HEPATOLOGY

HEPATOLOGY, VOL. 64, NO. 3, 2016

Lack of Immunological DNA Sensing in Hepatocytes Facilitates Hepatitis B Virus Infection

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Hepatitis B virus (HBV) is a major human pathogen, and about one third of the global population will be exposed to the virus in their lifetime. HBV infects hepatocytes, where it replicates its DNA and infection can lead to acute and chronic hepatitis with a high risk of liver cirrhosis and hepatocellular carcinoma. Despite this, there is limited understanding of how HBV establishes chronic infections. In recent years it has emerged that foreign DNA potently stimulates the innate immune response, particularly type 1 interferon (IFN) production; and this occurs through a pathway dependent on the DNA sensor cyclic guanosine monophosphate-adenosine monophosphate synthase and the downstream adaptor protein stimulator of IFN genes (STING). In this work we describe that human and murine hepatocytes do not express STING. Consequently, hepatocytes do not produce type 1 IFN in response to foreign DNA or HBV infection and mice lacking STING or cyclic guanosine monophosphate-adenosine monophosphate synthase exhibit unaltered ability to control infection in an adenovirus-HBV model. Stimulation of IFN production in the murine liver by administration of synthetic RNA decreases virus infection, thus demonstrating that IFN possesses anti-HBV activity in the liver. Importantly, introduction of STING expression specifically in hepatocytes reconstitutes the DNA sensing pathway, which leads to improved control of HBV *in vivo*. *Conclusion:* The lack of a functional innate DNA-sensing pathway in hepatocytes hampers efficient innate control of HBV infection; this may explain why HBV has adapted to specifically replicate in hepatocytes and could contribute to the weak capacity of this cell type to clear HBV infection. (HEPATOLOGY 2016;64:746-759)

Hepatitis B virus (HBV) is a human pathogenic virus that specifically infects hepatocytes, causes chronic hepatitis, and is a major cause of hepatocellular carcinoma. HBV is a DNA virus, and it is estimated that more than 2 billion people are or have been infected worldwide[1] and that 240 million are chronic carriers. Immune responses are detectable only weeks after HBV infection when adaptive immune responses become activated,[2,3] but there are data to support that an earlier response would benefit virus clearance by the host.[4] Chronic HBV infection is a strong risk factor for development of liver

Abbreviations: AdV, adenovirus; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDI, hydrodynamic injection; IFN, interferon; IHC, immunohistochemistry; ISG, IFN-stimulated gene; MAVS, mitochondrial antiviral-signaling protein; poly(I:C), polyinosinic:polycytidylic acid; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; STING, stimulator of IFN genes protein; TBK1, TANK-binding kinase 1; WT, wild type.

Received January 7, 2016; accepted May 23, 2016.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28685/suppinfo.

Supported by the Danish Medical Research Council (12-124330), Fabrikant Einar Willumsens Mindelegat, Fhv. Dir. Leo Nielsen og Hustru Karen Margrethe Nielsens Legat for Lægevidenskabelig Grundforskning, the Aarhus University Research Foundation, and a DFF-Mobilex postdoctoral fellowship (to M.K.T.) funded through the European Union's 7th Framework program.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28685

Potential conflict of interest: Nothing to report.

fibrosis and cirrhosis, which is strongly associated with development of hepatocellular carcinoma.[5] Administration of interferon-alpha (IFN-α) to patients infected with HBV is an established treatment in the clinic. IFN-α activates the Janus kinase signal transducer and activator of transcription pathway, which results in transcription of IFN-stimulated genes (ISGs).[6] ISGs are known to have antiviral properties, and IFN-α treatment can also inhibit the virus replication through epigenetic control[7] or deamination of the HBV covalently closed circular DNA.[8] However, IFNs can hardly be detected in HBV-infected patients,[9,10] and experimental HBV infection in chimpanzees or woodchucks has also failed to induce this classical response to viral infections.[11]

Intracellular DNA-sensing proteins constitute a new class of proteins, which recognize foreign DNA, including viral DNA to induce innate immune responses.[12] To date, several DNA sensors have been identified, and particular interest has been drawn to cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), which synthesizes cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) upon DNA binding in the cytosol.[13,14] cGAMP activates the adaptor protein stimulator of IFN genes (STING), which can bind and activate TANK-binding kinase 1 (TBK1).[15] The pathway downstream of STING/TBK1 leads to phosphorylation of different transcription factors such as IFN regulatory factor 3 and nuclear factor B, which are essential for expression of type 1 IFNs and thus activation of the Janus kinase/signal transducer and activator of transcription pathway in the surrounding cells.[12]

Given the lack of strong innate immune responses upon HBV infection, it seems as if this virus has adapted to a biological niche, where it escapes immune detection or that the virus efficiently inhibits innate immune responses. Data from *in vitro* studies demonstrate that HBV activates inflammatory cytokine but no IFN responses[16] and harbors mechanisms to inhibit IFN induction.[17] However, at this stage systematic *in vivo* studies of HBV infection have been hampered by the lack of small animal models supporting HBV infection.

We investigated the role of the DNA-sensing machinery in control of HBV infection *in vivo* in order to understand the impact of this pathway on viral control in the context of the liver. To this end, we used a model where mice are infected with adenovirus (AdV) containing a replication-competent, overlong HBV genome as mice cannot be infected with HBV. We demonstrate that murine hepatocytes do not express STING and therefore are unable to induce IFN in response to DNA or AdV-HBV infection. Interestingly, introduction of STING expression *in vivo* specifically in hepatocytes enabled these cells to sense DNA and led to improved control of HBV infection. Finally, we found that primary human hepatocytes also lack detectable expression of STING and responsiveness to foreign DNA, thus validating the human relevance of the findings in the murine model system. Collectively, this work has uncovered that the innate DNA-sensing pathway is not operative in hepatocytes and suggests that this contributes to the inability of the liver to mount protective innate immune responses against HBV infection, thus facilitating establishment of chronic infection.

Materials and Methods

ANIMALS

All mice were bred on a C57BL/6 background and kept in a pathogen-free housing facility. STING-Goldenticket (*Tmem173*$^{gt/gt}$) mice were obtained from Russell Vance,[18] cGAS$^{-/-}$ mice were from the European Conditional Mouse Mutagenesis Program, and *Mavs*$^{-/-}$ mice were from Stanislas Goriely. All experiments were carried out at the University of Aarhus and approved by Danish government authorities. All mice

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were male, and control C57BL/6 mice were age-matched (8-10 weeks of age). All mice used were males and age-matched (8-10 weeks of age) with control C57BL/6 mice.

VIRUSES, POLYINOSINIC: POLYCYTIDYLIC ACID, AND HYDRODYNAMIC INJECTION

HBV (genotype D) was purified from the supernatant of stably transfected HepG2 cells by heparin column and subsequent sucrose gradient. A 1.3-fold overlong HBV genome was cloned in the AdV 5 vector driven by a cytomegalovirus promoter, to give rise to functional HBV particles.[19] As control, an AdV 5 vector expressing green fluorescent protein from the cytomegalovirus promoter was used. AdV-HBV and AdV-green fluorescent protein were produced in HEK-293 cells, and 4 days after infection, cells were lysed by three cycles of snap freezing. After removing cell debris by centrifugation, AdV particles were purified and concentrated through two subsequent cesium-chloride gradients and thoroughly dialyzed. Mice were infected intravenously with 10^9 infectious units in saline buffer. Polyinosinic:polycytidylic acid (poly[I:C]; Sigma; 20 mg/mouse) was administered intraperitoneally in saline buffer. Mice were stimulated with 500 μg DNA (60 bp) in 200 μL saline buffer intravenously for 5 hours.

To complement STING, the coding sequence of murine STING (*Tmem173*) was cloned under the control of a hepatocyte-specific human alpha$_1$-antitrypsin promoter,[20] and a flag tag was added to the C terminus of STING. Mice were anesthetized with isoflurane (Abbott) and hydrodynamically injected with 25 μg plasmid in a saline buffer with a total of 10% volume of their body weight.

CELL CULTURE WORK

Primary hepatocytes and Kupffer cells were isolated from mice using a two-step perfusion of the liver: first, with perfusion buffer, followed by digestion buffer (Gibco). Single cells were rinsed through a 100-μm mesh, and hepatocytes were pelleted following three centrifugations at 50*g* for 4 minutes. The supernatant was collected for immune cells and added to tissue culture dishes for 8 minutes to allow Kupffer cells to adhere. Adherent cells were washed and incubated with Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and fetal calf serum (FCS). Live hepatocytes were isolated through gradient centrifugation in Percoll, washed, counted, and plated on precoated plates in DMEM containing antibiotics and FCS. Primary human hepatocytes were from 10 donors and were handled according to the supplier's protocol (HMCS10; Invitrogen). Low passage numbers of Huh7 and HepG2 cells were used, and they were grown in DMEM containing antibiotics and FCS.

Primary human hepatocytes were infected with HBV at a multiplicity of infection of 100 for 48 hours. Cells were first seeded in William's E medium supplemented with 10% FCS, penicillin/streptomycin, glutamine, insulin-transferrin-sodium selenite (Sigma), hydrocortisone (50 μM), and 1.8% dimethyl sulfoxide. Cells were incubated for 16 hours with HBV in the presence of 5% PEG8000. Cells were washed three times, and fresh medium was added. Primary murine hepatocytes were infected at a multiplicity of infection of 10 infectious units AdV-HBV in DMEM and 10% FCS overnight. Cells were stimulated *in vitro* with 5 μg DNA or poly(I:C) and 2 μL Lipofectamine/mL medium.

IMMUNOBLOTTING

Tissues from liver or spleen were lysed in radio immunoprecipitation assay buffer in the presence of protease and phosphatase inhibitors, followed by homogenization and centrifugation. The cleared supernatants were sonicated, and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The blots were blocked with 2.5% bovine serum albumin or 5% dry milk in phosphate-buffered saline containing 0.1% Tween 20 before being probed with the following primary antibodies: STING (R&D; AF6516), ISG15 (CS2743S), cGAS (Abiocode; R3251-1), phospho-TBK1 (CS5483S), glyceraldehyde 3-phosphate dehydrogenase (SC-25778), hepatitis B core antigen (HBcAg; Dako; B0586), Flag (Sigma; F1804), viperin (Millipore; MABF106), and vinculin (Sigma; V9131). Appropriate peroxidase-conjugated secondary antibodies were used and developed (Jackson ImmunoResearch).

IMMUNOHISTOCHEMISTRY

Tissues were fixed in 10% formaldehyde overnight, embedded in paraffin, and cut into 4-μm sections. Antigen retrieval was performed at 100°C in a citrate buffer, pH 6, for 20 minutes. Sections were blocked in 2.5% bovine serum albumin in phosphate-buffered

saline + 0.1% Tween 20, and the primary antibodies HBcAg, STING, Flag, and viperin were used, followed by secondary antibodies coupled to horseradish peroxidase. Counterstaining was performed with hematoxylin.

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA from liver and spleen was extracted with TRIzol (Invitrogen) and homogenized according to the manufacturer's instructions. RNA was treated with deoxyribonuclease (Invitrogen), and 50 ng total RNA was used per quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Complementary DNA was synthesized prior to qRT-PCR with site-specific primers in the same reaction tube for the qRT-PCR for 30 minutes at 48°C. SYBR Green III (Stratagene) and TaqMan Gene Expression Assay (Applied Biosystems) were used according to the manufacturers' recommendations for the qRT-PCR, and 40 cycles of amplification were performed. Data were analyzed with the delta delta cycle threshold method and normalized to β-actin. For primers see Supporting Table S1.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Hepatitis B surface antigen (HBsAg) was measured from serum with MONOLISA antibody to HBsAg enzyme-linked immunosorbent assay (ELISA; Bio-Rad), and HBsAg was used as a standard (ab91276; Abcam). Murine IFN-β was measured with an in-house ELISA. Plates were coated with antibody against IFN-β (sc-57201) and blocked with phosphate-buffered saline containing 10% FCS. A 50-μL sample was loaded and incubated overnight at 4°C. IFN-β detecting antibody (32400-1; R&D) was added to wash the plates, which were incubated overnight at 4°C. Secondary antibody coupled to horseradish peroxidase was added to the wash plates, and tetramethylbenzidine substrate was used to develop the signal. IFN-β (500 IU/mL) was used as a standard (PBL; 124000-1).

STATISTICS

For statistical analysis of data, an unpaired two-tailed Student *t* test was used. $P \leq 0.05$ was considered to reflect statistically significant differences between compared groups. The data are representative of one out of three or more experiments.

STUDY APPROVAL

All described animal experiments have been reviewed and approved by Danish government authorities and hence comply with Danish laws (Animal Experiments Inspectorate, Copenhagen, Denmark).

Results

NO IMPAIRMENT OF CONTROL OF HBV INFECTION IN MICE DEFICIENT FOR STING

To address the role of innate DNA sensing in control of HBV infection, we used mice deficient for STING. Mouse hepatocytes cannot be infected with HBV. Sodium taurocholate cotransporting polypeptide is a functional receptor for HBV; but human and murine sodium taurocholate cotransporting polypeptide exhibit only 32% sequence identity, and murine hepatocytes neither support HBV uptake through sodium taurocholate cotransporting polypeptide nor nuclear transport of HBV DNA.[21] Therefore, we used a model where an AdV vector was employed to transfer a replication-competent HBV genome into the nucleus, allowing infection of murine hepatocytes *in vivo* and production of functional progeny HBV particles without covalently closed circular DNA formation or the capability to reinfect murine hepatocytes.[22] Mice were infected with AdV-HBV and 1 day later analyzed for expression of ISGs in the liver and spleen. As expected, after infection with a DNA virus, at the very early stage postinfection STING-deficient mice had impaired induction of viperin and ISG15 in the liver and similar induction in the spleen from the infected mice but no changes in IFN-β (Supporting Fig. S1A,B).

In order to address the importance of the STING pathway in early innate control of HBV clearance, mice were analyzed for HBV infection 4 days postinfection. This time point was chosen because only limited adaptive immune responses are active at this stage. HBcAg was detected in hepatocytes of both wild-type (WT) and STING-deficient mice, but surprisingly, no difference in HBV core protein expression was observed between the two mouse strains (Fig. 1A). The apparent lack of requirement for STING in control of HBV infection was also observed when we

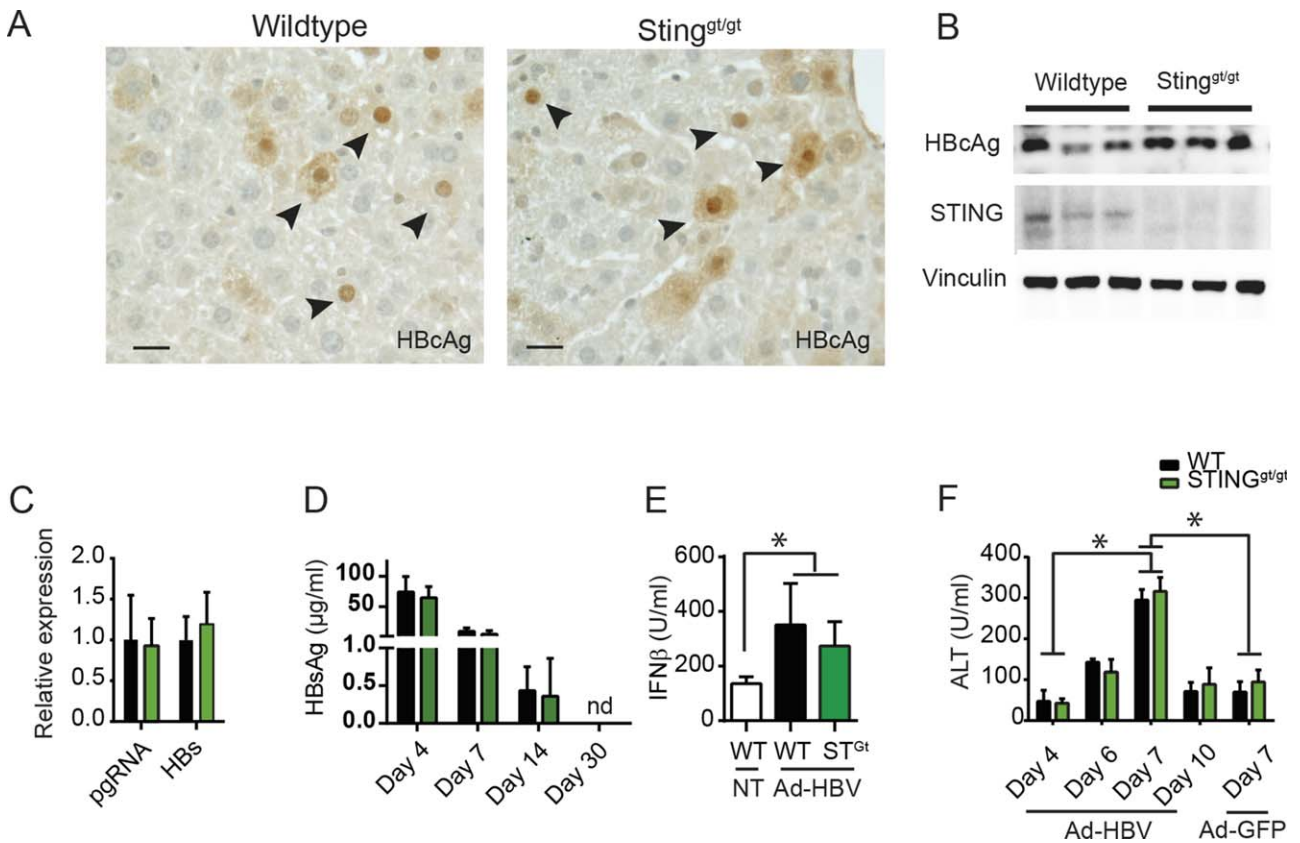

FIG. 1. No role for STING in control of HBV infection. WT and *Sting*$^{gt/gt}$ mice were infected with AdV-HBV, and the effect of innate immunity on virus load was addressed 4 days postinfection. (A) Liver sections were stained for HBcAg (brown), and black arrowheads mark positive hepatocytes (n = 6). Scale bar = 20 μm. (B) Western blotting for HBcAg and STING on whole-liver extracts. Vinculin was used as a loading control (n = 3). (C) Expression of pregenomic RNA and HBsAg in whole-liver extracts (n = 6). (D) ELISA for circulating HBsAg at the indicated days postinfection (n = 3-6 per measurement). (E) ELISA for IFN-β in the serum (n = 5). (F) Alanine aminotransferase levels in the serum at the indicated days postinfection (n = 3-6 per measurement, $^*P < 0.05$). Data are means standard deviation, and an unpaired two-tailed Student *t* test was used. Abbreviations: ALT, alanine aminotransferase; GFP, green fluorescent protein; pgRNA, pregenomic RNA.

measured HBcAg and expression of HBV pregenomic RNA or total HBV messenger RNA (Fig. 1B,C). ELISA for circulating HBsAg confirmed that infection was not affected by the lack of STING at day 4 postinfection or at later time points; up to 2 weeks after infection, no difference in HBsAg levels in the serum between WT and STING-deficient mice and identical kinetics of viral clearance were observed (Fig. 1D). Immunohistochemistry (IHC) on liver sections from mice infected for 1 month identified low but comparable numbers of hepatocytes positive for HBcAg in both control and STING-deficient mice (Supporting Fig. S2A).

IFN-β levels in the serum 4 days after infection were similar between WT and STING-deficient mice (Fig. 1E). This indicated that the DNA-sensing pathway in hepatocytes is not critical for the IFN response at a time point when HBV infection is established in the liver. In agreement with the lack of a difference in viral load, we also observed that serum alanine aminotransferase levels, as a measure of liver damage, were comparable in infected WT and STING-deficient mice (Fig. 1F). These data also revealed that the highest degree of liver damage was found around day 7 postinfection. Finally, the AdV-HBV induced significantly higher alanine aminotransferase levels than the AdV-green fluorescent protein virus, thus demonstrating that the liver damage was indeed caused mainly by HBV and not AdV (Fig. 1F).

cGAS is the DNA sensor upstream of STING. We infected mice deficient for cGAS with AdV-HBV and analyzed the virus load 14 days postinfection. Virus

load was not different between cGAS-deficient and WT mice, similar to what was observed for STING-deficient mice (Supporting Fig. S2B). A similar analysis was done with mice deficient for mitochondrial antiviral-signaling protein (MAVS), which cannot signal in response to sensing of RNA virus infection, which may be relevant as HBV replicates through an RNA intermediate.[23] Two weeks postinfection, the levels of circulating HBsAg were comparable between WT and MAVS knockout mice, indicating that RNA sensing is also not instrumental in HBV clearance *in vivo* (Supporting Fig. S2B). In conclusion, although the DNA-stimulated, STING-dependent pathway is activated to a modest degree immediately after infection with AdV-HBV, this does not have an impact either on clearance of the virus or on development of disease.

PROTEINS OF THE cGAS-STING PATHWAY ARE EXPRESSED AT LOW LEVELS IN THE LIVER

To seek insight into the surprising lack of a role for innate DNA sensing in host defense against HBV, we analyzed the expression of factors in the DNA-sensing pathway and stimulation of IFNs/ISGs 4 days after infection with HBV. Interestingly, expression of STING was very low in the liver compared to the spleen (Fig. 2A). Regarding ISG expression, we observed that both ISG15 and viperin were induced by the infection on day 4 in WT mice (Fig. 2A). While ISG15 was partly dependent on STING, viperin was expressed in a STING-independent manner. Interestingly, expression of the genes encoding cGAS and STING was very low in samples from the liver compared to the spleen (Fig. 2B). By contrast, the transcript for the messenger RNA encoding MAVS of the RNA-sensing pathway as well as the downstream transcription factor IFN regulatory factor 3 were readily detectable and comparable between the two organs. These data suggest that the components of the innate DNA-sensing pathway are expressed at only low levels in the liver, which may have an impact on the activation of innate defense mechanisms during infection in this organ.

HEPATOCYTES DO NOT EXPRESS STING AND FAIL TO RESPOND TO FOREIGN DNA

The low levels of STING in whole-liver extracts suggested either general low expression by all cell types or differential expression of STING between cell types of the liver. To delineate the expression of STING in hepatocytes and liver macrophages (Kupffer cells), these cell types were isolated from whole mouse livers. Western blot analysis revealed that STING was not detectable in hepatocytes but was strongly expressed in Kupffer cells, the primary resident immune cell in the liver (Fig. 2C,D). *In vitro* stimulation with DNA to activate the cGAS-STING pathway confirmed that hepatocytes do not respond to cytoplasmic DNA as no expression of IFN-β of ISGs was induced (Fig. 2C). In contrast, *in vitro* DNA stimulation of Kupffer cells induces ISGs and phosphorylation of TBK1 (Fig. 2D).

Next, primary murine hepatocytes were infected with AdV-HBV, and 2 days later the supernatant was analyzed for levels of HBsAg and IFN-β. Media from infected hepatocytes contain HBsAg but no detectable IFN-β, confirming that hepatocytes do not produce IFN-β *in vivo* or *in vitro* in response to HBV infection (Fig. 2E,F). Transfection of poly(I:C) stimulated toll-like receptor 3 and the cytoplasmic RNA sensor melanoma differentiation-associated protein 5, but transfection of DNA did not induce IFN-β (Fig. 2E). In summary, the innate DNA-sensing pathway is not operative in hepatocytes, while it is intact in liver macrophages, the Kupffer cells.

STIMULATION OF IFN RESPONSES IN HEPATOCYTES *IN VIVO* ALLOWS CLEARANCE OF HBV INFECTION

IFNs stimulate expression of ISGs, many of which encode proteins with direct antiviral activity.[6] Given the lack of a functional DNA-sensing pathway in hepatocytes, we were interested in whether the stimulation of IFN responses in hepatocytes would promote clearance of the virus. Because MAVS was expressed in the liver (Fig. 2C) and *in vitro* stimulation of hepatocytes with the synthetic RNA poly(I:C) induced expression of IFN-β and ISGs (Fig. 2D,F), we hypothesized that poly(I:C) treatment *in vivo* could be used to address this question. Mice treated with poly(I:C) showed abundant expression of viperin (Fig. 3A). Expression analysis showed that other ISGs were also induced 24 hours after poly(I:C) stimulation in the liver (Fig. 3B,C). Furthermore, we noted that poly(I:C) treatment led to dramatic induction of cGAS, but not STING, in the liver.

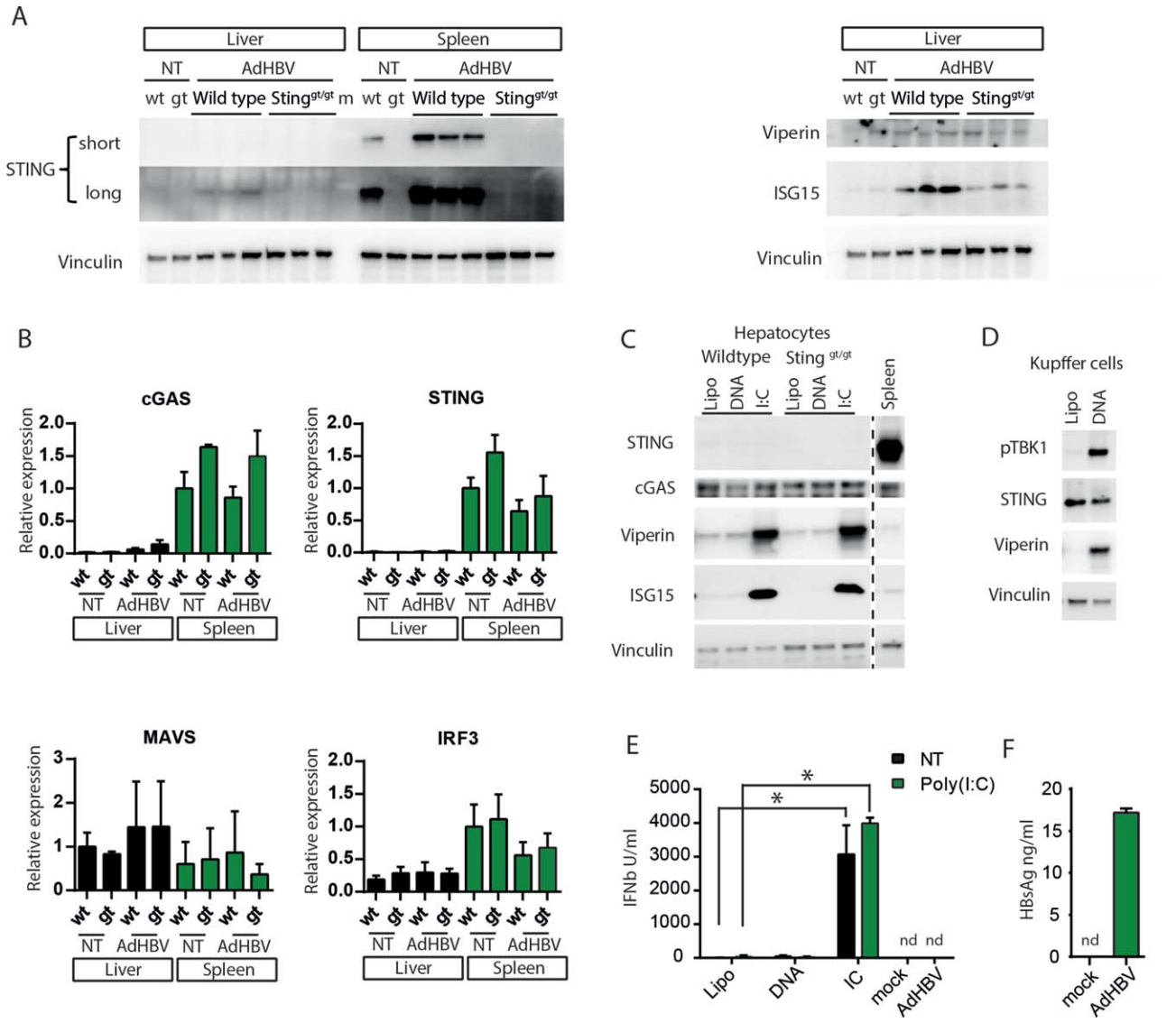

FIG. 2. Hepatocytes do not express STING and fail to respond to DNA. (A) Western blotting for, first, STING on samples from liver and spleen and, second, viperin and ISG15 on liver samples harvested 4 days post infection or nontreated controls (NT). Vinculin was used as loading control (n = 6). (B) Expression of cGAS, STING, MAVS, and IFN regulatory factor 3 in noninfected mice or 4 days postinfection on samples from liver and spleen (n = 6). (C) Hepatocytes were isolated from WT and STING-deficient mice and stimulated *in vitro* for 24 hours with Lipofectamine, DNA, or poly(I:C). Lysates were analyzed by western blotting for STING, cGAS, viperin, and ISG15. Vinculin was used as a loading control and spleen extract as a positive control (n = 3). (D) Kupffer cells were isolated from WT mice and stimulated *in vitro* for 5 hours with Lipofectamine or DNA before analysis by western blotting for pTBK1, STING, and viperin. Vinculin was used as a loading control (n = 3). (E,F) Medium was collected from hepatocyte cultures, which were treated as indicated for 24 or 48 hours, respectively, prior to ELISA for IFN-β and HBsAg (n = 3-6, $^*P < 0.05$). Data are means ± standard deviation, and an unpaired two-tailed Student *t* test was used. Abbreviations: I:C, polyinosinic:polycytidylic acid; IRF3, IFN regulatory factor 3; Lipo, Lipofectamine; NT, not treated.

To assess the impact of a stimulated IFN pathway in the liver on clearance of HBV infection, mice were treated with poly(I:C) 1 day postinfection with AdV-HBV to first allow establishment of the HBV transcription template. Four days after infection, we determined the number of HBV replicating hepatocytes by IHC, virus particles in the serum by measuring HBsAg, and expression of pregenomic RNA and total HBV RNA (Fig. 3D-F). Poly(I:C)-treated mice showed reduced HBV replication at the levels of gene expression, protein expression, and circulating viral antigen. These data demonstrate that AdV-HBV-infected hepatocytes can

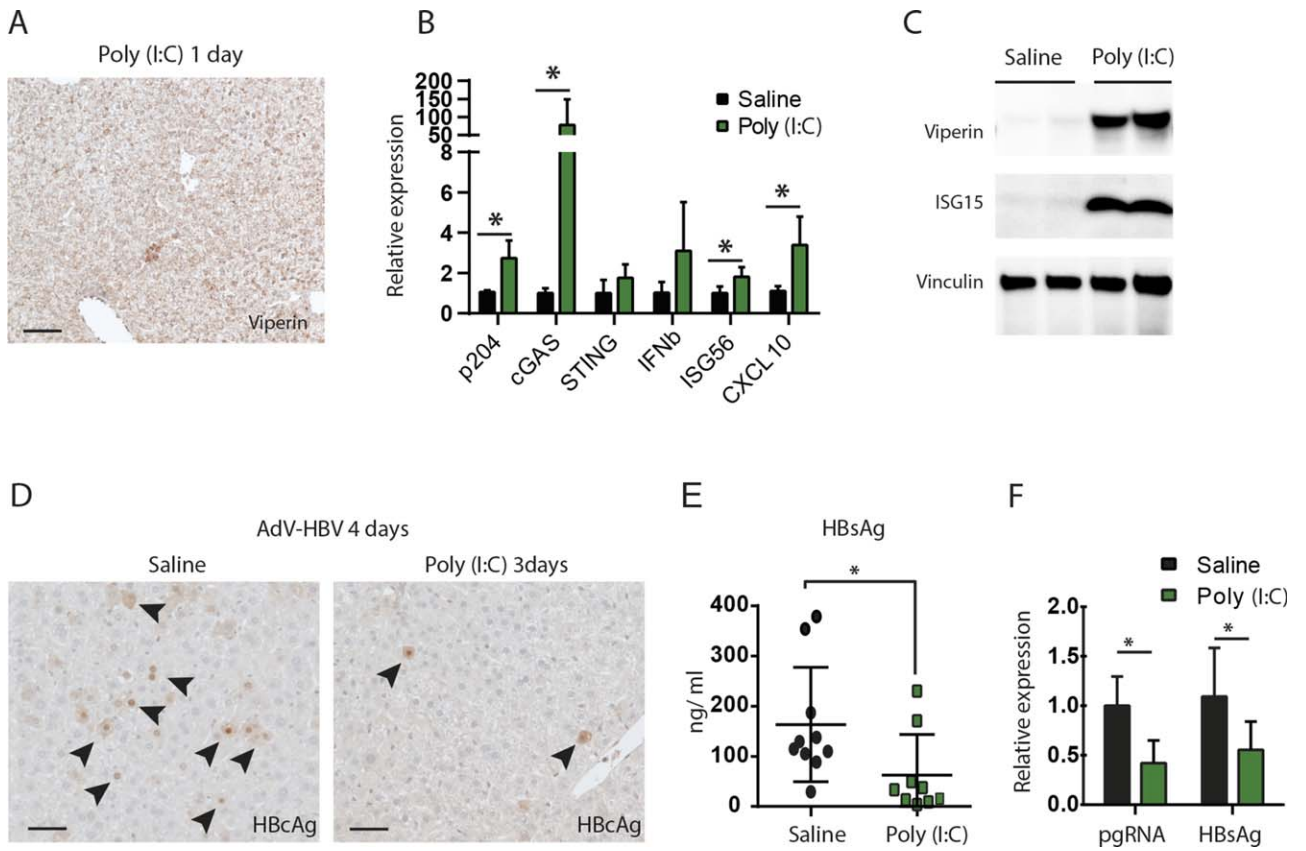

FIG. 3. Stimulation of IFN responses in the liver leads to restriction of HBV. Mice were treated with poly(I:C) for 1 day. (A) Liver section staining for viperin (brown, n = 3). Scale bar = 100 μm. (B) Quantitative PCR analysis for gene expression in whole liver from nontreated or poly(I:C)-treated mice (n = 5). (C) Western blotting for viperin and ISG15 on liver samples isolated from mice treated with poly(I:C) for 24 hours (n = 5). (D-F) Mice were infected with AdV-HBV and treated with poly(I:C) 1 day postinfection. Four days postinfection mice were sacrificed, and (D) liver sections were stained for HBcAg. Black arrowheads mark positive cells (n = 6). Scale bar = 50 μm. (E) HBsAg in serum was measured by ELISA (n = 9, 10), and (F) expression of pregenomic RNA and HBsAg in whole-liver extracts was evaluated by qPCR (n = 6, $^*P < 0.05$). Data are means ± standard deviation, and an unpaired two-tailed Student *t* test was used. Abbreviations: CXCL10, chemokine (C-X-C motif) ligand 10; pgRNA, pregenomic RNA.

express ISGs and respond to IFN, leading to control of infection, thus suggesting that an IFN response in the acute phase of HBV infection would confer protection.

CELL TYPE-SPECIFIC EXPRESSION OF STING IN HEPATOCYTES *IN VIVO* RESTORES DNA SENSING AND CONFERS PROTECTION AGAINST HBV INFECTION

Hepatocytes can produce ISGs to clear virus infection but lack STING expression and a functional DNA-sensing pathway to sense HBV infection. We thus designed a plasmid expressing murine STING under a liver-specific promoter and performed hydrodynamic injection (HDI) of the plasmid into the tail vein of STING-deficient mice to restore STING expression in hepatocytes. IHC analysis confirmed that approximately one third of the liver cells from mice undergoing this treatment expressed STING (Supporting Fig. S3A,B). *Per se*, overexpression of STING in hepatocytes did not lead to phosphorylation of TBK1 or elevation of ISG15 expression (Supporting Fig. S3C). We therefore stimulated with DNA *in vivo* for 5 hours and analyzed for expression of ISGs. Mice reconstituted with STING in the liver showed upregulation of ISG15 and viperin 5 hours after stimulation (Fig. 4A).

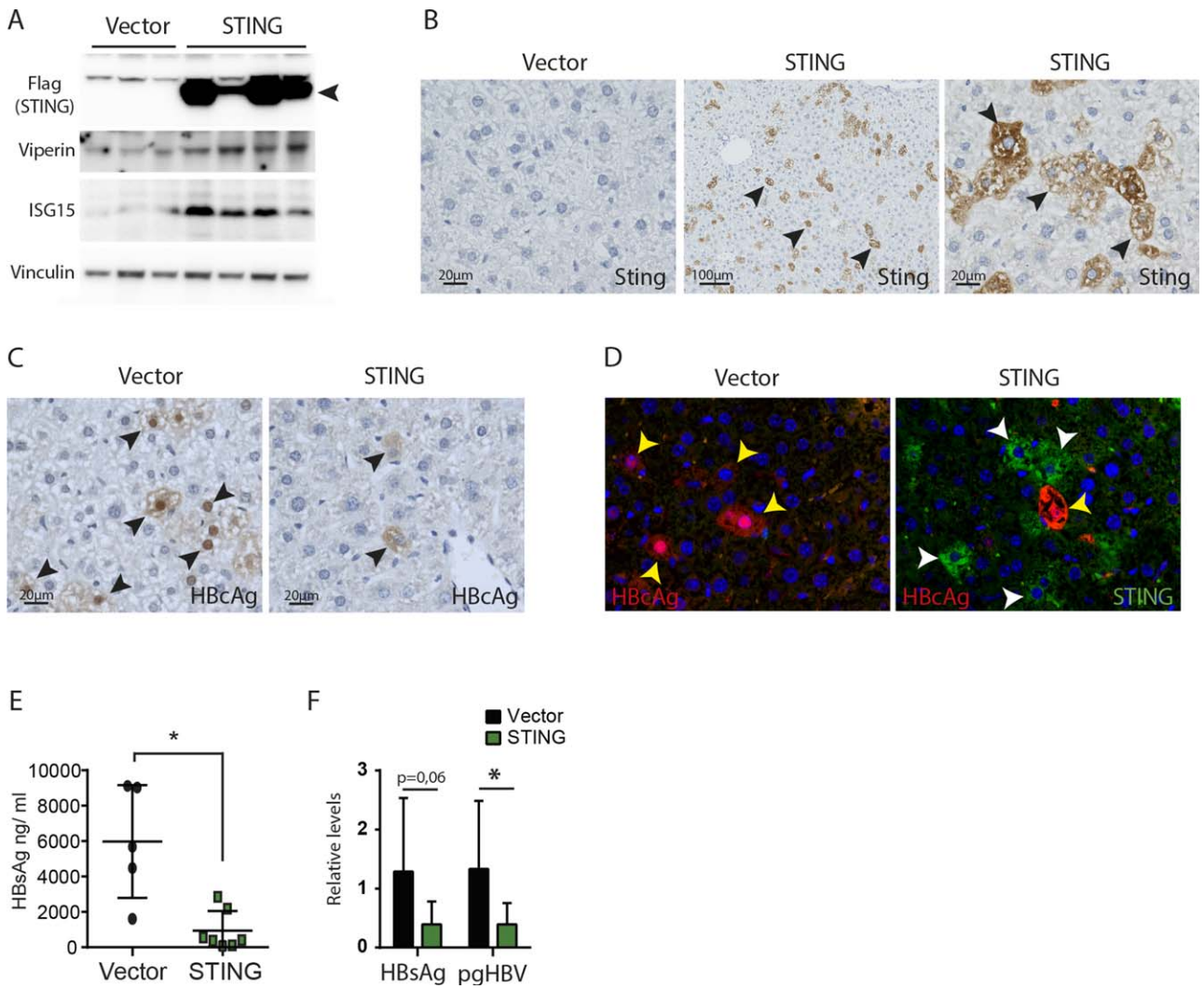

FIG. 4. Hepatocyte-specific expression of STING *in vivo* enables antiviral control of HBV infections. STING-deficient mice underwent HDI with 25 μg of empty plasmid or vector encoding STING-Flag under a hepatocyte-specific promoter. (A) HDI-treated mice were treated with double-stranded DNA 3 days postinjection. Liver extracts were isolated 5 hours later and analyzed for Flag, viperin, and ISG15 by western blotting. Vinculin was used as a loading control (n = 4). (B-E) Mice were subjected to HDI with plasmid and 3 days later infected with AdV-HBV. Four days postinfection, liver sections were stained for STING (B) and for HBcAg (C). Black arrowheads mark positive cells (n = 5, 7). (D) Mice were subjected to HDI with an empty vector or one encoding STING. After 3 days, mice were infected with AdV-HBV; and 4 days later, liver sections were costained for HBcAg and STING. Yellow arrowheads mark HBcAg-positive cells, and white arrowheads mark STING-positive cells (n = 5, 7). (E) HBsAg in serum was measured by ELISA (n = 5-7). (F) Expression of pregenomic RNA and HBsAg in whole-liver extracts was evaluated by qPCR (n = 6). Data are means standard deviation, and an unpaired two-tailed Student *t* test was used. Abbreviation: pg, pregenomic.

To find out whether DNA sensing in hepatocytes can promote defense against HBV replication *in vivo*, we infected STING-deficient mice with AdV-HBV 3 days after HDI of the STING expression plasmid or an empty vector. Four days postinfection, mice were analyzed for the expression of STING and HBcAg in the liver. The transfection efficiency with about one third of hepatocytes being positive for STING was retained over the 4 days of infection, and we did not observe HBV affecting the levels of STING, which was localized to the cytoplasm (Fig. 4B). Importantly, IHC on liver sections demonstrated that mice expressing STING in hepatocytes displayed a lower number of infected cells (Fig. 4C). Moreover, immunofluorescence revealed that

STING-positive cells did not costain with HBcAg (Fig. 4D). ELISA demonstrated that these mice also had reduced levels of HBsAg in the plasma (Fig. 4E). Total RNA expression and pregenomic HBV RNA were also reduced in mice overexpressing STING, showing that a DNA-sensing pathway can sense HBV and subsequently reduce HBV gene expression and replication (Fig. 4F). These data show that reconstitution of the STING pathway in hepatocytes allows a more efficient innate antiviral defense against HBV infection.

HUMAN HEPATOCYTES DO NOT EXPRESS STING AND LACK A FUNCTIONAL DNA-SENSING PATHWAY

The lack of DNA sensing in murine hepatocytes could contribute to the inefficient clearance of HBV infection. We were interested in determining whether human hepatocytes also failed to induce ISGs in response to foreign DNA. First, we examined Huh7 cells, which is a well-characterized human liver carcinoma cell line. No STING protein could be detected in Huh7 cells, and the cells did not respond to DNA stimulation but responded to poly(I:C) stimulation with an ISG signature (Fig. 5A). For another hepatocyte-derived cell line, HepG2, STING expression was observed, as well as responsiveness to both double-stranded DNA and poly(I:C) (Fig. 5A). This was also seen after AdV-HBV infection of the two cell lines, where HepG2 showed an innate immune response (Fig. 5B). Importantly, overexpression of STING in Huh7 cells enabled responsiveness to DNA measured by phosphorylation of TBK1 after DNA stimulation (Fig. 5C). Thus, similar to the *in vivo* transfection of murine hepatocytes, introduction of STING in a human hepatocyte cell line leads to functional DNA sensing. Finally, we examined the expression of STING in primary human hepatocytes. We analyzed a well-characterized pool of human primary hepatocytes from 10 donors for STING expression and activation of the DNA-sensing pathway. The cells were permissive for HBV infection, and HBV replicated efficiently in these cells (Fig. 5D,E). Notably, we did not detect STING protein in primary human hepatocytes or induction of ISG expression after DNA transfection (Fig. 5F). Infection of primary human hepatocytes with HBV did not induce ISG15 expression above a marginal elevation evoked by the mock infection (Fig. 5F). Altogether, these data show that both murine and human primary hepatocytes do not express STING and hence fail to respond to DNA, potentially delaying the onset of antiviral defense against HBV infection.

Discussion

In this study we report that hepatocytes do not have a functional DNA-sensing pathway due to lack of STING expression and therefore cannot respond to the presence of foreign DNA in the cytosol with a type 1 IFN response. The lack of DNA sensing seems to impair the ability of hepatocytes to control HBV infection, and the virus seems not to actively interfere with DNA sensing because introduction of STING expression in hepatocytes reduced viral gene expression and replication. Thus, we propose that hepatocytes represent a beneficial biological niche for DNA viruses. This is of particular advantage for a small DNA virus like HBV, which is likely to have fewer mechanisms to evade immune responses than larger viruses such as herpesviruses and poxviruses.[24]

Until now, nearly all primary cell types investigated have functional DNA sensing. The lack of DNA sensing in hepatocytes seems to be evolutionarily conserved between humans and mice, and probably in chimpanzees and woodchucks, as HBV infection does not induce innate immune response in these species.[9] The question as to why the DNA-sensing pathway is not operative in hepatocytes is interesting and may represent an evolutionary compromise. Hepatocytes have a great proliferative potential, which is activated upon liver damage, and it is known that cell proliferation leads to accumulation of DNA in the cytosol.[25] Accumulation of host DNA in the cytoplasm leads to DNA-driven inflammatory diseases.[26,27] Thus, it is conceivable that cell types with a high proliferative potential do not harbor a strong capacity to mount immunological responses to DNA. In agreement with this idea, T cells do not produce IFN in response to intracellular DNA.[28] In contrast, the resident macrophages of the liver, Kupffer cells, did express STING and responded to DNA in the cytosol. Consistent with this, we registered STING-dependent innate immune signaling early after infection with AdV-HBV, but this response was not able to alter virus load. This suggests that the response was either too

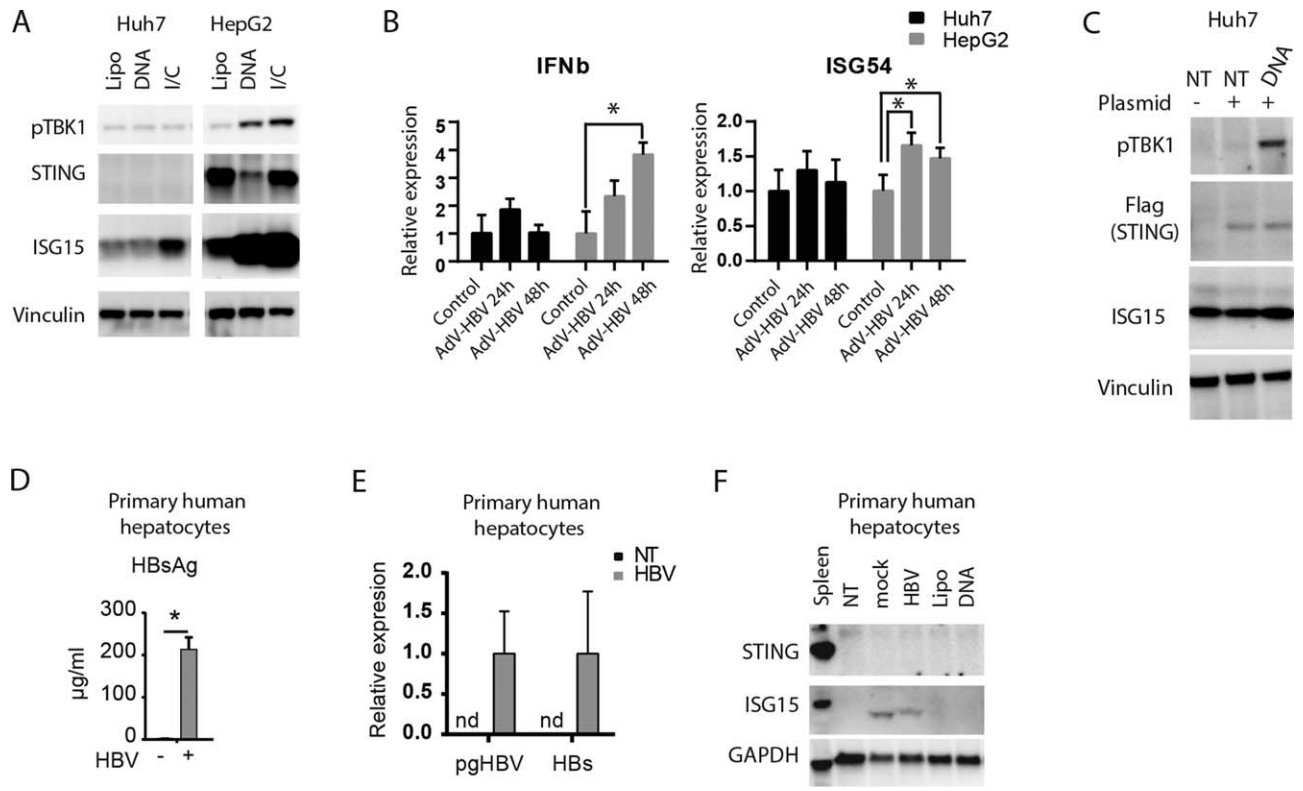

FIG. 5. The STING-dependent DNA-sensing pathway is not operative in human hepatocytes. (A) Two human hepatocyte cell lines were analyzed for expression of STING, phospho-TBK1, and ISGs 24 hours after stimulation with either double-stranded DNA or poly(I:C). Vinculin was used as a loading control. A representative experiment is shown (n = 3). (B) Huh7 and HepG2 cells were infected with AdV-HBV (multiplicity of infection = 10) for 24 and 48 hours, and expression of IFN-β and ISG54 was measured (n = 3, $^*P < 0.05$). (C) Huh7 cells were transfected with an empty vector or STING-Flag-encoding plasmid. One day later, cells were stimulated with DNA or only Lipofectamine for 5 hours, and a protein extract was analyzed by western blotting for phospho-TBK1, Flag, ISG15, and vinculin. Media from primary human hepatocytes infected for 48 hours with HBV (multiplicity of infection = 100) were analyzed for (D) HBsAg by ELISA and (E) expression of pregenomic HBV and HBsAg (n = 3, $^*P < 0.05$). (F) A pool of primary human hepatocytes was analyzed by western blotting for expression of STING and ISG15 after stimulation with double-stranded DNA (5 μg/mL, 24 hours) or infection with HBV (multiplicity of infection = 100, 48 hours). Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control and a murine spleen as a positive control. Data are means ± standard deviation, and an unpaired two-tailed Student *t* test was used. Abbreviations: Lipo, Lipofectamine; NT, not treated; pg, pregenomic; pTBK1, phospho-TBK1.

weak or of a too short duration and that it therefore did not exert sufficient paracrine antiviral action on the infected hepatocytes. It is also possible that recognition of HBV during infection would be different from AdV-HBV infection. However, in our model the cells are presented with large amounts of virus particles at once, inducing a robust innate immune response; but the activation of other pathways apart from the DNA-sensing pathway cannot be ignored.

Activation of the innate immune response by poly(I:C) increased expression of ISGs, such as chemokine (C-X-C motif) ligand 10 and ISG56, and reduced virus load. Similarly, systemic activation of the STING pathway by the agonist 5,6-dimethylxanthenone-4-acetic acid could reduce HBV load in an HDI model of HBV infection, emphasizing that innate immune responses can reduce HBV replication.[29] More than 250 ISGs have been identified,[30] and there is now an emerging understanding of the mechanistic basis for the anti-HBV activity of ISGs.[31] For instance, tripartite motif 22 inhibits HBV core promoter activity,[32] and indoleamine 2,3-dioxygenase inhibits translation of viral RNAs through tryptophan depletion.[33]

HBV replicates through a pregenomic RNA transcript, and cytosolic sensing of viral RNA has been indicated to inhibit HBV replication.[34,35] Mice lacking MAVS, the adaptor protein in the RNA-sensing pathway, however, did not display an impaired viral

load. This result is consistent with a similar study, where HBV DNA was delivered through HDI instead of AdV delivery.[36] HBV probably escapes sensing of its RNA by readily packing it into a capsid preventing contact with RNA sensors[37] as our data suggest that the RNA-sensing pathway is highly operative in hepatocytes, as measured both by expression of the factors involved in the pathway and by induction of the innate immune response after stimulation. Further possible explanations for this include that the viral RNA intermediate does not harbor 5-triphosphates or extensive secondary structures, which are the two main signatures of RNA stimulating the MAVS pathway. It is intriguing that the pathway for sensing RNA, but not DNA, is operative in hepatocytes; and future work will shed light on this, including the impact on other infectious diseases. In this respect, it is interesting that the RNA-sensing receptor melanoma differentiation-associated protein 5, which acts upstream of the MAVS pathway, plays an important role in the induction of innate immune responses to infection of the liver with *Plasmodium* sporozoites, the malaria parasites.[38]

Our analysis *in vivo* and *in vitro* showed that STING is not expressed in hepatocytes, resulting in dysfunction of DNA sensing. *In vivo* transfection of hepatocytes with STING restored the pathway to detect both naked DNA and HBV infection. This demonstrates that other components of the DNA-sensing pathway, including cGAS, are expressed in hepatocytes. Future work will identify whether these proteins serve other functions in the liver or whether the DNA-sensing pathway in the liver is latent, with only one component not being constitutively expressed. Because STING is an IFN-inducible protein, it is possible that STING is induced in the liver upon infection of, e.g., liver macrophages or dendritic cells and that this leads to restoration of the pathway.[39] However, data mining of published data sets did not indicate increased expression of STING upon liver inflammation.[40-42] *In vivo* transfection of murine hepatocytes resulted in STING expression in about one third of the cells. This was sufficient to allow detection of DNA and HBV infection and furthermore to decrease the viral load in the organ. This indicates that hepatocytes can signal to the surrounding cells to activate antiviral responses but also induce them themselves. This could include secretion of IFN and direct crosstalk such as delivery of cGAMP between cells through the gap junctions to activate a STING-dependent innate immune response in neighboring cells.[43]

In gene therapy, the liver has been a primary target because genes, especially those involved in metabolism, can be altered in hepatocytes. It has also been observed that gene expression could occur for months after HDI in mice or DNA vector-mediated gene transfer using AdV or adeno-associated virus vectors.[44,45] The lack of DNA sensing may explain the persistent gene expression in hepatocytes and open the perspective for long-term gene therapy without genomic integration of the DNA in diseases that can be corrected in hepatocytes. Future studies should address the link between innate DNA sensing and the potency of different gene therapy strategies, including adeno-associated virus, which has shown promising results as a vector *in vivo*.[46]

Collectively, in this work we demonstrate that the innate DNA-sensing pathway is not operative in hepatocytes due to lack of STING expression, and this allows HBV to infect this cell type without evoking expression of an antiviral program. Introduction of STING expression specifically in the liver strongly reduced the levels of HBV. Because the virus apparently did not evolve to actively interfere with DNA sensing, liver-directed gene delivery of STING combined with cGAMP treatment could serve as a means to treat HBV infection. Although an operative cGAS-STING pathway in hepatocytes most likely would be detrimental over time, transient stimulation of the pathway could enable a localized antiviral IFN response.

Acknowledgment: The technical assistance of Kirsten Stadel Petersen is greatly appreciated.

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