Review

Host Innate Immunity against Hepatitis E Virus and Viral Evasion Mechanisms

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Hepatitis E virus (HEV) infections cause epidemic or sporadic acute hepatitis, which are mostly self-limiting. However, viral infection in immunocompromised patients and pregnant women may result in serious consequences, such as chronic hepatitis and liver damage, mortality of the latter of which reaches up to 20–30%. Type I interferon (IFN)-induced antiviral immunity is known to be the first-line defense against virus infection. Upon HEV infection in the cell, the virus genome is recognized by pathogen recognition receptors, leading to rapid activation of intracellular signaling cascades. Expression of type I IFN triggers induction of a barrage of IFN-stimulated genes, helping the cells cope with viral infection. Interestingly, some of the HEV-encoded genes seem to be involved in disrupting signaling cascades for antiviral immune responses, and thus crippling cytokine/chemokine production. Antagonistic mechanisms of type I IFN responses by HEV have only recently begun to emerge, and in this review, we summarize known HEV evasion strategies and compare them with those of other hepatitis viruses.

Keywords: Hepatitis E virus, immune evasion, innate immunity, interferon

Introduction

The hepatitis E virus (HEV) is one of the major causes of viral hepatitis. According to the World Health Organization, 20 million people are infected with HEV worldwide. Acute self-limiting hepatitis, frequent epidemic occurrence, and a high mortality rate in affected pregnant women (20–30%) are hallmarks of hepatitis E infection [1]. HEV variants are classified into seven genotypes (GT 1–7) and all are members of the recently suggested species *Orthohepevirus A* of the *Orthohepevirus* genus [2, 3]. Among the seven genotypes, GT 1–4 have been associated with human infections. GT1 and GT2 infections, predominantly occurring in developing countries, are known to be restricted only to humans and non-human primates. On the contrary, GT3 and GT4 are transmitted zoonotically from pigs, deers, and wild boars in developed countries [4, 5], which accounts for a majority of HEV outbreaks and sporadic cases in the area. On the other hand, GT5 and GT6 were discovered in wild boar and GT7 was isolated from camel. However, their zoonotic potential is not yet known.

HEV is a non-enveloped virus with a positive-sense single-stranded RNA genome. The 7.2 kb genome is capped and consists of the 5′ untranslated region (UTR), three open reading frames (ORFs 1–3), and the 3′ UTR followed by poly A [6–8]. ORF1 encodes seven nonstructural (NS) proteins, including methyltransferase, Y-domain, papain-like cysteine protease, hypervariable region (HVR), X-domain, RNA helicase, and RNA-dependent RNA polymerase (RdRp) [9–11], which are involved in viral genome replication and protein processing. ORF2 encodes the viral capsid protein, which plays an important role in binding to cells, viral assembly, and regulation of host immune responses [12–14]. ORF3 encodes the small protein of 113 or 114 amino acids, which is necessary for virion release from infected cells [15–18]. HEV genotype 1 was reported to express another gene, ORF4, upon endoplasmic reticulum stress. ORF4 protein was shown to interact with multiple viral proteins, stimulating viral RdRp activity [19].

HEV infection induces innate immune responses in cells mainly through pathogen recognition receptors (PRRs), including retinoic-acid-inducible gene I (RIG-I)-like receptors

and Toll-like receptors (TLRs) [20, 21]. Upon recognition of viral RNAs, those receptors activate downstream signal cascades, involving various signaling molecules such as mitochondrial antiviral-signaling protein (MAVS) [22–24], TIR-domain-containing adapter-inducing interferon- β (TRIF) [25, 26], I kappa B kinase epsilon (IKK ϵ)/TANK-binding kinase 1 (TBK1) [27–29], interferon regulatory factor 3 (IRF3), IRF7 [30–36], and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [23, 37, 38]. Activation of those factors leads to expression of type I IFNs and IFN-stimulated genes (ISGs), which mount antiviral immune responses in infected cells and uninfected neighboring cells as well as mobilization of adaptive immune responses.

Interestingly, cells, chimpanzees, and human subjects infected by HEV display notable virus survival in the presence of sustained IFN responses, indicating that HEV may encode potent immune evasion strategies [39]. However, the lack of efficient HEV cell culture and small animal models has greatly hampered detailed analysis of the mechanisms of viral immune evasion strategies. In this comprehensive review, we provide current understanding of type I IFN-induced antiviral immune responses against HEV infection and viral evasion mechanisms for survival and replication.

Type I IFN-Induced Antiviral Immunity in HEV Infection

Type I IFN-induced antiviral immunity is the first line of defense against virus infection in cells. A large subgroup of molecules comprise human type I IFNs depending on their effects on cellular functions, mainly IFN- α and IFN- β , IFN- ϵ , IFN- κ , IFN- ω , and IFN- ν and other molecules [40–42]. IFN- α and IFN- β have antiviral activities, counteracting viral infection and replication in infected cells and also activating other immune cells, such as natural killer cells, dendritic cells, and Kupffer cells [43–47].

During HEV infection, recognition of viral RNAs by PRRs triggers activation of downstream signaling molecules, leading to expression of type I IFNs and a myriad of ISGs [20, 48, 49]. RNA recognition receptors, such as RIG-I, melanoma differentiation-associated protein 5 (MDA5), and Toll-like receptor 3 (TLR3), have been shown to elicit type I IFN production through different signaling cascades [50, 51] with RIG-I being a major specific sensor for viral dsRNA. RIG-I contains a RNA helicase-DEAD box and two repeats of caspase recruitment domain (CARD) for the

recognition of the viral RNA and the recruitment of MAVS, respectively [52–54].

RIG-I-Mediated Recognition of HEV Infection

The transcription level of RIG-I was initially low, but increased markedly after 48–72 h upon HEV infection in human lung epithelial cells (A549), while levels of MDA5 and TLR3 were not changed [55]. Similarly, initial expression of IFN- β was low, but significantly increased at 72 h post-infection. In addition, inflammatory cytokines and chemokines, such as chemokine (C-C motif) ligand 20 (CCL20), CCL5/RANTES, interleukin-6 (IL-6), IL-8, tumor necrosis factor alpha (TNF- α), TNF- α family member LTA, and TNF-related apoptosis-inducing ligand (TRAIL)/TNF superfamily member 10 (TNFSF10) were upregulated in HEV-infected A549 cells. Of note, expression of IL-6 and RANTES was dampened by the silencing of myeloid differentiation primary response gene 88 (MyD88), but not by that of TRIF. As TLR7 induces NF- κ B activation through MyD88, but not TRIF, these data suggest that TLR7 is involved in recognition of the viral RNA and triggering of NF- κ B-dependent cytokine production [55].

Subsequent studies showed that ORF3 is responsible for transcriptional activation of IFN- β through upregulation of expression and activity of RIG-I (Fig. 1) [56]. Interestingly, ectopic expression of ORF3 enhanced K63-linked ubiquitination by interaction with the N-terminal CARD of RIG-I, which was mediated by two ubiquitin ligases, tripartite motif-containing protein 25 (TRIM25) and Riplet/Ring finger protein 135 (RNF135). Ubiquitination-mediated RIG-I activation leads to its interaction with MAVS on the mitochondria, activating downstream signaling cascades [57, 58]. Domain mapping of ORF3 demonstrated that the C-terminal domain of ORF3 was sufficient for the activation of RIG-I signaling. Furthermore, analysis of the ubiquitination activity of ORF3 cloned from four HEV genotypes showed that GT1- and GT3-derived ORF3 activated RIG-I-mediated IFN expression at comparable levels, whereas that of GT2 and GT4 had a negligible effect, suggesting that ORF3-mediated IFN induction is genotype-specific [59]. Molecular differences among HEV genotypes remain largely unknown. GT1 and GT2 HEV infect only humans and non-human primates and GT3 and GT4 infect both humans and animals. However, what factors are involved in differential host tropisms of various HEV genotypes remains an open question. It is noteworthy to mention that analysis of a cell culture-adapted GT3 strain of HEV (Kernow-C1) isolated from feces of an HIV-1/HEV co-infected patient indicated

integration of the host S17 ribosomal RNA sequence in the HVR of the HEV genome [60]. Furthermore, HEV replication efficacy was increased by S17 insertion into the HEV RNA genome in cell culture using an infectious cDNA clone [61, 62]. Similarly, the host S19 ribosomal protein sequence integrated in the HEV RNA, an insertion that was previously found in HEV from the serum of a liver transplant patient, only partially improved viral growth in cell culture [63], suggesting that the sequence of an insert may be important for viral replication *in vitro*. Taken together, these results suggest that genotype-specific variations may have important implications in the host tropisms of HEV. Elucidation of detailed molecular differences in the genome sequences of various HEV genotypes and strains await further scrutiny. Of note, RIG-I seems to stimulate type I IFN responses upon HEV infection both through canonical and non-canonical signaling pathways, as RIG-I-mediated ISG expression could also be independent of IRF3 and IRF7 activation [59]. HEV replication efficacy was inversely correlated with levels of RIG-I as shown by both gain- and loss-of-function studies in Huh7.5, A549, and HepaRG cells. As a result, ISG expression, but not that of IFN, was accordingly regulated.

It is interesting to note that RIG-I-mediated antiviral responses play a major role in HCV infection in a similar manner. Recognition of the polyuridine motif of the 3'UTR of the HCV RNA genome by RIG-I triggers expression of IFNs and ISGs, counteracting viral replication in cells [64]. Therefore, levels of RIG-I protein are closely correlated with those of type I IFN induction in a particular cell type. In hepatoma cell lines, such as Huh7 and Huh7.5, endogenous levels of RIG-I are low owing to mutations. Thus, IFN induction is largely defective. Cells with substantial RIG-I expression, such as LH86 cells, can mount sufficient innate immunity against HCV infection to suppress viral replication [65, 66]. In fact, it was shown that the expression level of RIG-I in LH86 is comparable to that in primary hepatocytes. These results attest to the importance of RIG-I in the induction and activation of antiviral immune responses to liver-tropic viruses, such as HEV and HCV.

HEV-Mediated IRF Activation

During virus infection, IRF1 serves as a strong transcriptional regulator in the IFN signaling network. Overexpression of IRF1 effectively inhibited HEV replication in a Huh7-based p6-luc replicon as well as a full-length infectious model (Fig. 1) [67]. It was shown that antiviral immune effects of IRF1 were comparable to those of high-

dose recombinant IFN- α (1,000 U/ml) treatment on HEV-harboring cells. Interestingly, IRF1 did not trigger IFN- α , - β , and - γ gene expression in Huh7, HepaRG, A549, and MRC-5 cells. Moreover, the expression level of IRF1 mRNA was not increased by treatment with recombinant IFN- α . Thus, IRF1 may not be involved in induction of IFN nor a target of IFN-regulated antiviral immune responses. Molecular analyses showed that IRF1 binds to the signal transducer and activator of transcription 1 (STAT1) promoter region, driving transcription of STAT1, but not STAT2 and janus kinases (JAKs). As a result, levels of total and phosphorylated STAT1 proteins increase accordingly, stimulating induction of ISGs in hepatoma and non-hepatoma cells. In addition, when combined with ribavirin, IRF1 demonstrated higher ISG induction and stronger inhibition of HEV replication than ribavirin alone. These results suggest that modulation of IRF1 activity may provide an effective means to abate HEV replication and virus-induced pathologies.

IL-8 is a multifunctional proinflammatory chemokine produced mainly through activation of TLR signaling [68, 69]. Upon virus infection, IL-8 recruits immune cells, such as macrophages, neutrophils, and dendritic cells, to the site of infection, inducing phagocytosis, apoptosis, and cell death [70, 71]. HEV infection in Huh7 cells significantly upregulated the IL-8 promoter activity, and a HEV-encoded ORF1 was subsequently shown to heighten IL-8 expression (Fig. 1) [55, 72]. ORF1-mediated IL-8 expression entails activation of activator protein 1 (AP-1), a transcription factor that regulates various cytokines and growth factors [73, 74]. Other cytokines, such as IFN- β and IFN- γ , displayed an antagonistic effect on the expression of IL-8 [75–77]. Analogously, HCV-encoded E2 and NS5A inhibited expression of IFN- β and downstream ISGs through upregulation of IL-8 [78–80]. In addition, as shown in HEV infection, AP-1 was required for IFN- β gene transcription as it binds to the PRD IV element of the IFN- β promoter, inducing assembly of IFN- β enhanceosome [81]. However, the detailed mechanisms of IL-8 and AP-1 on IFN- β expression remain largely elusive.

Several human hepatoma cell lines (Huh7, Huh7.5, and HepG2/C3A) display differential capacities to support HEV replication, likely due to varying levels of endogenous antiviral molecules, such as PRRs, IRFs, and ISGs. As shown by TaqMan Low Density Array, at early stages of HEV infection, HepG2/C3A cells express substantial levels of PRRs (RIG-I, IFIH1, and TLR3) and ISGs (ISG15, ISG20, ISG54, ISG56, IFN-induced GTP-binding protein MX1, 2'-5'-oligoadenylate synthetase 1 (OAS1), TRAIL, *etc.*) whereas Huh7.5 and S10-3 cells did not [82]. In fact, Huh7.5 cells

contain a mutation in RIG-I, which might be responsible for the low levels of antiviral immune responses. When Huh7.5 cells were reconstituted with wild-type RIG-I, significant levels of RIG-I-mediated immune responses were observed, leading to inhibition of HEV replication. Furthermore, inhibition of TBK/IKK ϵ , downstream of RIG-I, suppressed IRF3-mediated type I IFN induction, enhancing HEV replication in HepG2/C3A cells [27, 83]. These data provide a hint on the crucial roles played by RIG-I-mediated sensing of the HEV RNA genome for the induction of antiviral immune responses and viral clearance.

When phosphorylated, IRF3 homodimerizes or heterodimerizes with IRF7, subsequently translocating into the nucleus and activating IFN- α / β production. Similarly, phosphorylation of NF- κ B p65 on serine 468 and 536 is required for its nuclear translocation and induction of the type I IFNs [84, 85]. Phosphorylation of IRF3 and NF- κ B p65 requires, in turn, autophosphorylation of upstream kinases (TBK1 and IKK ϵ) on serine 172. The importance of TBK1 S172 phosphorylation in the induction of type I IFNs was highlighted when a RIG-I-specific inhibitor (BX795) was employed. BX795 treatment blocked RIG-I activation, leading to impairment of TBK1 S172 phosphorylation and subsequent IFN expression. In contrast, acitretin, a synthetic retinoid approved by the US Food and Drug Administration, functions as an agonist of RIG-I signaling and was shown to induce preferential apoptosis of HIV-infected cells [86–88]. It would be intriguing to examine if acitretin has similar therapeutic effects on HEV infection in vitro and in vivo.

HEV Evasion of Type I IFN-Induced Antiviral Immunity

Many pathogenic viruses have evolved molecular evasion mechanisms to escape the host immune defense in direct and indirect manners. For example, HCV encodes several proteins, such as core, E2, NS3/4A, NS4B, and NS5A, to incapacitate host immune responses (Fig. 1) [89, 90]. Those immune regulators are believed to play pivotal roles in the development of drug resistance and chronic infection induced by HCV (cirrhosis and/or liver cancer). In the case of HEV infections, the majority of them are self-limiting and acute infections and are quickly resolved in healthy subjects. However, in immunocompromised patients, HEV infection may progress to chronic phases, inducing persistent hepatitis and liver damage. These data suggest that immune responses are an important determinant of outcomes of HEV infection, and its genome may likely

encode antagonists and/or modulators of host immune responses.

In this regard, it is interesting to note that IFN- α treatment only partially inhibited HEV replication, whereas it markedly blunted that of HCV in vitro and in vivo, implying for HEV-encoded antagonists of IFN- α -mediated antiviral immunity [91]. In addition, other IFNs, such as IFN- β , - γ , and λ 1, λ 2, and λ 3, had little, if any, suppressive effect on HEV replication, highlighting the importance of IFN- α in the antiviral immunity. This notion was corroborated by the observations that silencing of STAT1 and IRF9 resulted in significant increase of the HEV RNA levels in cells. Furthermore, IFN- α -induced STAT1 phosphorylation and subsequent ISG expression were significantly decreased upon HEV infection in A549 cells [92] at early time points (15–30 min), while those of JAK1, tyrosine kinase 2, and STAT2 were unchanged. These data suggest that HEV encodes strong IFN- α antagonists in its genome. In fact, HEV ORF3 protein interacts with STAT1 as shown by co-immunoprecipitation assay, impairing IFN- α -induced STAT1 phosphorylation and down-modulation of ISGs, PKR, 2',5'-OAS, and MxA (Fig. 1). These observations are analogous to those in HCV infections, as HCV core protein also downregulates IFN signaling by the inhibition of STAT1 tyrosine phosphorylation and its dimerization with STAT2 [92]. In addition, HCV core protein suppresses activation of the JAK-STAT pathway by induction of suppressor of cytokine signaling 3 in HepG2 cells [93], a powerful inhibitor of JAK-STAT signaling [94, 95]. Moreover, ORF3 protein (HEV GT4) inhibited IFN- β gene expression in poly I:C-stimulated A549 cells by upregulation of signal regulatory protein- α , a cell transmembrane glycoprotein known to be involved in the negative regulation of innate immunity (Fig. 1) [96]. Some of the nonstructural proteins encoded by HEV emerge as potential IFN antagonists as well. When the ORF1 of HEV was expressed in the absence of ORF2 and ORF3 expression in S10-3 cells, poly I:C-induced IFN- β expression was notably inhibited [97], which was likely due to the reduction of IRF3 phosphorylation and activation. Molecular analyses indicated that PCP and HPX protein are responsible for the ORF1-mediated inhibition of type I IFN-induced antiviral immunity. PCP is a papain-like cysteine protease, responsible for proteolytic cleavage of the ORF1 polyprotein. Mutation of the catalytic domain, cysteine 1152 of PCP, deprived its proteolytic activity, blocking ORF1 processing. However, recent studies have shown that PCP plays important roles in the modulation of host immune responses as well. It is intriguing to note that PCP carried out deISGylation of the ISG15-conjugated

Fig. 1. Activation and evasion of innate immune responses by hepatitis E virus (HEV).

Upon HEV infection, the viral genome is recognized by pattern recognition receptors (PRRs), such as retinoic-inducible gene-I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and Toll-like receptor 3 (TLR3), subsequently triggering activation of type I interferon (IFN) signaling cascades. IFN regulatory factor 1 (IRF1) restricts HEV replication by activating STAT1 and IFN-stimulated gene (ISG) expression after HEV infection. In addition, HEV ORF3 induces RIG-I expression and its activation by interacting with the RIG-I N-terminal domain. HEV has evolved molecular evasion mechanisms to escape the host immune defense in direct or indirect manners. (i) HEV papain-like cysteine protease (PCP) inhibits RIG-I and TANK-binding kinase 1 (TBK1)-induced IFN- β induction by downregulating K63-linked ubiquitination. (ii) HEV X protein inhibits TBK1-induced IRF3 phosphorylation. (iii) HEV ORF3 protein activates signal regulator protein alpha (SIRP- α) to hijack the negative feedback mechanism of innate immune responses, suppressing IRF3 phosphorylation. (iv) ORF3 also inhibits IFN- α -induced STAT1 phosphorylation and ISG expression. HCV-encoded immune evasion mechanisms are also indicated.

cellular proteins, presumably by its deubiquitination activity [98–100]. Furthermore, the deubiquitinase activity of PCP mediates deubiquitination of RIG-I and TBK1 (Fig. 1) [97, 98], the ubiquitination of which is crucial for IRF3 phosphorylation and subsequent IFN- β expression. PCP protein from severe acute respiratory syndrome coronavirus has deubiquitinating enzyme activity as well [101]. These data provide compelling evidence that HEV PCP protein functions as a powerful antagonist of type I IFN-induced antiviral immunity.

Last but not least, HPX also exhibits immunomodulatory activity, which consists of three putative domains: HVR, Pro, and X. Ectopic expression of HPX inhibited poly I:C-induced activation of RIG-I, MDA5, TBK1, and IKK ϵ [97].

In addition, poly I:C-induced IRF3 phosphorylation was also decreased by HPX protein (Fig. 1). Individual expression of the three domains of HPX showed that X domain was responsible for HPX-mediated inhibition of cellular antiviral immunity.

In conclusion, since its discovery over 30 years ago, HEV infection-related pathology has presented itself as one of the major causes of hepatitis, affecting more than 20 million people annually. However, virus-specific therapeutics and preventive vaccines are still elusive. The lack of efficient cell culture and small animal models has greatly hampered detailed analysis of viral replication mechanisms and host-pathogen interactions. Emerging data from available cell culture models and infectious molecular clones strongly

suggest that several viral proteins are involved in HEV evasion of type I IFN-induced antiviral immune responses. Further studies are required to understand HEV-induced pathobiology by improving an efficient cell culture system and/or a small animal model.

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