Inhibition of hepatitis B virus replication by activation of the cGAS-STING pathway

Jing He,1 Ruidong Hao,1 Dan Liu,2 Xing Liu,1 Shaoshuai Wu,2 Shuting Guo,3 Yuan Wang,2 Po Tien1 and Deyin Guo1,2

1State Key Laboratory of Virology and Modern Virology Research Center, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, PR China
2School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei 430072, PR China
3College of Life Sciences, Wuhan Institute of Bioengineering, Wuhan, Hubei 430415, PR China

Cyclic GMP-AMP (cGAMP) synthase (cGAS) senses cytosolic DNA and catalyses synthesis of the second messenger cGAMP, which activates the downstream signalling adaptor protein STING, leading to the expression of type I interferons. Hepatitis B virus (HBV) is a small DNA virus, and the cGAS-STING pathway may inhibit HBV RNA synthesis and viral assembly in cell culture, but the exact roles of the cGAS pathway in the restriction of HBV replication in infection systems remain to be elucidated. In this study, replication of HBV was significantly inhibited both in cell culture and in vivo in a mouse model when the cGAS-STING pathway was activated by dsDNA or cGAMP. In contrast, the presence of enzymatically inactive cGAS mutant did not influence HBV replication. Moreover, knockdown of cGAS in human peripheral blood monocytes led to a higher level of intracellular HBV DNA. Collectively, our data indicate that the cGAS-STING pathway plays a role in the surveillance of HBV infection and may be exploited for development of novel anti-HBV strategies.

INTRODUCTION

Hepatitis B virus (HBV) belongs to the family Hepadnaviridae. It is a major cause of liver disease, with both transient and chronic infections recorded (Seeger & Mason, 2000). HBV contains a 3.2 kb, partially double-stranded genomic DNA (relaxed circular, RC-DNA), which is converted into a plasmid-like, covalently closed circular DNA (cccDNA) inside the host cell nucleus during infection. The cccDNA is the transcription template of pregenomic RNA (3.5 kb) and other mRNAs (Beck & Nassal, 2007; Grimm et al., 2011; Guidotti & Chisari, 2006). Although the liver is the main site of HBV replication, HBV DNA is also detected in peripheral blood mononuclear cells (PBMCs) of patients with acute or chronic hepatitis B (Trippler et al., 1999). PBMCs are believed to be an extrahepatic HBV reservoir according to a series of studies (Michalak et al., 1994; Cabrerizo et al., 1997). It has been demonstrated that innate immune responses profoundly affect the replication or infection of HBV, via Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and cyclic GMP-AMP (cGAMP) synthase (cGAS) (Guo et al., 2009; Zhang & Lu, 2015; Dansako et al., 2016). However, it remains to be determined how HBV DNA is sensed by the innate pattern recognition receptors and whether DNA-sensing signalling pathways contribute to the host defence in HBV infection. DNA is usually confined within the nucleus and mitochondria of eukaryotic cells; therefore, the existence of DNA in cytoplasm is a signal of infection or cellular damage. The innate immune system utilizes a number of DNA sensors to detect DNA from cellular or tissue damage, and pathogens. Recognition of intracellular DNA triggers activation of multiple pathways, which are characterized by AIM2-dependent secretion of IL-1β (Atianand & Fitzgerald, 2013) and STING-mediated activation of type I interferons (IFNs) (Cavlar et al., 2012). Recently, cGAS was identified as a new cytosolic DNA sensor that activates the type I interferon pathway (Sun et al., 2013). cGAS contains a poorly conserved and highly positively charged N-terminal fragment, followed by a conserved motif found in the nucleotidyltransferase (NTase) family, which partially overlaps with a C-terminal male abnormal 21 (Mab21) domain (Civril et al., 2013; Sun et al., 2013). The NTase and Mab21 domains of cGAS reveal a bilobal scaffold, and a unique zinc-binding motif named ‘zinc thumb’ located between the two lobes, which is essential for DNA binding (Kranzusch et al., 2013). cGAS binds dsDNA and to some extent also ssDNA, and utilizes its positively charged surface and the zinc thumb to interact with the DNA sugar-phosphate backbone (Civril et al., 2013; Kranzusch et al., 2013). Upon binding to DNA, the NTase catalytic pocket undergoes large conformational changes, which opens up the catalytic
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results in the cGAS-STING pathway profoundly suppressed replication in HepG2 cells. The results showed that the expression level of cGAS changed slightly after the cells were treated with either IFN-α or IFN-β (Fig. 1b). This suggests that the expression of cGAS was not markedly enhanced by type I IFNs in HepG2 cells.

Activation of the cGAS-STING pathway inhibits HBV replication in cell culture

To investigate whether activation of the cGAS-STING pathway could affect HBV replication, HepG2 cells were co-transfected with a HBV replication-competent plasmid (pHBV1.3) and control vectors or plasmids expressing cGAS and STING. An IFN-β reporter was co-transfected with plasmids for measuring IFN responses. Expression of desired proteins in transfected cells was confirmed by Western blot analyses (Fig. 2a, lower panel). The steady-state level of HBV RNA in HepG2 cells was markedly reduced when the cells were co-transfected with both cGAS and STING (Fig. 2a), but not with cGAS alone (Fig. 2a). Consistent with this, the levels of core-associated HBV RNA and the secreted HBV e antigen (HBeAg) were decreased up to five-fold in proportion to HBV RNA reduction in the cells under the corresponding treatment conditions (Fig. 2b, c). Notably, the reduction in HBV DNA, RNA and HBeAg was correlated with the enhanced activity of the IFN-β promoter (Fig. 1b). Similar results were obtained with the human liver cell line L02, as shown for IFN-β promoter activity and HBeAg expression (Fig. 2d). These results indicate that activation of the cGAS-STING pathway suppressed the replication and gene expression of HBV.

A series of site mutations have been reported to abolish the cGAS function of triggering the expression of type I IFNs (Civril et al., 2013; Kranzusch et al., 2013; Li et al., 2013). We generated three cGAS-inactive mutants, including the NTase active site mutant (E225A/D227A), Zn-thumb mutant (C396A/C397A) and the DNA-binding pocket for ATP and GTP binding and subsequent cGAMP synthesis (Gao et al., 2013b).

cGAMP, a cyclic dinucleotide, which can be recognized by the protein STING, is identified as a second messenger of the innate immune response during DNA virus infection (Wu et al., 2013). cGAMP has two isoforms: the canonical c-dinucleotide of microbial cGAMP with conventional 3′–5′ linkages, and the non-canonical c-dinucleotide generated by cytoplasmic dsDNA sensor cGAS with non-canonical 2′–5′ linkage (Ablasser et al., 2013; Zhang et al., 2013). The stimulator of IFN genes (STING) is a central adaptor protein of the DNA-induced IFN expression signalling pathway (Ishikawa & Barber, 2008). It is the adaptor of cGAMP, c-di-GMP, c-diAMP and cGAMP (Tamayo et al., 2007; Römling, 2008; Gao et al., 2013b). When activated, STING re-localizes from the endoplasmic reticulum to the Golgi complex and is assembled into a structure that contains TANK-binding kinase 1 (TBK1) (Ishikawa et al., 2009; Saitoh et al., 2009), and promotes TBK1-dependent phosphorylation of IRF-3 by direct interaction with both proteins, leading to the induction of the expression of type I interferons (IFNs) and antiviral responses (Tanaka & Chen, 2012).

It has been reported that cGAS is the DNA sensor of large DNA viruses and human immunodeficiency virus (HIV) (Gao et al., 2013a). Very recently, the cGAS pathway was shown to be involved in the inhibition of HBV RNA and viral assembly in cell culture (Dansako et al., 2016). However, the exact roles and mechanisms of the cGAS pathway in the restriction of HBV replication in infection systems remain to be elucidated. In this study, we show that activation of the cGAS-STING pathway profoundly suppressed HBV gene expression and replication both in vitro and in vivo, and knockdown of cGAS in primary human cells enhanced HBV replication. These results indicate that the cGAS-STING pathway may play an important role in the host defence in HBV infection.

RESULTS

Induction of the cGAS-STING pathway in HepG2 cells

The cell line HepG2 derived from human hepatoma is commonly used for HBV replication, but it is not completely competent in innate immune responses. To study the function of the cGAS-STING pathway in HBV replication, HepG2 cells were transfected with a combination of plasmids including an IFN-β reporter (which contains a luciferase reporter under the control of the IFN-β-responsive promoter as an indicator for IFN response) in the presence or absence of cGAS and STING expression plasmids. When the cells were co-transfected with cGAS and STING plasmids, the activity of the IFN-β promoter was markedly enhanced by the transfected plasmids (Fig. 1a), and addition of any type of DNA including HBV, pUC18 plasmid or salmon sperm DNA did not further enhance IFN-β promoter activity (Fig. 1a), suggesting that cGAS and STING plasmids per se already activated the cGAS pathway. Furthermore, noncanonical cGAMP, the second messenger synthesized by cGAS, but not the canonical cGAMP synthesized by bacterial enzymes, activated the IFN-β promoter in the presence of STING (Fig. 1b). These results indicate that activation of the cGAS-STING pathway in HepG2 cells requires exogenous expression of both cGAS and STING, and the DNA recognition by cGAS is not sequence-specific.

As the NF-κB pathway is also relevant to HBV infection (Hösel et al., 2009), we also tested whether the NF-κB pathway was activated by cGAS-STING activation. As shown in Fig. 1(c), NF-κB reporter activity was not significantly elevated by the activation of the cGAS-STING pathway in HepG2 cells (Fig. 1c).

As cGAS is functionally deficient in HepG2 cells, we further explored whether type I IFNs can induce cGAS expression in HepG2 cells. The results showed that the expression level of cGAS changed slightly after the cells were treated with either IFN-α or IFN-β (Fig. 1d). This suggests that the expression of cGAS was not markedly enhanced by type I IFNs in HepG2 cells.
site mutant (K407A/K411A). The expression levels of desired proteins in the transfected cells were determined by Western blot with a cGAS antibody (Fig. 3a, lower panel). The three cGAS mutants did not reduce the level of HBV RNA and DNA as efficiently as wild-type cGAS in HepG2 cells (Fig. 3a). The secreted HBeAg levels in the extracellular culture medium were correlated with the levels of HBV DNA and RNA (Fig. 3b). These results suggest that enzymatic activity is required for cGAS-mediated suppression of HBV replication, and confirm that the anti-HBV function observed in our system is indeed cGAS-dependent.

**Inhibition of HBV replication is cGAS dose-dependent**

As activity of the cGAS-STING pathway may be affected by the expression level of cGAS, we measured the dose-dependent effect of cGAS on IFN-β promoter activity and HBV replication. As shown in Fig. 4(a), co-transfection of the cGAS expression plasmid at 20 ng markedly reduced HBV RNA and replication intermediates, and the inhibition effect increased gradually with higher amounts of cGAS. The levels of secreted HBeAg in the culture medium showed a similar profile (Fig. 4b). IFN-β promoter activity was inversely correlated with HBV replication.

**Fig. 1.** Induction of the cGAS-STING pathway in HepG2 cells. HepG2 cells were transfected with the indicated amounts of control vector or plasmids expressing cGAS or STING for 24 h, and stimulated with 4 µg ml⁻¹ salmon sperm DNA, 8 µg ml⁻¹ canonical or non-canonical cGAMP, or co-transfected with an HBV replication-competent plasmid (pHBV1.3) and control vectors for 48 h. NC indicates that transfection included only those plasmids indicated at the bottom of the graph. The cells were lysed and subjected to luciferase activity assay. pRL-TK was included as control for transfection efficiency. (a, b) Effects of cGAS-STING pathway on the IFN-β promoter in HepG2 cells under different stimulations. (c) Effects of cGAS-STING pathway on the NF-κB promoter in HepG2 cells. (d) qRT-PCR analysis of cGAS, IFI6 and IFI27. HepG2 cells were treated with 2.5 ng ml⁻¹ IFN-α or 20 ng ml⁻¹ IFN-β for 8 h and harvested for qRT-PCR. Data are the mean of three independent experiments and were statistically analysed with a t-test and one-way ANOVA. *, P<0.05; **, P<0.01; ***, P<0.001. Data are presented as mean±SD (n=3) and are representative of at least three independent experiments.
Fig. 2. Activation of the cGAS-STING pathway inhibits HBV replication in cell culture. HepG2 and L02 cells were transfected as indicated and harvested 48 h after transfection [a (RNA and proteins), c and d] or 96 h post-transfection [a (DNA) and b]. (a) HepG2 cells were co-transfected with control vectors or plasmids expressing cGAS and/or STING (200 ng each) and pHBV1.3 (800 ng) in 35 mm-diameter dishes. For Northern blot analysis of HBV transcripts, each lane was loaded with 2 µg total RNA. The rRNA (18S and 28S) was presented as loading controls. RC, relaxed circular DNA; SS, single-stranded DNA. Expression of intended proteins by the transfected plasmids was confirmed by Western blot analysis with the antibodies described in Methods, and the levels of β-actin serve as loading controls (lower panel). (b) The levels of HBV-DNA in the cells were measured by qPCR; (c, d) HBeAg in the culture medium of HepG2 or L02 cells was measured by ELISA. (d) IFN-β-luciferase activity was measured in L02 cells. Data are the mean of three independent experiments and were statistically analysed with a t-test and one-way ANOVA. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (b–d) Data are presented as mean±SD (n=3) and are representative of at least three independent experiments.
replication (Fig. 4c). These results suggest that cGAS inhibits HBV replication in a dose-dependent manner.

Knockdown of endogenous cGAS leads to a higher level of intracellular HBV DNA in human peripheral blood mononuclear cells (PBMCs)

We further studied the effects of the cGAS pathway on HBV replication, by knockdown of endogenous cGAS in PBMCs with small interfering RNA (siRNA). PBMCs were challenged by HBV virus collected from the culture supernatants from HBV-producing HepG2.2.15 cells, which produced $8.029 \times 10^6$ copies ml$^{-1}$ HBV. As shown in Fig. 5(a), the mRNA levels of cGAS were reduced by about 70% in cells transduced with sicGAS1, and by 50% with sicGAS2 relative to the control siRNA (Fig. 5a). The efficie (nt depletion of cGAS by sicGAS1 decreased the level of IFN-$\beta$ mRNA expression fivefold (Fig. 5b), and increased the level of intracellular HBV DNA by more than twofold (Fig. 5c). These results indicate that cGAS is involved in the defence of HBV replication in human primary cells.

cGAMP inhibits HBV replication in HepG2 cells

We further tested whether the di-nucleotide cGAMP could suppress HBV replication. We adopted canonical and non-canonical cGAMP, the latter being the product of cGAS, both of which can associate with STING. As shown in Fig. 6(a), both canonical and non-canonical cGAMP reduced the level of HBV transcripts and the HBV DNA in the presence of STING (Fig. 6a). The level of secreted HBeAg in the culture medium was correlated with the levels of HBV RNA and DNA (Fig. 6b). These results demonstrate that the dinucleotide cGAMP can induce anti-HBV activity and that STING is crucial for the suppression of HBV replication. Taken together with the above results, these data suggest that the cGAS-cGAMP-STING axis contributes to the suppression of HBV replication.

cGAS-STING pathway inhibits HBV gene expression and replication in mice

As the cGAS-STING pathway suppressed HBV replication in HepG2 cells, we continued to study the effects of this pathway on HBV replication in vivo using a mouse model dependent on the hydrodynamic injection-based transfection of

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**Fig. 3.** cGAS active site mutations led to reduced HBV gene expression and replication. HepG2 cells were co-transfected with 200 ng STING expression vector, 1600 ng pHBV1.3 and 200 ng wild-type or a series of cGAS mutants [NTase active site mutant (E225A+D227A), Zn-thumb mutant (C396A+C397A) and DNA-binding site mutant (K407A+K411A)] in 35 mm-diameter dishes. (a) The cells were harvested at 48 h post-transfection, 2 μg total RNA was loaded in each lane for Northern blot analysis, and the expression level of cGAS and its mutants was determined by Western blot with a cGAS antibody; 96 h after transfection, the HBV DNA levels were detected by Southern blotting. RC, relaxed circular DNA; SS, single-stranded DNA. (b) The levels of HBeAg in the culture medium were measured by ELISA. Data are the mean of three independent experiments and were statistically analysed with a $t$-test. **,$P<0.01$; ***,$P<0.001$. Data are presented as mean±SD ($n=3$) and are representative of at least three independent experiments.
HBV-replicating plasmid pHBV1.3. The pHBV1.3 and cGAS or STING expression plasmids or their control vector were co-delivered into mice. Immunohistochemical staining showed that the cGAS-STING pathway inhibited the expression of HBeAg and HBcAg in mouse livers (Fig. 7a). Western blot analysis confirmed the expression of cGAS in murine liver (Fig. 7b, lower panel). Northern and Southern blot results showed that HBV transcription and replication were reduced significantly upon cGAS and STING expression (Fig. 7b). Similarly, the titres of HBeAg, HBsAg and HBV-DNA in mouse sera were reduced significantly upon cGAS and STING injection (Fig. 7c, d). We also found that the expression of IFN-β mRNA in mouse liver was twofold higher when cGAS and STING were co-delivered (Fig. 7e). These results indicate that the cGAS-STING pathway inhibited HBV gene expression and replication in vivo.

**DISCUSSION**

cGAS is identified as a cytoplasmic dsDNA sensor, and its activation leads to the expression of type I IFNs. In the presence of DNA in the cytoplasm, cGAS is activated and forms a dimeric cGAS-DNA complex which synthesizes 2’ 3’-cGAMP from ATP and GTP (Zhang et al., 2014). 2’ 3’-cGAMP is a high-affinity ligand for STING. By binding with 2’ 3’-cGAMP, STING undergoes a series of structural changes and recruits TBK1 to phosphorylate IRF3 and activate IKK to phosphorylate IκBα, leading to IκBα degradation (Ishii et al., 2006). The dimerized IRF3 then translocates into the nucleus along with NF-κB and triggers the expression of type I IFNs and other cytokines (Cai et al., 2014). In our study, we found that the activity of the IFN-β promoter was enhanced by overexpression of cGAS and STING (Fig. 1a), indicating that transfection of plasmid DNA in the absence of HBV DNA can activate the cGAS pathway. Thus, in the transfection system, the cGAS pathway may be induced by both HBV DNA and plasmid DNA, and this system can only be used for observation of the effect of cGAS activation. The non-canonical cGAMP is the cGAS product, and activated the IFN-β promoter when STING alone was transfected (Fig. 1b). Our results also show that activation of the cGAS-STING pathway inhibited the transcription, gene expression and replication of HBV both in human liver cell lines and in vivo in a mouse model (Figs 2 and 7). As a DNA sensor, cGAS is in possession of two main functions: recognition of foreign DNA and activation of its downstream adaptor. These two functions require three domains: the NTase and Mab21 domains and a unique zinc-binding motif named ‘zinc thumb’ (Kranzusch et al., 2013). It has been shown that cGAS is deprived of the ability of DNA sensing or cGAMP synthesis by site-directed
mutations and, as a consequence, these mutants are incapable of stimulating the innate immunity responses (Civril et al., 2013). By taking advantage of this, we found that several loss-of-function mutants were deficient in the suppression of HBV replication (Fig. 3). Therefore, activation of the cGAS-STING pathway plays a role in inhibiting transcription, gene expression and replication of HBV.

The cGAS-STING pathway activates the expression of type I (IFN-α/β) IFNs that are considered the primary antiviral IFNs. By binding cell surface receptors, type I IFNs initiate a signalling cascade through the Janus kinase (JAK) and signal transducer and activator of transcription (JSTAT) pathways, leading to the transcriptional regulation of a large number of different IFN-stimulated genes (ISGs) (Stark et al., 1998). ISGs control viral infection by directly targeting various stages of different viral life cycles. For instance, ISG56, also known as IFIT1, disrupts the translation of viral mRNA by binding eIF3e or eIF3, and can also directly recognize the type 0 cap structure (without 5’-O-methylation) on viral RNA, or viral RNA containing the 5’-ppp (Diamond & Farzan, 2013). Another ISG, the 2’–5’ oligoadenylate synthetases (OAS) family, activated RNAse L to degrade viral genome (Kristiansen et al., 2011), and ISG15 to the ISGylation of viral proteins, inhibiting virus viral translation, replication and egress stage (Morales & Lenschow, 2013; Schneider et al., 2014). Recently it was reported that the cGAS-STING pathway inhibited HBV replication by negatively regulating HBV RNA and viral assembly (Dansako et al., 2016). In addition, our study showed that the HBV 3.5 kb, 2.4/2.1 kb RNA level, the relaxed circular DNA (RC-DNA) and the single-stranded DNA (SS-DNA) level were markedly reduced by activation of the cGAS-STING pathway (Figs 2, 4 and 7). These results suggest that inhibition of HBV replication may be regulated during the viral RNA synthesis stage because pre-genomic RNA is the template of viral DNA, and the reduced level of intracellular HBV DNA may be caused by reduction in 3.5 kb RNA synthesis. These phenomena may be related to the expression of some of the ISGs in response to type I IFNs activated by the cGAS–STING pathway. Detailed mechanisms on how cGAS-induced ISGs inhibit HBV replication need to be elucidated in future work.

Human PBMCs contain a series of immune cells, and are believed to be an extra-hepatic HBV reservoir with detectable HBV DNA from patients with acute or chronic hepatitis B (Michalak et al., 1994; Cabrerizo et al., 1997; Trippler et al., 1999). In this study, activation of the cGAS–STING pathway in HepG2 and L02 cells required exogenous expression of both cGAS and STING, which suggests that the pathway was not intact in these two human liver cell lines (Figs 1a and 2d). In contrast, quantitative reverse transcription polymerase chain reaction (qRT-PCR) showed that the mRNA expression of cGAS and STING is detectable in human PBMCs, suggesting that the cGAS–STING pathway is functional in PBMCs. The depletion of cGAS led to a lower level of IFN-β response during HBV infection, and an elevated level of intracellular HBV DNA (Fig. 5), indicating that the presence of cGAS is critical in the activation of IFN-β expression and the inhibition of HBV replication. These results may in part explain why HBV mainly replicates in liver cells with a low cGAS expression level in comparison with PBMCs.

STING is critical for regulating the production of IFNs in response to cytoplasmic DNA (Sun et al., 2009). It is the adaptor of cyclic dinucleotides (CDNs), including c-di-GMP, c-di-AMP and cGAMP (Tamayo et al., 2007; Römling, 2008; Gao et al., 2013b). These CDNs are bacterial second messengers, except for 2’-3’-cGAMP, one special non-canonical isofom of cGAMP generated by cGAS. In our study, we found that stimulating cells with non-canonical cGAMP led to the activation of the IFN-β promoter

![Fig. 5. Knockdown of endogenous cGAS in human primary cells enhances HBV replication.](image-url)
Fig. 6. Non-canonical and canonical cGAMP inhibits HBV replication in HepG2 cells. HepG2 cells were cotransfected with 200 ng control vector or plasmids expressing STING and 1600 ng pHBV1.3 per 35-mm-diameter dish, and 24 h post-transfection, the cells were stimulated with 8 μg ml⁻¹ canonical or non-canonical cGAMP using Lipofectamine 2000 for 0, 4, 36 and 72 h. (a) Northern blot analysis of HBV transcripts with the rRNA as loading controls and Southern blot analysis of HBV DNA. RC, relaxed circular DNA; SS, single-stranded DNA. (b) The levels of HBeAg in the culture medium were measured by ELISA. Data are presented as mean±SD (n=3) and are representative of at least three independent experiments.
(Fig. 1b) and reduction in HBV replication in the presence of STING (Fig. 6). This suggests that activation of the cGAS–STING pathway may be used as a new strategy in defence against HBV infection, and non-canonical cGAMP could be developed as a potential inhibitor of HBV replication.

METHODS

Plasmids and reagents. The HBV (genotype D, GenBank accession number V01460.1) replication-competent plasmid pHBV1.3 was generously provided by Dr Guangxia Gao (Institute of Biophysics, Chinese Academy of Sciences, China). The plasmid expressing N-terminally Flag-tagged-cGAS was generously provided by Dr Zhijian J. Chen (Department of Molecular Biology, University of Texas Southwestern Medical Center, USA). The plasmid pRK-STING was generously provided by Dr Hong-Bing Shu (College of Life Sciences, Wuhan University, China). The pTRE2-HBV and the HBV DNA fragments pGL3-EnhI/X (nt 950–1375), pGL3-EnhII/C (nt 1415–1815), pGL3-SP1 (2707–2849) and pGL3-SP2 (2937–3182) were described previously (Hao et al., 2015). The cGAS mutants (E225A+D227A, C396A+C397A and K407A+K411A) were generated by site-directed mutagenesis using Phanta Max Super-Fidelity DNA Polymerase (Vazyme).

IFN-α and IFN-β were purchased from Preprotech. Canonical cGAMP (3′ to 5′ cGAMP) and non-canonical cGAMP (2′ to 5′ cGAMP) were purchased from InvivoGen.

Virus, cell cultures and transfection. Human liver L02 and human hepatoma cell-derived HepG2 and HepG2.2.15 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin. HepG2 and L02 were transfected with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. PBMCs were collected from...
healthy volunteers, isolated with an solution of human lymphocytes (TBD Science) and cultured in RPMI 1640 medium after washing twice in phosphate-buffered saline (PBS). PBMCs were transfected with siRNA by electroporation with an Amaxa 4D-Nucleofector device (Lonza) according to the manufacturer’s protocols. HBV was collected from the culture supernatants of HepG2.2.15 cells, which contained 8.029×10⁶ copies ml⁻¹ of HBV.

**Nucleic acid analysis.** To purify HBV DNA from intracellular core particles, the cells from one 35 mm-diameter dish were washed with cold PBS and lysed in 0.5 ml NP-40 lysis buffer [50 mM Tris-HCl (pH 7.0) and 0.5% NP-40] at 4°C at 96 h post-transfection. After centrifugation, the supernatants were digested with RNase A and DNase I (Promega) at 37°C for 1 h, and DNase I was inactivated at 70°C for 15 min in the presence of 10 mM EDTA. The core-associated DNA was then isolated by digestion with 0.5 mg ml⁻¹ proteinase K and 1% SDS overnight at 50°C, purified by phenol/chloroform (1:1) extraction and ethanol precipitation and resolved in 20μl Tris-EDTA (TE) buffer. HBV DNA was quantified by real-time quantitative PCR and analysed by Southern blot analysis. For Southern blot analysis, half of the extracted DNA was loaded and separated in a 1% agarose gel, denatured and neutralized following standard procedures, and then transferred to a positively charged nylon membrane (GE Healthcare) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer.

Total cellular RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Five micrograms of total RNA were resolved in 1% agarose gel containing 2.2 M formaldehyde, and then transferred to a positively charged nylon membrane (GE Healthcare) in 20× SSC buffer. The 28S and 18S rRNAs were used as loading controls.

For the detection of HBV DNA and RNA, the membrane was probed with a digoxigenin (DIG)-labelled HBV RNA probe. Probe preparation and subsequent DIG detection were conducted with a DIG Northern Starter kit (Roche) according to the manufacturer’s protocol.

**Western blotting.** Cells were washed once with PBS and lysed in RIPA buffer [50 mM Tris (pH 7.6), 1% NP-40, 140 mM NaCl and 0.1% SDS]. The protein samples were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked with PBS containing 0.1% Tween 20 and 5% skimmed milk and probed with antibodies against anti-cGAS (Santa Cruz), anti-flag (Sigma) and anti-β-actin antibodies (Proteintech).

**Real-time quantitative PCR.** For mRNA detection, total RNA was isolated from cells with TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Five micrograms of total RNA from each mouse were subjected to Northern blot analysis. The RNA was reverse transcribed with M-MLV reverse transcriptase (Promega). SYBR Green master mix (Roche Diagnostics) was used for real-time PCR. GAPDH mRNA was analysed to serve as an internal control. The primers used in this study were: 5¢-GGGAGGCCGTCGTAAACCTCCTTAT-3¢ and 5¢-CTCTTGGATGTTGGTAGTA-3¢ for e-gas detection; 5¢-ACTGTTGGTGTGGCTACTA-3¢ and 5¢-TGCCCAAACAGTCGCAAG-3¢ for STING detection; 5¢-AGGAGGATGATCACTTTCGAC-3¢ and 5¢-TGATAGACATTAGCCAGC-3¢ for IL27 detection; 5¢-AGTGGGGTGCCTGATAAC-3¢ and 5¢-GCAGTGGGGTGGAGGCAGGTAAG-3¢ for IFI6 detection; 5¢-TCCTGGTGTCGTCATTAGAGGA-3¢ for IFI27 detection; 2RC/CCS 5¢-ACCCTGTTGCTGTAGCC-3¢ and 5¢-ACCCCTGGTCTGATGACCA-3¢ for GAPDH detection.

**Analysis of secreted HBV antigens.** At the indicated time points, cell supernatants or mouse sera were collected to detect the levels of HBV E antigen (HBsAg) and surface antigen (HBsAg) with a commercial ELISA kit (Kehua Bio-engineering).

**Immunohistochemical detection of HBV proteins.** Paraffin-embedded liver sections were treated with 3% hydrogen peroxide and blocked with 5% bovine serum albumin (BSA). The sections were then incubated sequentially with anti-HBs antibody (Sigma), anti-HBc antibody (Sigma), biotin-labelled secondary antibody and avidin-biotin complex. Peroxidase stain was developed with 3,3'-diaminobenzidine solution and counterstained with haematoxylin.

**Dual-luciferase assays.** HepG2 cells were co-transfected with IFN-β reporter, NF-κB reporter and HBV plasmids (100 ng) together with indicated amounts of cGAS and STING expression plasmids or control vectors. pRL-TK (50 ng) was included as a transfection efficiency control. At 48 h post-transfection, the cells were lysed and subjected to luciferase activity assay using the Dual-Glo system (Promega).

**Hydrodynamics-based transfection of HBV in mice.** Four-week-old female Balb/c mice were used and separated into four groups (eight mice each). pHBV1.3 (10 µg) and pSV-β-gal (5 µg) were injected into the tail vein together with pcDNA3 plasmid or cGAS expression vector (10 µg) and pRK or STING expression vector (10 µg), within 5 s in a volume of saline equivalent to 10% of mouse body weight. Four days after the hydrodynamic injection, sera were taken for analysis of HBsAg, HBsAg and HBsDNA. For Northern blot analysis, a piece of liver tissue was homogenized in 1 ml TRIzol reagent and total RNA was isolated according to the manufacturer’s instructions. Five micrograms total RNA of each mouse were subjected to Northern blot analysis. The level of β-gal mRNA in the liver was determined by real-time PCR and served as a control for injection efficiency. For Southern blot analysis, a piece of liver tissue was homogenized in 800 µl lysis buffer [50 mM Tris-HCl (pH 7.0), 10 mM EDTA, 1% NP-40 and 1% SDS] and digested with RNase A (Promega) at 37°C for 1 h, then incubated at 50°C overnight with 1 mg ml⁻¹ proteinase K. Total DNA was isolated by phenol/chloroform (1:1) extraction and ethanol precipitation. The DNA pellet was rinsed with 70% ethanol and re-suspended in 30 µl TE buffer. Five micrograms of total DNA from each mouse were subjected to Southern blot analysis. All mice were housed in pathogen-free conditions. The animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998) and were approved by the Experimental Animal Ethics Committee of Wuhan University School of Medicine.

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