressing this question are sparse. It is possible that these viruses traverse the gut epithelium by transcytosis across microfold cells (M cells) associated with Peyer's patches in the distal small intestine, a mechanism suggested previously for both poliovirus and HIV (Hocini and Bomsel 1999; Ouzilou et al. 2002). There is some evidence that HAV can undergo transcytosis, particularly if facilitated by immunoglobulin A (IgA) antibody to the virus (Counihan and Anderson 2016). The recent development of stem-cell-derived human enteroids should provide an excellent tool to study the interactions between enteric hepatitis viruses and gastrointestinal tissues. Human enteroids phenotypically and functionally recapitulate the human intestinal epithelium in vivo, and support infection by a number of enteric viruses, including human norovirus, a virus known to be notoriously difficult to propagate in cell culture (Ettayebi et al. 2016).

Innate Immunity to Enteric Hepatitis Viruses

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