Hepatitis B Virus-Hepatocyte Interactions and Innate Immune Responses: Experimental Models and Molecular Mechanisms

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Short title: HBV Innate Immunity in Hepatocytes

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Abbreviations: IFN, interferon; PAMP, pathogen associated molecular pattern; NF-κB, nuclear factor-kappa B; IRF, interferon regulatory factor; IKK, I□B kinases; TBK1, TANK-binding kinase 1; Poly(I:C), polyinosinic:polycytidylic acid; Poly(dG:dC), poly(deoxyguanylic-deoxycytidylic) acid; Poly(dA:dT), poly(deoxyadenylic-deoxythymidylic); ISD, interferon stimulatory DNA; 2'3'-cGAMP, cyclic [G(2',5')pA(3',5')p]-=; ISG, interferon stimulated gene; PHH, primary human hepatocyte; CXCL10; C-X-C motif chemokine 10, interferon gamma inducible protein 10; IL, interleukin; MDA5, melanoma differentiation-associated gene 5; AIM2, absent in melanoma 2; STAT, signal transducer and activator of transcription; CCL, chemokine (C-C motif) ligand; STING, Stimulator of interferon genes; cGAS, cyclic GMP-AMP

synthase; cccDNA, covalently closed circular DNA; GEq; Genome equivalent; RIG-I, retinoic acid-inducible gene.

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Abstract

Chronic hepatitis B virus (HBV) infection is a major cause of liver disease and cancer worldwide. While current therapeutic approaches can efficiently control viral infection, efficient curative antivirals are absent. The understanding of virus-hepatocyte interactions and sensing of viral infection is an important prerequisite for the development of novel antiviral therapies for cure. Hepatocyte intrinsic innate immunity provides a rapid first line of defense to combat viral infection through the up-regulation of antiviral and inflammatory genes. However, the functional relevance of many of these antiviral signaling pathways in the liver and their role in HBV pathogenesis is still only partially understood. The recent identification of intracellular RNA and DNA sensing pathways and their involvement in disease biology, including viral pathogenesis and carcinogenesis, is currently transforming our understanding of virus-host interactions. Here we review the current knowledge on intrinsic antiviral innate immune responses including the role of viral nucleic acid sensing pathways in the liver. Since HBV has been designated as a "stealth virus," the study of the impact of HBV on signaling pathways in the hepatocyte is of significant interest to understand viral pathogenesis. Characterizing the mechanism underlying these HBV-host interactions and targeting related pathways to enhance antiviral innate responses may open new strategies to trigger noncytopathic clearance of cccDNA to ultimately cure patients with chronic HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) infection is a significant cause of morbidity that includes the development of cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC)[1]. Furthermore, death from HBV-related liver disease remains one of the highest causes of mortality worldwide even though a functional vaccine has existed for decades[2]. Given that there are more than 240 million individuals with chronic HBV infection globally, the mechanisms underlying disease pathogenesis are of significant impact[3].

HBV pathogenesis involves the activation of the immune system as a host cytolytic response generated to clear infected hepatocytes. In addition, virus control can also be achieved through non-cytolytic mechanisms. Failure of these usually potent antiviral immune responses can lead to chronic HBV infection and subsequently clinically significant hepatitis and liver disease[4]. Due to the existence of a robust and specific host immune response to HBV in humans, manipulation through delivery of targeted and non-targeted therapies, represents a viable approach for the development of a "sterilizing" cure[5]. With implications for both our understanding of pathogenesis and toward the realization of curative therapies, studying antiviral immune mechanisms are of paramount importance[6].

Initial breakthroughs to generate an effective vaccine and to understand HBV pathogenesis leveraged our understanding of the adaptive immune response[7]. More recently, the role of innate responses, that are not pathogen specific, have garnered significant attention[8]. With regards to host defense in general, the liver is enriched with immune cells, particularly, cells of the innate immune system including myeloid cells[9]. Furthermore, intrinsic innate responses to hepatitis viruses, within the hepatocyte, have been implicated in both protective direct antiviral and inflammatory responses[10]. In the

case of the hepatitis C virus (HCV), that also causes significant liver disease and is generally considered a non-cytopathic virus, many studies have demonstrated that the virus both activates and blocks intrinsic innate antiviral responses within infected cells[11]. However, HCV can also evade host immunity, given its ability to bypass the adaptive immune response, making it very challenging to develop an effective vaccine as was developed for HBV several decades ago[12]. Although early success with the generation of a vaccine for HBV was realized, HBV cure will be a challenging future endeavor[13]. In the case of HBV, that may be considered as a more complex virus than HCV given the presence of both DNA and RNA viral products and the existence of replication intermediates in multiple cellular compartments (e.g. nuclear and cytoplasmic)[14], the data are much less clear regarding interactions with the hepatocyte host defense machinery[15]. This review summarizes the current literature on cell culture model systems and the absence or presence of functional cell-intrinsic host defense mechanisms. The focus here is on antiviral and hepatocyte responses within HBV infected cells while other aspects of innate immunity including natural killer cells and Toll-Like Receptors (TLRs) are not discussed in detail. In addition, published data are summarized from reports that provide experimental insight, from the characterization of innate responses within hepatocytes, to address the following questions: 1. Does the hepatocyte contain the molecular machinery that would be needed to detect or sense HBV or isolated components of the virion and are the associated signaling pathways functional? 2. Does HBV inhibit functional intrinsic innate immune signaling pathways? 3. Does HBV or isolated components of the virion activate innate signaling?

HBV Virology and Life Cycle

HBV is a member of the hepadnaviridae family with a partially double-stranded genome that infects hepatocytes. After entry and following uncoating, the viral nucleocapsid is released into the cytoplasm and its relaxed circular (rcDNA) genome is subsequently released at which point its genome translocates to the nucleus. The viral genome is imported into the nucleus through mechanisms that are still only partially understood. In the nucleus, its genome is modified to the covalently closed circular (cccDNA) form that exists stably as an extrachromosomal viral genome. This "minichromosome" serves as a template for both pregenomic RNA (pgRNA) and viral mRNA transcription. Viral RNA is transcribed from the DNA genome through a RNA polymerase II-dependent mechanism and is translated into the major proteins that make up the virion[14]. Reverse transcription of the pregenomic RNA by the viral reverse transcriptase, within the nucleocapsid with subsequent envelopment, results in newly formed virions (Figure 1). HBV proteins including the HBsAg are extremely potent antigens that have facilitated the development of effective vaccines[7]. Currently licensed nucleos(t)ide analogues efficiently inhibit viral replication but fail to stop pgRNA and viral protein production and they do not facilitate clearance of cccDNA from infected hepatocytes.

Hepatocyte Cell-Intrinsic Innate Immunity and HBV

Hepatocyte cell-intrinsic innate immunity provides a first line of defense to thwart invading pathogens including hepatitis viruses[11, 16]. Production of type I and III interferon (IFN) are potent effectors of this initial response with type III/IFNL being a significant

145 component of the antiviral response in the hepatocyte; however, the production of IFN is
146 only one component of a multipronged cell-intrinsic antiviral response[10].

147 Significant progress has been made recently in our understanding of how cells
148 trigger host defense responses through recognition of pathogen associated molecular
149 patterns (PAMPs). Of the many PAMPs that have been identified, viral RNA has emerged
150 as a major stimulator of intrinsic cellular defense mechanisms. In the case of an RNA virus
151 like HCV, the viral genome itself is recognized through several distinct pathways that will
152 be discussed in upcoming sections. Briefly, once the virus uncoats and the RNA is released
153 into the cytoplasm, significant secondary structure in the viral genome, particularly in the
154 untranslated region (UTR), or double stranded RNA (dsRNA) intermediates, produced
155 during viral replication, are potent activators of pattern recognition receptors (PRRs)
156 including the RIG-I like receptors (RLRs) and TLR3[17-21]. However, it has been less clear
157 how cells in the liver trigger innate immune signaling in response to DNA species
158 originating from hepatitis viruses[22].

159 Recent breakthroughs in our understanding of RNA and DNA dependent signaling
160 processes arose through the discovery of proteins that have been implicated in the sensing
161 of these nucleic acids[23]. DNA has been shown to directly or indirectly, through RNA
162 intermediates arising from RNA Polymerase III activity, induce cytokines through the
163 activation of transcription factors including IFN regulatory factor 3 (IRF3) and nuclear
164 factor-kappa B (NF-κB)[24, 25]. However, these DNA sensing mechanisms which can be
165 tissue specific, have yet to be fully characterized regarding their functional role in
166 hepatocytes, contribution to HBV antiviral immune responses and the development of
167 hepatitis[26-29].

HBV has a DNA genome that is converted to RNA intermediates through the activity of cellular RNA polymerases[30-32]. Although HBV can replicate to high titers in hepatocytes, that can appear as "ground-glass" with haematoxylin and eosin staining due to large load of viral proteins in infected cells, HBV is not a strongly cytopathic virus[33]. Rather, HBV-associated liver damage is thought to be the consequence of chronic cytolytic immune responses, targeting infected hepatocytes, by liver-infiltrating immune cells[34].

Using experimentally infected chimpanzees, microarray analyses, performed at early time points, suggested that HBV does not activate innate antiviral responses in hepatocytes nor inhibit other intrahepatic innate immune responses[35]. After this study, HBV was designated as a "stealth virus"[36]. Interestingly, another study demonstrated that HBV might be cleared from infected hepatocytes before any detectable adaptive immune response is mounted[37], thus suggesting that innate immunity or antiviral responses at the level of infected cells could play an important role in viral clearance. In addition, inflammatory chemokines can be upregulated to detectable levels in HBV infected patients. Initial studies demonstrated that these chemokines were mainly produced and detected only after an adaptive immune response[38-40]. However, more recent studies have shown that HBV can also stimulate production of chemokines at earlier time points[41-43]. Recent publications have also demonstrated that hepatocytes and macrophages, stimulated with HBV in vitro, can produce inflammatory cytokines eluding to the possibility that HBV does not completely evade immune recognition and that the virus may directly modulate cell-intrinsic innate immune pathways that are involved in the production of inflammatory cytokines and chemokines[26-28, 44]. PRRs that have been implicated in HBV sensing are discussed in upcoming sections and include cGAS and RLRs[26, 45, 46].

Overall, antiviral innate immunity against HBV, that occurs very early after virus contact with hepatocytes, is an area that has been less studied until recently and necessitates further investigation in appropriate models. In addition, as compared to RNA viruses such as HCV, less is known about how human hepatocytes recognize DNA viruses such as HBV and their replicative RNA intermediates. Given that HBV replication, in humans, is generally not detectable until about one month after HBV infection[1, 47, 48], cell-intrinsic innate immunity may be important for controlling early virus replication.

Cell Culture Models to Study Hepatocyte Cell-Intrinsic Innate Immunity to HBV

The understanding of HBV-host interactions, including cell-intrinsic innate immune responses after infection, has been hampered for many years by the paucity of robust and physiologic cell culture model systems[49, 50]. This is specifically true for the study of HBV infection in hepatocyte models possessing functional host defense pathways that may also require the inclusion of non-parenchymal liver cells to be fully biologically relevant[27, 50]. As for most viruses, tumor-derived cells lines have been useful in increasing our understanding of HBV biology. Gene editing approaches have been used to overexpress viral proteins and to generate cell lines that continuously express hepatitis viral genomes. Specifically, HBV DNA genomes and plasmids encoding viral proteins have been delivered intracellularly through various transfection techniques and viral transduction systems[49]. Overall, the use of transformed cell lines has relied on the fact that several of them harbor defects in intrinsic innate immune antiviral pathways in cells that would normally restrict expression of viral genomes and proteins [20, 51]. These cell lines,

214 although abnormal, enabled these viruses to be studied without major interference from
215 host defense signaling pathways. Specifically, a distinguishing characteristic of hepatocyte
216 derived cell lines is the ability to detect extracellular and endosomal pathogen-derived
217 RNAs through the TLR3 pathway that is important for antiviral responses[10]. However, the
218 use of transformed cells may be suboptimal for studies focused on understanding innate
219 hepatocyte responses to HBV given the variance in functional TLR3 signaling or other
220 components of intracellular host defense[28].

221 To investigate the pathways involved in sensing of HBV, including its DNA and
222 associated RNA intermediates in human hepatocytes within minutes to hours after viral
223 cell entry, several models have been described[49, 50]. These include primary human
224 hepatocytes (PHH), that have functional intrinsic innate immune responses, and the
225 HepaRG cell line, that is immortalized with some host defense pathways intact, as opposed
226 to other transformed hepatoma cell lines[52, 53]. Interestingly, owing to the differences in
227 antiviral innate immunity within the HepG2 cell line when compared with Huh7 cells, this
228 model has proven to be useful in the study of host defense pathways and to validate results
229 obtained in PHHs. Specifically, the genetically altered HepG2 cell line was capable of
230 producing large amounts of type III IFNL in response to HCV infection, which also has
231 been observed in PHHs[10, 54].

232 With regards to the hepatoma cells lines that can support HBV infection, the
233 development of sodium-taurocholate cotransporting polypeptide (NTCP)-overexpressing
234 hepatoma cells, such as HepG2-NTCP cells, facilitates the study of the full HBV life cycle
235 in a robust and easy-to-use cell culture model[49]. As previously mentioned, HepG2 cells
236 are capable of mounting an efficient innate immune responses after infection by HCV[54];

however, the level and breadth of activation of antiviral responses is far less that that observed in PHHs[10]. Another study utilized the HBV-infected HepG2-NTCP cells for studying the interaction between RIG-I and HBV RNA, suggesting that this cell line can be useful for the study of innate immune responses after HBV infection[46]. The PH5CH8 cell line has also been utilized for related studies, albeit less frequently, because it has a functional TLR3 system[19, 55, 56].

These data suggest that results from immortalized and transformed cell lines should be validated in HepaRGs, PHHs as well as in vivo models, if available, that may include humanized mice[26, 50, 57]. Unfortunately, even these models also have limitations. HepaRG cells are bipotent hepatic progenitor cells that can differentiate into both biliary and hepatocyte-like cells and can divide indefinitely[58]. To be fully capable of supporting infection with HBV, these cells must also be treated with dimethyl sulfoxide to foster additional maturation into more differentiated hepatocyte-like cells. According to head-to-head comparisons, HepaRG cells have many similarities to PHHs that are considered to be the gold standard for cell-based models[59, 60]. Initially, the HepaRG cell line was demonstrated to be capable of supporting HBV infection and replication[58]. Unfortunately, HBV infection is optimized through the use of polyethylene glycol (PEG) that is employed during the infection to enhance virion uptake. Infection may be further optimized through continuous administration of PEG to facilitate viral entry by increasing interactions between the HBV virion and the cell membrane[61] but the overall infection rate is low with minimal cell-to-cell virus spread[62]. HepaRG may best support HBV infection when they are engineered to over- express NTCP[63]. However, the use of overexpression models has several important caveats: First, it is clear that introduction of the DNA plasmid itself to

deliver the target protein will activate cell-intrinsic innate immunity. Second, cell lines that are able to produce high levels of the target protein will rely on a blunted antiviral response given the propensity for these host defense pathways to shut down the translation of foreign proteins that is a major mechanism of pathogen resistance[64, 65].

As previously mentioned, PHHs are considered the gold standard for laboratory studies of hepatocyte function. PHHs are obtained from patients undergoing liver resection (usually for a metastasis from a non-liver cancer) and they are isolated from the adjacent "healthy" parenchymal tissue. In addition, PHH can also be obtained from the fetal livers of aborted embryos and serve as a substrate for hepatitis virus infection[66]. However, since these fetal hepatocytes, often a combination of both hepatocytes and hepatoblasts depending on the gestational age of the donor fetus, are utilized and discarded, their use is much less controversial than that of embryonic stem cells, which offer the prospect for long-term biomedical applications that includes cloning[67]. Unlike HepaRG cells, PHHs once plated do not replicate and therefore have a limited life span in tissue culture usually between 1 and 2 weeks although fetal hepatocytes can be stable for several more weeks[66]. Once in culture, these cells rapidly dedifferentiate, concomitantly down regulating biological characteristics found in mature hepatocytes[68]. Although these cells have a limited life span in culture, they readily support infection by HBV. However, viral replication is usually limited when compared with levels seen in cell lines since these PHHs presumably have intact host defense pathways that combat the infection[10, 26]. Many studies have also demonstrated productive infection of PHHs with serum-derived and cell-culture-derived HBV[33, 69-71]. A challenge with using PHHs is the presence of contaminating cells from lymphoid and myeloid lineages that also have functional and distinct cell-intrinsic

innate immune pathways[27]. Ideally, single cell analysis or studies using immunofluorescence are optimal to ensure that any innate immune responses that are observed are arising from an infected hepatocyte. These techniques may be dispensable for experiments with HepaRG cells were there are no contaminating immune cells.

Induced pluripotent stem cells (iPSCs) are a newly developed source of hepatocytes, named hepatocyte like cells (HLCs) or induced hepatocytes (iHeps) that can be used for studies on viral hepatitis[72, 73]. These cells, once generated can be a reliable source of cells that can be differentiated into partially mature hepatocytes. The advantages of stem-cell-derived hepatocytes over PHHs includes the ability to obtain an unlimited supply of pure normal hepatocytes and these cells would be less variable when compared with PHHs that are obtained from different donors that can vary by gender, age, exposure to medications/chemicals, genetic polymorphisms and the presence or absence of underlying liver disease[74, 75]. These human stem-cell-derived hepatocytes have proven to be a useful substrate to successfully support infection by HBV[72]. In addition, HLCs/iHeps would offer the benefit of not containing contaminating white blood cells that are often present in PHH cultures. Unfortunately, stem-cell-derived hepatocytes do not fully differentiate into functional mature hepatocytes. However, various approaches have been taken to overcome this limitation through the use of small molecules that promote differentiation and manipulation of the microenvironment used in the in vitro culture systems[76-78]. Since HBV has a DNA genome that is transcribed into viral RNAs[30-32], careful characterization of nucleic acid sensing pathways are needed in more advanced models including HepaRG cells, PHHs and HLCs/iHeps. This would facilitate additional insight into the mechanisms by which HBV can possibly regulate innate responses in

human hepatocytes to promote the liver inflammation that is observed in infected patients.

Does the hepatocyte contain the molecular machinery to sense HBV and are the associated signaling pathways functional?

The liver is a major immunologic organ in the human body[9] being a site of initial hematopoiesis in fetal development and also a site where viruses and associated particles may accumulate[79]. As a consequence of these observations, it would not be unexpected that hepatocytes, the major parenchymal cell of the liver, have developed adequate machinery to detect and combat foreign pathogens. Furthermore, as epithelial cells, hepatocytes can respond to the major IFN proteins including IFN-α, β, γ and λ (IFNL)[10, 80]. This opens up the exciting possibility that pretreatment of hepatocytes first with IFN, to upregulate levels of proteins involved in innate immune signaling, may facilitate the detection and characterization of pathways that can sense HBV and components of the virion[28]. These pathways may subsequently be amenable to therapeutic interventions in efforts to achieve a sterilizing cure for HBV. One draw back of this approach would be with regards to HBV infection in vitro where pretreatment with IFN may render cells less likely to be infected due to up-regulated host defense mechanism.

Given their earlier initial discovery and importance in immune responses that are conserved throughout multicellular organisms, Toll-like receptor (TLR) expression and functionality has been extensively studied in hepatocytes. Hepatocytes express TLRs and upon stimulation with their cognate ligands, they activate downstream antiviral and inflammatory pathways[81]. These responses can be broadly classified as those involving foreign nucleic acids versus other types of molecular patterns[82]. Since HBV has a DNA

genome and produces RNA intermediates, these nucleic acid detection pathways are pertinent. For nucleic acid detection, hepatocytes express TLR3, 7, 8 and 9 and these pathways are functionally activated upon ligand stimulation[83]. Specifically poly(I:C) that is added to the cell culture media directly, not delivered intracellularly, is able to up-regulate well characterized viral stimulated genes as previously described[10, 21]. With regard to non-nucleic acid detection, both the TLR 2 and 4 pathways are functional[84]. In addition, hepatocytes express the major signal adaptor molecules for TLRs that are TRIF/TICAM1 and MYD88. Given that the expression of TLRs is mainly on membranes associated with the extracellular space including endocytic vesicles, they represent an important surveillance pathway for the presence of components of foreign pathogens[85].

More recently, the identification of intracellular cytoplasmic signaling pathways that recognize nucleic acids have yielded great insight into intrinsic cellular innate immune responses. In regards to RNA species, clearly both the RIG-I/MDA5 pathways are present and functional in hepatocytes as has been demonstrated through studies involving HCV[10]. In addition, targeted analysis of the presence and functionality of these pathways has been demonstrated through the use of specific viral RNA mimetics. Transfection of poly(I:C), typically performed using lipofection, to deliver the molecule to the cytoplasmic compartment is able to up-regulate well characterized viral stimulated genes as previously described[10].

Since HBV has a DNA genome, hepatocyte defense pathways that can detect this form of nucleic acid are of paramount interest. However, there has been much more variance in reported data regarding the presence and functionality of innate immune pathways within the hepatocyte that sense DNA species. Recently, the cyclic GMP-AMP

(cGAMP) synthase (cGAS) was identified and characterized as a DNA sensor exhibiting antiviral activity against a broad range of DNA and RNA viruses[86, 87]. cGAS is encoded by the MB21D1 gene and directly binds to cytoplasmic dsDNAs inducing the production of cGAMP that is an intracellular, second messenger to activate cell-intrinsic innate immunity. Importantly, cGAS is an IFN stimulated gene (ISG) that is significantly induced following exposure of the cell to IFN[65]. A recent report has demonstrated that cGAS is expressed in both in human liver and PHH and is also able to activate host defense pathways in hepatocytes after stimulation with DNA[26]. Furthermore, it would be expected that cGAS dependent pathways would be more active following IFN treatment given that cGAS itself is an ISG.

To further understand the recognition of foreign DNA in hepatocytes, studies have been carried out focusing on the Stimulator of Interferon Genes (STING) pathway that has been shown to be important for DNA responses and innate responses to HBV[23, 88, 89]. cGAMP, that is produced by cGAS, is subsequently recognized by STING that is encoded by TMEM173 gene. This triggers the expression of antiviral and inflammatory genes through TBK1 activation and subsequent nuclear translocation of IRF3 and NF-κB. Recent reports have demonstrated that STING is expressed in both the human liver and PHHs and is also able to activate host defense pathways in hepatocytes[26, 28, 90]. In contrast to cGAS, one recent study has reported that hepatocytes lack the DNA signaling molecule STING[29]. In addition, STING itself may not be significantly induced by IFN giving more importance to baseline expression of this molecule. Ultimately, STING is highly expressed in macrophages/kupffer cells and hepatocytes may express lower levels in comparison[90].

Given the likelihood that hepatocytes possess functional intracellular sensing pathways for pathogen derived DNA that can also be further upregulated by IFN, studies have sought to characterize responses to diverse species of DNA molecules. The innate immune response mounted following stimulation with double-strand DNA (ds-DNA) in human hepatocytes has been studied using ds-DNA viral mimetics to stimulate in vitro cell culture models including PHHs and HepaRG cells. Transfection of DNA species, typically done through lipofection, including poly(dA:dT) (b-DNA) and poly(dG:dC) (z-DNA) have been shown to produce robust antiviral responses in hepatocytes. poly(dG:dC) and poly(dA:dT) have also been shown to activate the downstream signaling proteins TBK1 and IRF3 to stimulate IRF3 nuclear translocation that can be blocked using selective TBK1 inhibitors (e.g. BX795)[28].

Additional pathogen DNA mimetics including IFN stimulatory DNA (ISD) and 2'3'-cGAMP have been shown to stimulate robust innate immune responses in transfected cells[91, 92]. ISD is a 90-base pair non-CpG oligomer that can be easily synthesized. In both PHHs and HepaRG, both ISD and 2'3'-cGAMP can activate intracellular sensing pathways similar to cells treated with poly(dA:dT) and poly(dG:dC). However, transfected ISD and 2'3'-cGAMP may not be as potent stimulators when compared to poly(dA:dT) and poly(dG:dC)[28]. Certain DNA mimetics including poly(dG:dC) and poly(dA:dT), when transfected, are potent stimulators of IFN production in hepatocytes; however, this is thought to occur through the production of an RNA intermediary species that is generated from intracellular RNA polymerase III activity. These RNA species would subsequently activate RIG-I/MDA5 signaling[25].

Interestingly, DNA mimetics that stimulate antiviral responses independent of an

RNA intermediary, including ISD and 2'3'-cGAMP, resulted in significant upregulation of antiviral and proinflammatory genes at both the mRNA and protein level. However, IFNL2/3 was not detected at the protein level suggesting that RNA independent DNA sensing pathways do not result in secretion of IFNL2/3 at early time points. Interestingly knockdown of cGAS, STING and downstream signaling proteins TBK1 and IRF3 had dramatic effects on gene induction by ISD demonstrating almost complete inhibition[28]. Overall, through the utilization of siRNAs targeting several DNA sensors, the importance of the cGAS-STING-TBK1-IRF3 pathway in the recognition of DNA in hepatocytes has been demonstrated[26]. In addition, the NF-κB pathway has also been demonstrated to play an important role in the upregulation of proinflammatory cytokines following stimulation with DNA in hepatocytes[28].

As previously mentioned, recent data has demonstrated that the hepatocyte cGAS-STING pathway is functionally active even without first pretreating cells with IFN. With regards to HBV infection, this was demonstrated in hepatocytes by reduction of viral cccDNA levels in gain-of-function studies. In addition, CRISPR/Cas9-mediated downregulation of cGAS resulted in a marked increase whereas overexpression of cGAS resulted in a marked decrease in HBV infection and HBV cccDNA levels[26]. The importance of these findings is underscored by the fact that cccDNA is the key viral nucleic acid species responsible for HBV persistence. Furthermore, silencing of cGAS, STING and TBK1 expression significantly increased HBV infection, demonstrating their importance as viral restriction factors in hepatocytes[26].

To further address the mechanisms by which DNA can activate host defense pathways in hepatocytes, other proteins that have been implicated in foreign DNA sensing

have been studied. Since some DNA sensors can demonstrate tissue specific expression, there has been some variance in data from published reports. Gamma interferon inducible protein 16 (IFI16), a known DNA sensor[93], was found to be absent in hepatoma cells lines except HepaRG cells. After inducing a marked decrease in IFI16 protein levels using siRNA, there was no detectable role for this protein in the upregulation of proinflammatory genes by transfected DNA[28]. Also, the role of absent in melanoma 2 (AIM2), another known DNA signaling molecule[94], was studied and similar results were obtained with minimal impact being observed in hepatocyte DNA responses. Overall, with regards to DNA sensors that are expressed in hepatocytes, in contrast to studies performed with cGAS and STING, the silencing of IFI16 or AIM2 had minimal impact on the sensing of DNA and HBV infection[26, 28]. A list of PRR ligands that have been used to study the sensing of nucleic acids in hepatocytes is presented in Table 1. Overall, there is experimental evidence to support the possibility that hepatocytes do contain the molecular machinery needed to sense HBV and mount a subsequent antiviral response.

Does HBV inhibit functional intrinsic innate immune signaling pathways?

HBV has been described as a "stealth virus". As a consequence, there has been a lot of interest in studying the intriguing possibility that HBV actively blocks antiviral and inflammatory signaling within hepatocytes. Clearly, HCV has the ability to block antiviral signaling pathways. Strong experimental evidence has been published that the HCV NS3/4A protease cleaves the cytoplasmic adaptor protein MAVS as a major viral

442 mechanism for blocking RIG-I/MDA5 signaling[11]. With regards to HBV, the data has been
443 less clear and many genes have been reported to be regulated by this virus (Table 2).

444 HBV has been reported to specifically block the downstream IFN signaling
445 pathway[95] but others have failed to replicate these results[96]. IFN itself has been used as a
446 therapeutic agent for chronic HBV infection for decades. Unfortunately, due to its
447 pleiotropic effects as a cytokine, the precise mechanistic understanding of the antiviral
448 actions of IFN, that specifically impact HBV infection, remain to be fully characterized[97].
449 IFN may contribute to the stimulation of cytotoxic T-lymphocyte and NK cell-mediated
450 killing of HBV infected hepatocytes[98]. In addition, there is evidence that IFN has
451 significant direct antiviral effects in hepatocytes and that this activity may depend on the
452 upregulation of ISGs in hepatocytes[97, 98]. Treatment with IFN-α was able to decrease levels
453 of both HBV DNA and cccDNA[28]. Furthermore, activation of the lymphotoxin-β receptor
454 was reported to alter the specific degradation of cccDNA within the nucleus through a
455 cytidine-deamination mechanism via APOBEC3B upregulation. This resembles activation
456 of APOBEC3A by IFN that has been extensively studied[99]. However, even though several
457 cellular mechanisms have been proposed that could limit the number of cccDNA
458 molecules, including the deaminase function of APOBEC3 family members, their role in
459 actually affecting cccDNA quantity in vivo is still being investigated[100]. Given the
460 evidence of the efficacy of therapeutic IFN on HBV infection and recent data from
461 mechanistic studies involving the APOBEC family of proteins, the possibility that
462 endogenous IFN is released by resident hepatic cells or infiltrating immune cells, is of
463 interest. Endogenous IFN-mediated induction of ISGs may result in subtle antiviral
464 activity; however, it remains to be determined how much ISGs directly contribute to viral

clearance in humans or if they slightly decrease virus replication or viral protein translation until more robust adaptive immune responses take affect[100]. This is in contrast to HCV infection, were IFN responses have been strongly implicated in viral clearance[101].

Currently, it is unclear whether HBV possesses molecular mechanisms to directly block the direct sensing of pathogen associated molecular patterns (PAMPs) or suppress host defense responses once activated[26, 27, 96]. Recent studies have demonstrated that HBV has the ability to down regulate early antiviral responses[44] and that HBV proteins and replicative intermediates interfere with innate immune responses in different in vitro models[15]. Other studies suggest an active inhibition of innate immune responses by HBV[26]. Furthermore, regions of the HBV pgRNA are double-stranded and may activate dsRNA-dependent host defense mechanisms[46]. To block this host defense pathway, HBV has been reported to induce parkin-dependent recruitment of the linear ubiquitin assembly complex (LUBAC). The downstream consequence of this was attenuation of signal transduction through MAVS-dependent pathways[102]. More recently, HBV infection has been shown to decrease the levels of cGAS as a potential mechanism for interfering with innate signaling. Specifically, HBV infection suppressed cGAS expression and function in cell culture models and humanized liver chimeric mice[26]. In addition, the authors reported that HBV represses both expression of cGAS and its effector genes in humanized mice and studies are being conducted to further characterize the underlying mechanisms.

HBV has also been reported to possess numerous other additional strategies to counteract host innate immune responses. It was reported that the HBV polymerase protein, in addition to functioning as reverse transcriptase for pgRNA, inhibits host defense to

foreign nucleic acids by interfering with STING-mediated IFN-β induction and also by preventing the activation of IRF3[103]. The HBV polymerase has also been shown to directly interfere with pattern recognition (PRR) signaling via interaction with DDX3 and subsequent inhibition of downstream signaling through TBK1[104]. Additionally, the enzyme was shown to prevent the activation of IKKs through interaction with HSP90. Furthermore, the polymerase can mask the antigenic step of reverse transcription by delaying reverse transcription until the polymerase/pgRNA complex is successfully encapsidated[100].

With a more undefined role in the viral life cycle, the HBV X protein (HBx) has been reported to negatively regulate multiple aspects of host defense in hepatocytes. HBx has been shown to specifically block innate immune signaling by MAVS and TRIF and subsequent IRF3 induced gene expression[100, 105]. In addition, data has been generated that suggests that HBx specifically inhibits the transcription of TRIM22 that has been shown to be a host restriction factor for HBV [106]. HBx has also been shown to target multiple downstream signaling pathways including IRF3 and IRF7 nuclear translocation through the NF-κB essential modulator (NEMO) by the RUN domain Beclin-1 interacting cysteine-rich-containing (RUBICON) protein[107]. The HBx protein was also reported to interact with damaged DNA binding protein 1 (DDB1), which is a component of the cullin 4 (CUL4)-DDB1 ubiquitin ligase complex[108]. Building on these initial studies, it was subsequently demonstrated that HBx facilitates the CUL4-DDB1 E3 ligase to degrade the structural maintenance of chromosomes (Smc) 5/6 complex. The Smc 5/6 complex appears to bind to only episomal HBV DNA and thereby inhibits transcription. However, it does not appear to bind to chromosomally integrated HBV DNA. Thus, Smc 5/6 acts as a host

restriction factor and HBx targets the host ubiquitin-proteasome system to degrade the Smc 5/6 complex, thereby enabling HBV gene expression from cccDNA[109, 110]. Lastly, the HBV proteins core (HBVc) and pre-core (HBVpc) can inhibit the expression of MxA at the transcriptional level based on in vitro experiments[111].

It is important to point out that many of these mechanisms of innate immune evasion by HBV encoded proteins stem from overexpression studies that require artificial introduction into hepatoma cell lines. In general, overexpression of viral proteins may not provide physiologically relevant data when considering natural infection in humans with HBV infection as previously mentioned. Indeed, new studies utilizing more efficient in vitro HBV infection models demonstrate that HBV may not interfere with PRR mediated activation of innate responses in the hepatocyte[27, 96]. Furthermore, using ex vivo cultured human liver biopsies, PRR-mediated IFN and ISG induction was not suppressed in HBV-infected hepatocytes[112]. Overall, HBV may employ multiple strategies to evade sensing and antiviral activity of PRRs and their effector pathways and it may also block IFN production and signaling. However, additional data from in vivo and in vitro models suggest that HBV has a mild or even undetectable effect on the activation and downstream signaling of cell-intrinsic innate immune pathways.

Does HBV or isolated components of the virion activate innate signaling?

Given that hepatocytes have numerous mechanisms to sense multiple components of invading pathogens and there have been numerous reports of inhibition of these pathways by HBV, the dynamic interplay between HBV and its host cell has been intriguing. HBV can replicate to high titers, produce large amounts of viral antigens but

still not cause overt cytopathicity to infected hepatocytes. Published reports are presented here to discuss nuances within experimental findings that characterize this complex interaction.

Components of the HBV Virion

The HBV virion is a complex macromolecular structure composed of several distinct components. Purification of distinct components with subsequent stimulation of hepatocytes is a routinely used strategy to determine if there is functional innate immune sensing of HBV in the liver. In regards to its nucleic acid products, since HBV utilizes the hepatocyte RNA Polymerase II enzyme to complete its life cycle, its mRNA transcripts resemble cellular mRNAs reducing the likelihood of immune recognition of these viral transcripts. In addition, regulation of viral transcription by the HBx and core proteins may also play a role in shaping the transcriptional activity of the HBV genome from cccDNA to avoid triggering innate responses. This would be accomplished by allowing the viral gene expression to proceed in a controlled manner that is more similar to endogenous gene expression[100]. However, recent studies have suggested direct sensing of the pgRNA or other HBV RNAs by either MDA5[113] or RIG-I[17]. Naked rcDNA has also recently been reported to be sensed in a cGAS-dependent manner in hepatoma cell lines and PHHs[26]. The study concluded that in human hepatocytes, exposure to naked HBV genomes leads to the activation of innate antiviral immune responses. Collectively, these data suggest that HBV-derived nucleic acid, including non-encapsidated HBV DNA and pgRNA, is sensed by cGAS and other PRRs; however, this sensing is minimized or absent during natural HBV infection when HBV DNA is encapsidated.

HBV viral proteins have also been reported to stimulate cell-intrinsic innate immunity. Specifically, HBV the nucleocapsid protein may be a ligand for TLR2 that can stimulate proinflammatory chemokine production PHHs. However, there may not be high levels of non-enveloped HBV nucleocapsid structures in patients, as may be found in cell culture derived HBV isolates, so the biologic significance of this remains to be clarified[15]. The use of HBV virions purified from patients would support the relevance of these experiments[72]; however, many laboratories continue to use cell culture derived virions. UV-inactivated HBV has also been used to stimulate HepaRG cells and PHHs. UV irradiated virus can typically be inactivated following a standard dose of approximately 12 J/cm^2 [44]. Upregulation of inflammatory chemokines has been reported using UV inactivated virus[28] demonstrating that components of the intact virion may contribute to activation of host defense pathways in hepatocytes.

HBV infectious virions and innate immunity

As mentioned previously, physiologically relevant studies of direct HBV infection and cell-intrinsic innate immunity are challenging. This comes from the fact that in vitro models used to study the HBV life cycle are suboptimal arising from either the virions employed or the cells that are infected. Specifically, to generate a strong and productive infection in PHHs, up to 1000 virus-genome-equivalents (GEq) per cell of recombinant HBV virions are needed[58, 62, 70]. This may stem from hepatocyte host defense pathways that limit virus replication in vitro. Moreover, HBV inefficiently spreads through a monolayer of hepatocytes in vitro, unless additional modifications are made to standard infection protocols[61]. The prevention of viral spread may be due to the activity of unknown host defense pathways or mechanisms that do not lead to global changes in gene expression

580 as is observed with HCV infection in PHHs[10].

581 Another challenge is that the HBV inoculum, used for in vitro experiments, may
582 not resemble virus obtained from patients with chronic infection. Most researchers use
583 HBV produced by HepG2.2.15 or HepAD38 cells, HBV serotype ayw, genotype D, in
584 order to easily infect PHHs, differentiated HepaRG cells, or NTCP overexpressing
585 hepatoma cells. It is possible that these viral preparations may contain contaminants that
586 are not found in normal HBV containing patient sera that can contribute to non-physiologic
587 results[15]. The use of functional HBV virions purified from a patient may be the most
588 biologically relevant[72]; however, this continues to be a challenge for the field.

589 Overall, HBV appears to only marginally activate and/or regulate innate immune
590 responses in cell culture hepatocyte models most likely without the production of IFN from
591 hepatocytes. These results have also been supported by experiments from in vivo
592 models[114]. Thus HBV behaves like a "stealth virus" avoiding strong viral DNA and RNA
593 sensing and also the activation of pathways involved with recognition of viral proteins
594 except when considering HBsAg that drives antiviral responses from the adaptive immune
595 response. In further support of this concept, the amount of intracellular HBV DNA
596 generated in cell culture models can approach approximately more than 10 million copies
597 which is robust, significant and relevant. These data confirm that the levels of HBV DNA
598 in HBV infected cells are most likely sufficient to activate host defense pathways and the
599 absence of HBV sensing in infected cells is not due to low MOIs[26]. The ability of the virus
600 genome to be packaged in a viral capsid, subside in the nucleus and the generation of viral
601 RNAs that are very similar to cellular RNAs may contribute to evasion of host defense
602 pathways in this study. This is also possibly due to the fact that most host defense pathways

603 are based in the cytoplasm and not the nuclear compartment[115-117].

604 However, studies have reported that HBV can be sensed by hepatocytes at early
605 time points post infection and even later. Although there was only moderate elevation
606 of some genes involved in inflammation, the results have been confirmed in independent
607 studies[28, 44, 72]. Given that this response was minimal and possibly even transient, it was
608 hypothesized that HBV subsequently blocks this stimulation of gene expression[26, 44].
609 Microarray data from published studies have also demonstrated that HBV causes
610 changes in the expression of genes involved in the inflammatory response; however, the
611 level of induction was much less than that observed with HCV from experiments in
612 similar cell culture models[10]. Figure 2 highlights possible stimulation of cytoplasmic
613 DNA and RNA sensing pathways by HBV generated nucleic acids. More recently,
614 several well executed and controlled studies have been unable to detect any effect of
615 HBV infection on gene expression in cultured PHHs and other relevant models such as
616 ex vivo liver tissue [27, 96, 112].

617

618 ## Conclusions

619 In patients and chimpanzees infected with HBV, previous studies have been unable
620 to demonstrate a robust intrinsic innate immune response [35] that can be seen with other
621 hepatitis viruses such as HCV [10] even though HBV can replicate to very high levels in
622 hepatocytes. Recent studies taking advantage of improved cell culture models for HBV
623 infection have provided evidence for activation of distinct components of innate immune
624 signaling[17, 44]. Intriguingly, reports have been published that demonstrate that foreign DNA
625 and HBV components can stimulate innate immune responses in hepatocytes in multiple

626 models. These models have utilized controls to rule out the possibility of stimulation
627 arising from contaminants in the viral preparation. In addition, data has been generated
628 demonstrating that HBV blocks innate immune signaling pathways in hepatocytes. These
629 observations have been studied in patient liver biopsies, where new hepatocytes are
630 constantly being infected. It is therefore possible that the HBV virion possesses the ability
631 to down regulate inflammatory gene induction through experimentally validated
632 mechanisms and also through mechanisms that have yet to be characterized. This ability
633 to down regulate innate response would support the characterization of HBV as a "stealth"
634 virus when compared to other viruses such as HCV.

635 We also considered studies that have reported changes in the transcriptome
636 following stimulation with HBV in in vitro models. When compared to cells stimulated
637 with HCV, it is apparent that HBV stimulates a much weaker response that may be difficult
638 to detect. It is evident that inflammatory pathways may be significantly stimulated by HBV
639 to cause hepatitis in patients but this is in contrast to that observed following HCV infection
640 where interferon pathways are most activated[10]. Comparative microarray results of HBV
641 and HCV-infected PHHs demonstrate a much more robust and broad stimulation of the
642 innate immune responses by HCV which may also support the notion that HBV is a
643 "stealth" virus when compared to HCV.

644 Overall, HBV may be able to stimulate innate immunity through NF-κB dependent
645 pathways whereas HCV mainly activate both IRF3 and NF-κB[10]. In addition, other recent
646 studies have demonstrated a role for the cGAS-STING-TBK1-IRF3 in detection of HBV[88,
647 89]. More recent data has provided evidence that in human hepatocytes, naked HBV genomic
648 rcDNA is sensed in a cGAS-dependent manner whereas the incorporation into

nucleocapsids appears to shield the ability of this host defense pathway to sense this viral component. Furthermore, the cGAS-STING pathway has been shown to exhibit antiviral activity against HBV infection including reduction of viral cccDNA levels[26]. The lack of strong IRF3 activation in hepatocytes could explain the difficulty in detecting a functional IFN response in humans. However, in humans, activation of an IFN-independent innate response in hepatocytes could drive the subsequent inflammatory responses that are responsible for recruitment of immune cells into the liver in HBV infected patients.

Further characterization of chemokine and cytokine responses to HBV using appropriate models will add crucial insight into the pathogenesis of HBV infection and possibly unravel novel innovative targets for curative strategies for HBV including the degradation of cccDNA. Specifically, targeted activation of functional antiviral pathways in hepatocytes may aid in the inactivation or degradation of nuclear cccDNA. Additionally, clearance of infected cells through adaptive responses may be facilitated through stimulation of cell-intrinsic inflammatory pathways that would expose infected hepatocytes as specific targets for elimination. Given the effectiveness of current suppressive antiviral therapy, it would be prudent to ensure that stimulation of host defense pathways in hepatocytes circumvents overt liver toxicity that can be seen during a "flare" that can result in a cure. It is anticipated that an increased understanding of host defense in hepatocytes may not only facilitate HBV cure but may also yield new preventive or therapeutic options for virus-induced HCC where perturbation of innate immune response have been shown to play a pathogenic role.

Main Concepts and Learning Points

672 -Hepatitis B infects hepatocytes and drives liver inflammation that can result in cirrhosis
673 and hepatocellular carcinoma
674 -Current efforts to study hepatocyte cell-intrinsic innate immune responses are limited by
675 the lack of a robust cell culture model with limitations from both the HBV viruses
676 utilized and the cellular systems that are available.
677 -Results from published data are highly variable spanning from studies reporting
678 complete lack of detection and influence of HBV on antiviral host defenses to detectable
679 and robust responses.
680 -Hepatocytes express pathogen recognition receptors that can recognize components of
681 HBV but their expression levels and functional significance in viral clearance and disease
682 progression in patients with chronic infection is unknown.
683 - A better understanding of HBV sensing and interference with innate immune responses
684 may contribute to the discovery and development of novel antiviral strategies for viral
685 cure.

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Figure legends

Figure 1. Model of the HBV Life Cycle. Schematic model depicting the major steps in the HBV life cycle in infected human hepatocytes. The life cycle of HBV, including attachment, entry, uncoating, trafficking to nucleus, cccDNA formation, integration, transcription, translation, encapsidation and secretion, is depicted. Initially, HBV particles attach to the cell membrane and enter the hepatocytes through mechanisms that involve NTCP. After internalization, viral capsids are released and subsequently directed to the nucleus where the HBV genomes are liberated. In the nucleus, rcDNA genomes are converted into cccDNA that persists in the nucleus of infected cells as a "minichromosome", which serves as template for viral RNA transcription. dsDNA is also produced that can be integrated into the cellular genome or also converted into cccDNA. Viral mRNAs are transported to the cytoplasm where they are translated into viral proteins and together with the viral polymerase, the pgRNA is encapsidated and reverse transcribed within the nucleocapsid into progeny rcDNA. Mature nucleocapsids are then either directed to the multivesicular body pathway for envelopment with HBV envelope proteins or re-directed to the nucleus to establish a cccDNA pool. Permission granted for publication from Nature Publishing Group © Thomas, E. *et al. Nat. Rev. Gastroenterol. Hepatol.* https://www.nature.com/articles/nrgastro.2016.37.

Figure 2. Proposed model that highlights the role of nucleic acid signaling in intrinsic innate immunity to HBV. First, HBV pgRNA may be sensed by the IFN-induced genes MDA5 and RIG-I, leading to activation of NF-kB and IRF3. Second, transfected poly(dG:dC) and poly(dA:dT) activates TBK1 leading to activation of IRF3, resulting in

production of inflammatory cytokines. Third, DNA mimetics, transfected into cells, such as ISD and 2'3'cGAMP and HBV rcDNA bind cGAS resulting in activation of STING dependent activation of IRF3 and NF-kB producing inflammatory cytokines.

Tables

Table 1. Examples of pathogen-associated molecular pattern (PAMPs) with potential relevance for hepatocyte host defense signaling pathways and HBV control.

PAMPs[@]	DNA[26, 28]	RNA[19, 21, 118]	Hepatocyte Recognition[19, 20, 26, 28, 44]
Poly dAdT	+ CDS*	+ RLR^	Yes
Poly dGdC	+ CDS*	-	Yes
ISD	+ CDS*	-	Yes
2'3'GAMP	+ STING		Yes
5'ppp-dsRNA	-	+ RLR^	Yes
PolyI:C	-	+ RLR^	Yes
ssPolyU	-	+ TLR8	Yes

*CDS: Cytosolic DNA Sensor
^RIG-I Like Receptors
[@]PAMPs May Require Transfection for Stimulation

Table 2. HBV Regulated Genes

Host Defense Genes Inhibited, Activated or Both by HBV		
APOBEC3, LymphotoxinB	IRF3	RIG-I
cGAS	IRF7	RUBICON
CUL4-DDB1	MAVS	STING
DDX3	MDA5	TBK1
Interferon	MxA	TLR2
HSP90	NFKB	TRIF
IKK	Parkin	TRIM22

Figure 1*

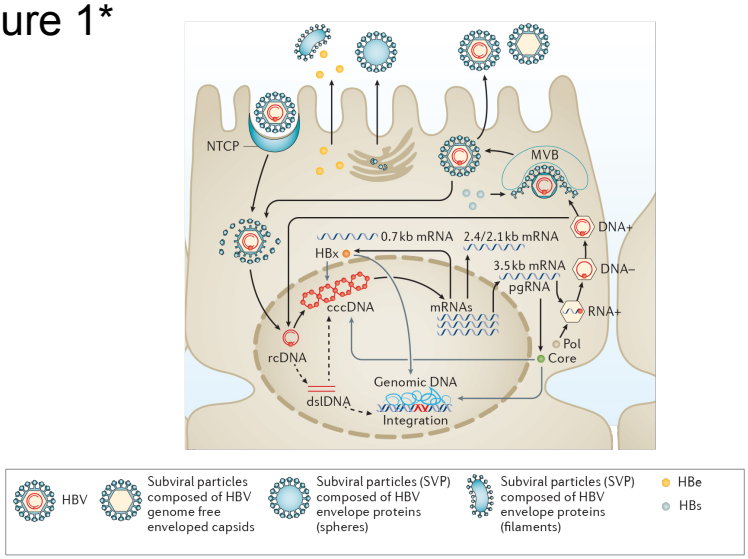

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Figure 2