学位論文

New insight into the host and virus interaction of HBV

（HBV に対する宿主応答の解析に関する研究）

2015 年 3 月

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List of publications and presentations

Publication(s):

Chean Ring Leong, Hiroyuki Oshiumi, Masaaki Okamoto, Masahiro Azuma, Hiromi Takaki, Misako Matsumoto, Kazuaki Chayama, Tsukasa Seya: "A MAVS/TICAM-1-Independent Interferon-Inducing Pathway Contributes to Regulation of Hepatitis B Virus Replication in the Mouse Hydrodynamic Injection Model" .J Innate Immun Vol. 7, No. 1, 2014

Presentations

1. **Chean Ring Leong**, Hiroyuki Oshiumi, Masaaki Okamoto, Masahiro Azuma, Hiromi Takaki, Misako Matsumoto, Tsukasa Seya: Inhibition of Hepatitis B virus replication by the Interferon- stimulated gene product of 20kDa protein (ISG20).The 79th Annual Meeting of the Japanses Society of Interferon & Cytokine Research, Sapporo, June 2014.

2. **Chean-Ring Leong**, Hiroyuki Oshiumi, Masaaki Okamoto, Masahiro Azuma, Hiromi Takaki, Misako Matsumoto, and Tsukasa Seya: Dispensable role of MAVS/TICAM-1 in IFN-inducing pathway to regulate HBV replication in the mouse hydrodynamic injection model .The 4th International Symposium on Carcinogenic Spiral, Sapporo, Feb 2014.

3. **Chean-Ring Leong**, Hiroyuki Oshiumi, Misako Matsumoto, Tsukasa Seya: The study of innate antiviral immune response to Hepatitis B virus using knock out murine models. The 61st Annual Meeting of the Japanese Society for Virology, Kobe, Nov 2013

Introduction

Hepatitis B virus

Hepatitis B is a major global health problem. More than 240 million people have chronic (long-term) liver infections. More than 780 000 people die every year due to the acute or chronic consequences of hepatitis B. It is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV). One major risk factor for the chronic infection with HBV is the development of Hepatocellular carcinoma (HCC) [1,2]. HBV infection is known to induce chronic necroinflammatory liver injury and to promote hepatocarcinogenesis. In fact, chronic HBV infection causes liver cirrhosis leading to over 50% of all HCC cases worldwide[3].

Structure and molecular virology

The hepatitis B Virus is a small non-cytopathic, parenterally transmitted, enveloped hepadnaviridae virus with circular, partially double-stranded DNA and represents the medically important prototype of enveloped DNA viruses[4].

The virus particle (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. These virions are 30-42 nm in diameter. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. The outer envelope contains embedded proteins that are involved in viral binding of and entry into susceptible cells.

The longer, (-) DNA strand is the coding strand for viral mRNA and viral pre-genomic RNA transcription. The shorter (+) strand is variable in length and is required for DNA synthesis during replication. There is a short cohesive overlap region stabilizing the circular structure of the genome at the 5' end. HBV genome is approximately 3200 bp in length and contains four overlapping open reading frames (ORFs). Each ORF encodes for specific structural proteins: The S gene which can be structurally and functionally divided into the pre-S1, pre-S2 and Surface region that encodes for the viral surface envelope proteins, HBsAg. The pre-core and core gene encodes for the nucleocapsid protein HBcAg and the secreted, non-structural pre-core protein, HBeAg. Multiple in-frame translation initiation sites in both the S and C gene bring to the related but functionally distinct proteins. The polymerase ORF encodes for the viral polymerase, which is functionally divided into three domains: the terminal protein domain involved in encapsidation and initiation of (-) strand synthesis. The reverse transcriptase domain responsible for genome synthesis and the ribonuclease H (RNase H) degrades the pre-genomic RNA in order to initiate the replication. There is also an ORF

encodes for HBxAg, a protein with multiple functions that are vaguely understood. HBx is known to be involved in signal transduction, transcriptional activation, DNA repair and protein degradation inhibition. In addition, HBx is known to be required for viral replication as well as HBV propagation [3,5].

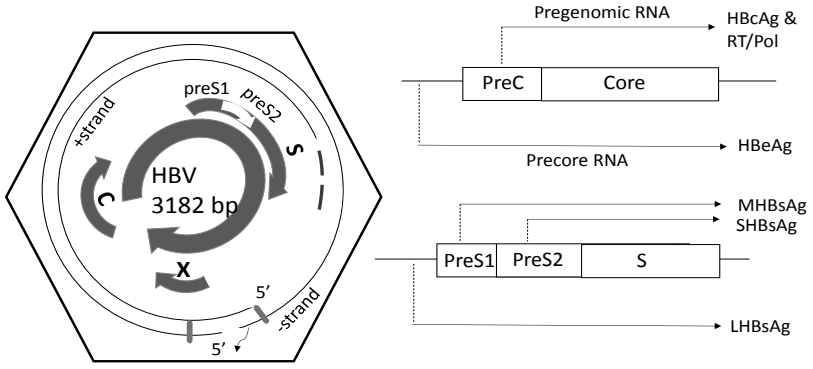


Figure: The HBV genome Genomic organization, RNA transcripts and gene products are shown on the left. HBV is a partially double-stranded enveloped DNA virus with four open readingframes,encoding for polypeptides of the surface antigen (S), the core protein (C), the polymerase (P) and the HBx protein (X). The transcription start sites of various HBV transcripts and their proteins are shown on the right. Figure was adapted from *Liang et al*. [6].

Life cycles

HBV infection into host hepatocytes follows a multiple step process [3,4,6,7].

Attachment

The virus gains entry into the cell by binding to a receptor on the surface of the cell and enters it by endocytosis. The cell surface receptor has been identified as the NTCP sodium/bile acid transporter.

Penetration

The virus membrane then fuses with the host cell's membrane releasing the DNA and core proteins into the cytoplasm.

Uncoating

Because the virus multiplies via RNA made by a host enzyme, the viral genomic DNA has to be transferred to the cell nucleus by host proteins called chaperones. The partially double stranded viral DNA that dissociate from the core proteins is then made fully double stranded and transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of four viral mRNAs.

Replication

The largest mRNA, which is longer than the viral genome, is used to make the new copies of the genome and to make the capsid core protein and the viral DNA polymerase.

Assembly

These four viral transcripts undergo additional processing and go on to form progeny virions which are released from the cell or returned to the nucleus and re-cycled to produce even more copies.

Release

The long mRNA is then transported back to the cytoplasm where the virion P protein synthesizes DNA via its reverse transcriptase activity.

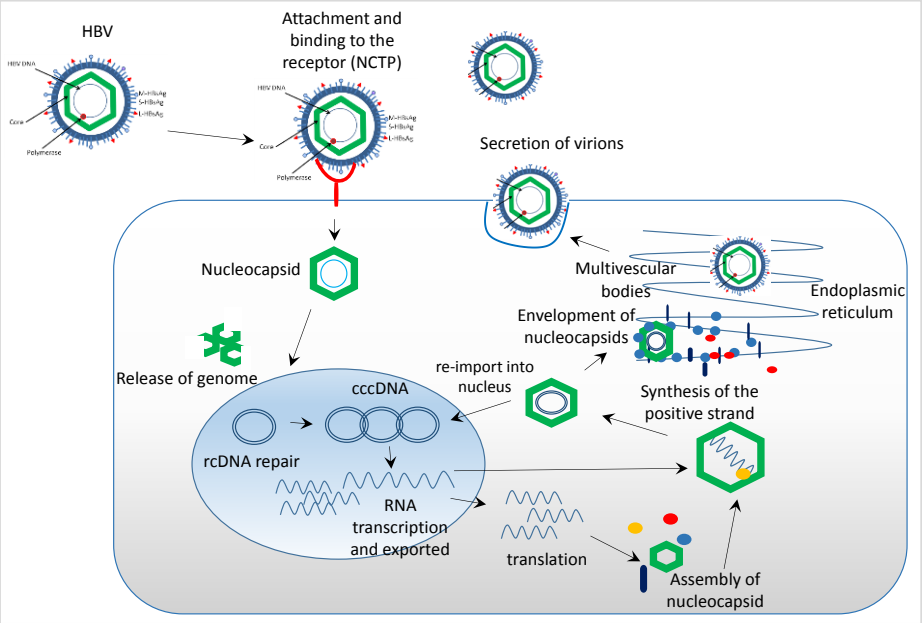


Figure: HBV life cycle. Figure was adapted and modified from *Grimm et. al*[3].

Hepatitis B virus infection

Viral hepatitis is a necroinflammatory liver disease that might vary in the severity. Upon infection, 95% of immunocompetent adults are likely to experience a self-limiting, transient liver disease while 5% of adults and 90% of newborns are persistently infected with HBV. Persistent infection is associated with chronic liver diseases as well as the high risk into the development of cirrhosis or HCC[8].

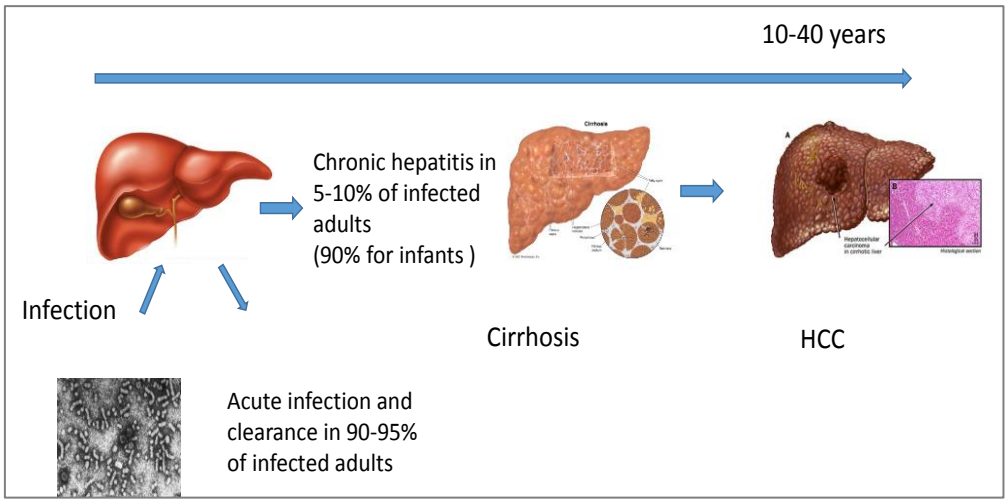


Figure: The overall pathogenesis of HBV upon infection

Acute and chronic HBV infection

Acute HBV infection can lead to fulminant hepatitis despite the fact that about 60% of the patients only show a mild, asymptomatic and subclinical illness whereby sometimes even went undetected. Acute fulminant hepatitis occurs only in 0.5% of patients and is characterized by sign of liver failure. During the HBV infection course of an immunocompetent individual, HBcAg-specific IgM appears earlier and persists life-long. Life-long protection is also given by the production of anti-HBs- and anti-HBe-specific antibodies [9].

While an acute HBV infection is characterized by a vigorous, polyclonal and multi-specific T cell response, patients with persistent and chronic HBV infection tend to show only a narrowly focused and weak HBV-specific T cell response. The failure of the immune system to clear and control the infection will lead to a subsequent liver inflammation, liver cirrhosis and finally HCC during the progression of chronic infection. Unlike the acute hepatitis, liver enzyme levels in chronic hepatitis B patients can be normal and no significant liver disease can be seen, particularly of the patients who were infected via vertical transmission [10].

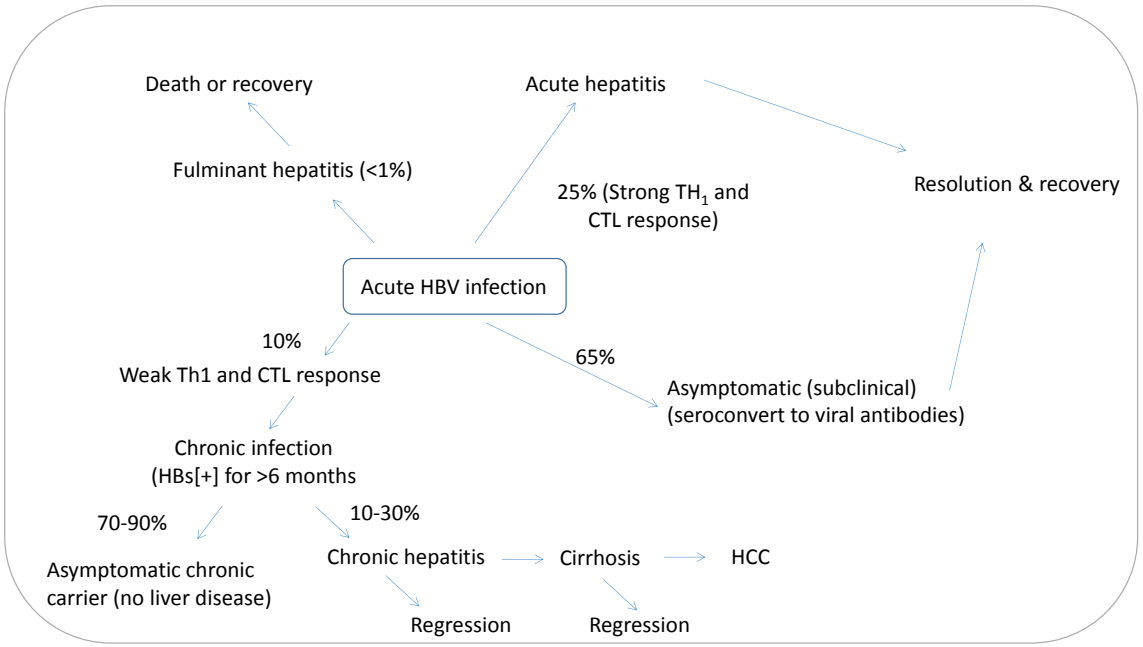


Figure: Pathogenesis and outcomes of HBV infection

Adaptive immune responses during HBV infection

Generally, adaptive immunity acts through functional maturation and expansion of distinct B and T cell clones that are able to specifically recognize the infectious agents. This process leads to the control of infection and elicits of a memory response which increases the host ability to block subsequent infections with the same pathogens. During HBV infection, HBV specific CD-4 and CD-8 mediated responses become detectable immediately after the start of the exponential increase in HBV replication. Therefore, the HBV specific T cell responses are generally considered to be timely activated.

In the self-limited infections, more than 90% of the HBV DNA is eliminated within the 2 ~ 3 weeks after the peak of viral replication. Under such circumstance, liver cell destructions by the non-cytopathic mechanisms sustained by cytokines secreted by CD8 T cells are absent. Unlike the self-limited infections, HBV persistence in the chronic HBV infection is always associated with defective HBV specific CD4 and CD8 T cell functions.

In the immune-competent adults, the release of cytokines or chemokine leads to clonal expansion of pathogen-specific T and B cells. Once activated, these adaptive immune cells contribute to tissue damage. Cytotoxic T cells ($CD8^+$ T cells) directly destroy virus-infected host cells through ligand dependent activation of cell death receptor-mediated apoptosis. Some CD4+ T cells boost a tissue-damaging inflammatory reaction, which can get chronic in the case of persistent virus infection. B cell-triggered antibody responses can also contribute

to tissue damage by the activation of the complement system. To prevent itself against immune system-mediated tissue damage, the host produces a subset of anti-inflammatory molecules, for example: IL-10 and TGF-β controlling the pro-inflammatory reactions [11].

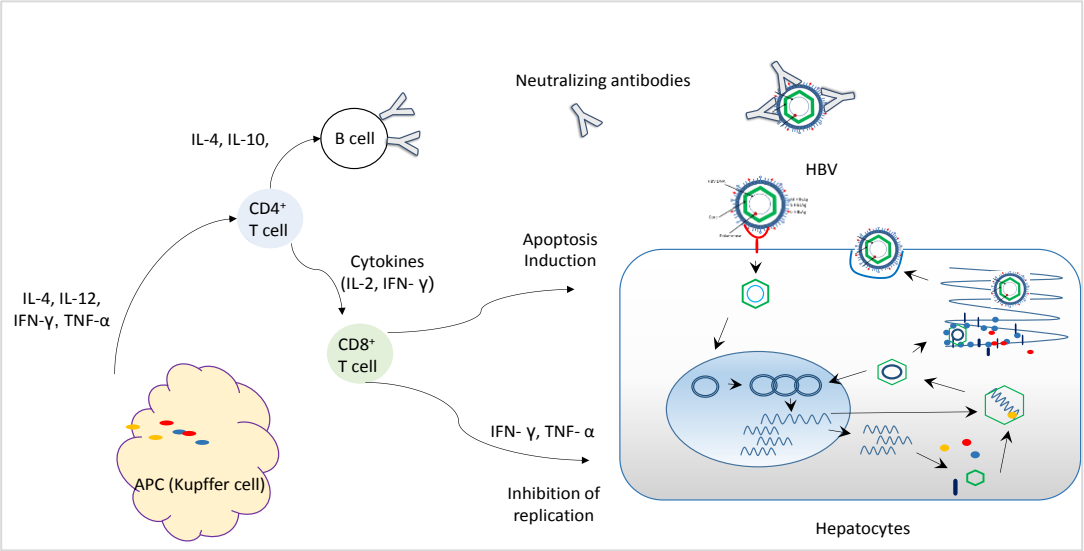


Figure: Cellular Immune response to HBV.

Innate immune response during HBV infection

In the decades long of studies on HBV, our knowledge of anti-HBV innate immune responses is still vague as hampered by technical limitations. This includes the difficulty in obtaining the data during natural infection in patients at the earliest pre-symptomatic stages of acute HBV infection. Despite the technical limitations, an overall scenario shows that limited or even absent activation of innate immunity seems to be the hallmark of acute HBV infection. Studies on the HBV infected chimpanzees showed a lack of induction of IFN-related genes during the phase of viral entry and expansion.

The apparent lack of type I IFN induction during HBV infection was interpreted as an ability of HBV to escape innate recognition. This is made possible by the replication strategy of HBV which uses a transcriptional template (cccDNA) that is formed in the nucleus of the infected cells. Under such circumstance, the viral template DNA might not be detected by the innate DNA sensing cellular machinery. On top of that, HBV produces polyadenylate viral mRNA that resembles the cellular transcripts of the host cells. And also, the newly transcribed HBV genomes are protected in viral capsids in the cytoplasm.

There are also numerous studies reporting on the HBV-mediated down regulation of the innate immune responses. Thus some believe that HBV facilitates initial infection and progression to persistence by actively manipulating the host innate immune response to its advantage. But there is increasing evidence that activation of innate host cell signaling pathways plays a major role in limiting the HBV infection in the immuno-competent adults. Indeed, *in vitro* studies using differentiated HepaRG cells transduced with recombinant baculovirus encoding the complete HBV genome showed that HBV elicited a strong and specific innate antiviral response in this cell line that resulted in a non-cytopathic clearance of HBV DNA, with up-regulation of IFN-β as well as other IFN-stimulated genes [12]. However, it remains to be further investigated if these data are truly reflective of the immunological events occur in the liver.

Abbreviation

cGAS	Cyclic GMP-AMP synthase
dsRNA	double stranded RNA
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMCV	Encephalomyocarditis virus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBcAg	Hepatitis B core-protein antigen
HBsAg	Hepatitis B surface-protein antigen
HBV	Hepatitis B virus
HCV	hepatitis C virus
IFN	interferon
IFNAR	interferon-α/β receptor
IRF	interferon regulatory factor
ISG20	interferon stimulated exonuclease gene 20kDa
ISGs	interferon stimulated genes
MAVS/IPS1	Mitochondrial antiviral-signaling protein
MDA5	Melanoma Differentiation-Associated protein 5
MyD88	Myeloid differentiation primary response gene (88)
N.D.	Not detected
NF-κB	nuclear factor-kappa B
NK	natural killer
NKT	natural killer T
NTCP	Sodium taurocholate cotransporting polypeptide
Poly (I:C)	polyriboinosinic polybiocytidylic acid
PRRs	pattern-recognition receptors
RAG2	recombination activating gene 2
RIG-I	retinoic acid-inducible gene-I
ssRNA	single stranded RNA
STING	STimulator of INterferon Genes
TICAM-1	TIR domain containing adaptor molecule 1
TLR	Toll-like receptor
VSV	Vesicular stomatitis virus
WT	Wild type

Chapter 1

Innate immunity against HBV: lack of recognition or active inhibition?

1. Introduction

Innate Immunity

In general, innate immunity is important in controlling infection immediately after contact with the pathogens, in order to limit the spread of the infection and to initiate efficient development of an adaptive immune response. The innate immune system is activated by pathogens by using pattern-recognition receptors (PRRs) that recognize specific structures on pathogens, such as double-stranded RNA and bacterial wall components[13,14]. The most important PRRs in viral infections are Toll-like receptors (TLRs), RNA helicases, such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), and double stranded RNA-dependent protein kinase (PKR). Following recognition of virus associated molecular patterns by cellular PRRs, recruitment of their distinct adaptor proteins will sequentially activate signaling cascades to induce cytokine production in virus-infected cells [13-15].

Main innate immune cells comprise natural killer (NK) cells, natural killer T (NKT) cells and macrophages. Sometimes dendritic cells especially plasmacytoid dendritic cells (pDCs) also play a significant role in the host innate immune defense. They consist of the first line defense against the invading pathogens. The early phase of viral infections is mainly characterized by the production of cytokines, type 1 interferon (IFN)-α/β, and the activation of natural killer (NK) cells. Production of type 1 IFNs can be triggered directly by virus replication through cellular mechanisms that detect the presence of viral RNA or DNA. The main sources of IFN-α/β are infected cells and plasmacytoid dendritic cells, whereas IFN-γ is produced primarily by NK and NKT cells. In the liver, the innate immune system consists of the non-parenchymal cells (Kupffer cells, sinusoidal endothelial cells) and parenchymal cells (hepatocytes and epithelial cells) with unique immunological characteristics[16].

Furthermore, PRRs particularly TLR and RIG-I are widely expressed in both parenchymal and nonparenchymal cells. Once activated, these PRRs signals the activation of IRF3 and 7 or NF-kB, leading to the induction of type I IFN and the expression of a variety of pro-inflammatory cytokines.

Recognition of viral DNA

Nucleic acid motifs are the main virus-derived PAMPs to be recognized by the innate immune system. Viral DNAs are recognized by the growing family of pattern recognition receptors

(PRRs) triggering the antiviral immune responses. In addition to cytosolic DNA receptors, like DAI, RIG-I, DHX9 (helicase) or AIM2, the recent discovery of immune interferon-16 (IFI16) in the nucleus, cGAS and STING as an innate immune DNA receptor present has spark an interesting progress in the study [15,17].

In the innate immune sensing of DNA, viral DNAs are recognized by cytoplasmic DNA receptors (as illustrated in the figure below); AIM2 mediates caspase-1 dependent pro-IL-1β and pro-IL-18 cleavage and secretion of their proactive forms. Four known cytoplasmic DNA sensors are represented; DAI, RIG-I, IFI-16, helicase DHX9/DHX36. These DNA sensors initiate the signaling cascade that leads to the activation of transcription factors such as IFN regulatory factor 3 (IRF3) and 7 (IRF7) and nuclear factor kB (NF-kB) that translocate in the nucleus to induce expression of interferons and pro-inflammatory cytokines. Recently, a new molecule, STING (stimulator of IFN genes), has been shown to be essential for the TBK1-IRF3- dependent induction of IFN-β by transfected DNA ligands and intracellular DNA produced by pathogens after infection. STING (also known as MITA, MPYS and ERIS) is a transmembrane protein that resides in the endoplasmic reticulum (ER). In response to cytosolic DNA, STING forms dimers and translocate from the ER to the Golgi then to punctate cytosolic structures where it co-localizes with TBK-1, leading to the phosphorylation of IRF3 [15,17].

On the other hand, extracellular DNA including CpG DNA is recognized in a distinct mechanism. The CpG-rich DNA is transported into endosome and bind to TLR9 in the compartment. It signals through MyD88 to induce IFN-α production [18].

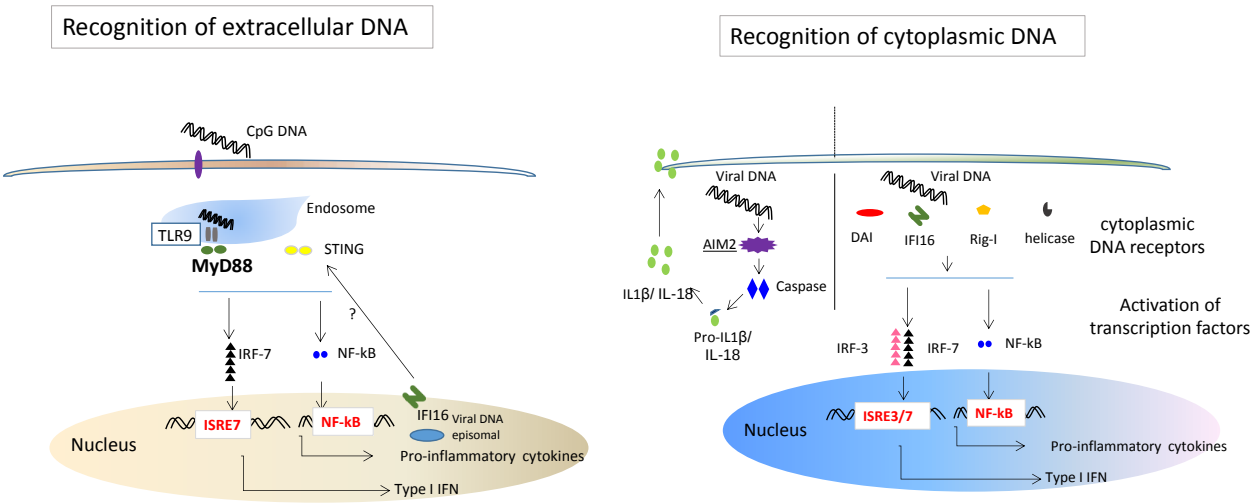


Figure: Innate immune sensing of DNA

Recognition of viral RNA

Viral RNA is recognized by innate immune receptors in both the endolysosomal compartment and cytoplasm. In the endolysosomal compartment, the viral RNA either double-stranded (dsRNA) or single-stranded (ssRNA) are recognized by the TLR3, 7 and 8. Whilst the DExD/H-box helicases RIG-I and MDA5 recognize the viral genomic RNA or transcriptional intermediates in the cytoplasm (as illustrated in the figure below).

The RIG-I-like receptors (RLRs) are cytosolic proteins recognizing viral RNA of different form and length. These receptors are expressed in most of the human cells and extensive studies of recent years revealed that RLRs were critical sensors of viral infection in most cell types except pDCs in which TLRs seems to be preferable detection of RNA virus infection. Binding to the RNA ligand will allow RIG-I to interact with the β-interferon promoter stimulator 1 (IPS-1 or MAVS) located at the outer mitochondrial membrane. Such association is crucial in initiating further signaling events which leads to the cytokine production in response to the viral infection. Although very homologous to RIG-I, MDA5 on the other hand has been reported to recognize long dsRNA. Upon the binding to viral RNA, MDA5 exposes a CARD and initiates cytokines and type I IFN production via MAVS in a manner similar to RIG-I. This subsequently led to the activation of IRF-3 and 7 or NF-kB followed by type I IFN release [13,19,20].

TLR-3 is localized to the intracellular compartment in macrophages, B lymphocytes, and cDCs. The Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1; also called TRIF) is the adaptor of TLR3, which recognize RNA virus infection and signals to protect host cells by inducing type I IFN. The adaptor TICAM-1 plays a pivotal role in the TLR3-mediated IFN induction [21]. TICAM-1 that being activated in the presence of viral ds RNA will recruit the kinase complex to activate IRF3 and 7 which induce type I IFN production. In addition, TLR7 and 8 in the intracellular compartment recognize the viral ss-RNA and trigger the innate immune responses via MyD88 as the adaptor.

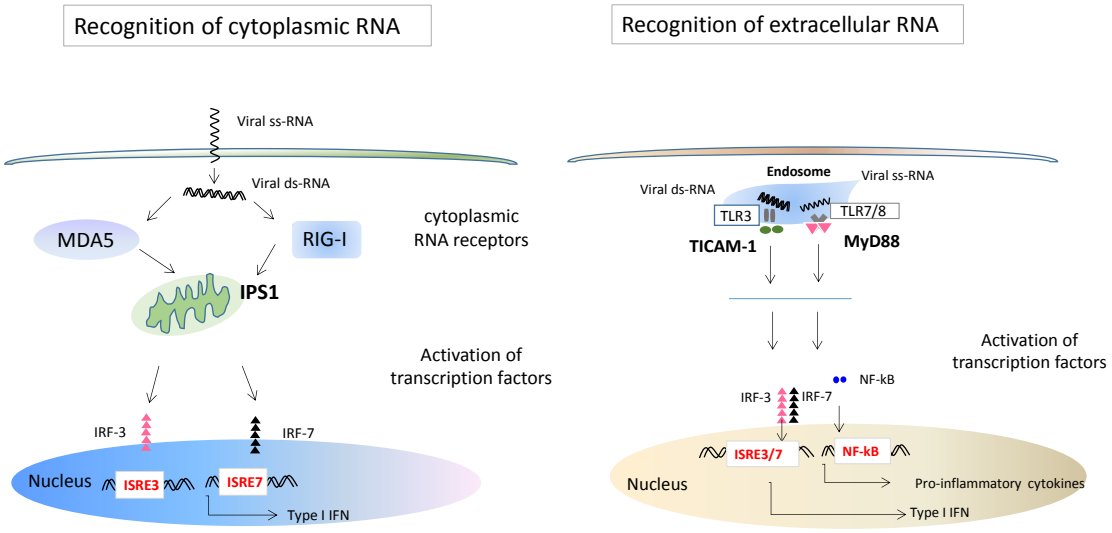


Figure: Innate immune sensing of RNA

Innate Immunity during HBV infection

In self-limited infections, HBV DNA falls by more than 90% within 2 to 3 weeks after the peak of viral replication and before detection of liver damage, indicating that a large quantity of virus eliminated without the need of liver cell destruction by non-cytopathic mechanisms sustained by cytokine[22]. Nevertheless, it still remains a question whether HBV triggers the onset of innate immune response during the course of infection.

It is generally believed that HBV does not induce an intrahepatic innate immune response because it acts like a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response, even though the precise mechanisms of which HBV accomplishes this are vaguely understood. However, in the recent years, different lines of evidence have challenged the view that there is a lack of recognition against HBV and argue that HBV can actually be sensed by the innate immunity. Indeed, *in vitro* studies using differentiated HepaRG cells transduced with recombinant baculovirus encoding the complete HBV genome showed that HBV elicited a strong and specific innate antiviral response in this cell line that resulted in a non-cytopathic clearance of HBV DNA, with up-regulation of IFN-β as well as other IFN-stimulated genes [12]. Moreover, acute infection with high dose of woodchuck hepatic virus (WHV) can induce immune genes immediately after infection[23]. A transient though slight activation of IFN-α gene was also detected in human hepatocytes infected with HBV in chimeric mice.

While on the contrary, a few other reports have suggested that the antiviral response

against HBV is mediated by the RIG-I/MAVS pathway in the cytosol and its activation is blocked by HBV polymerase in infected cells. For instance; recent works has demonstrated the potential ability of polymerase to inhibit IFN-β induction by interfering with the IRF signaling [24]. In addition, studies of overexpressing the HBV polymerase also shows that it can block the activation mediated by the recognition of TLR3 and RIG-I using poly I:C and Sendai virus as the heterologous inducers [25]. However, caution is needed in the evaluation of the physiological role of this mechanism. There are also studies indicating that the HBV x protein actively inhibit the innate immunity by interfering with signaling mediated by RIG-I through interaction with IPS-1/MAVS , which is essential for the induction of type I IFN [26]. Again, in these experimental setups, IFN-β production was activated by heterologous inducers including the poly I:C or vescicular stomatitis virus. And thus, no definitive evidence *in vivo* is available as analysis on the gene expression and effectors required for elimination of the replicative template has been especially made difficult with the technical limitation.

2. Aim(s) of the current study

What is the PAMP(s) of HBV?

There are many issues that remain unsettled with our current understanding on the innate immune response against HBV. It is important to bear in mind that analysis of the anti-HBV efficacy of IFN in the HepG2 or Huh7 hepatocyte derived cell lines may be influenced by the fact that such transformed cells have defects in the IFN-signaling pathways and as mentioned previously that over-expression of different pathogen recognition receptors in hepatocytes like cells can make the interpretation of the results obtained in such models difficult. It is clear that a better understanding of HBV infection awaits data generated in a more physiological system.

Despite numerous studies on HBV pathogenesis, the putative molecular patterns of HBV that trigger cellular responses remain unknown. It is time for us to shed an insight into questions such as whether the innate immunity against HBV is activated. In the event if innate immunity plays a crucial role at the early time point of the infection, which of the viral components: HBV DNA, RNA or viral proteins that are actually recognized by the pattern recognition receptors (PRRs) is an issue we would like to clarify in our current study.

Since viral clearance is a multifaceted process, it is thus crucial for the advancement of our understanding with a suitable laboratory animal to study the immunopathogenesis of HBV infection and the mechanisms of HBV persistence. The mouse model is the most suitable

laboratory animal for the study of the immunological events. Yet, mouse cannot be infected with HBV. As a result, majority of the studies on the immunopathogenesis of HBV were conducted in the HBV-transgenic mice. Since the HBV-transgenic mice are inherently tolerant to the transgene products, we still lack of the appropriate tool to address the immunological events upon the onset of HBV.

Herein, we hydrodynamically injected a naked HBV plasmid DNA into wild-type (WT) and gene-disrupted mice deficient in TICAM-1, MAVS, TICAM-1/MAVS, IRF-3/7, IFNAR, MyD88 or RAG2 to identify and characterize the immunological events for HBV clearance. We took advantage of the liver-targeting manner of hydrodynamic injection that enables us to study the role of different intracellular viral DNA or RNA sensing pathways of the immune systems response upon the onset of HBV pathogenesis. With the availability of various gene-disrupted mice, our study allows the identification of pathways crucial for the clearance of HBV.

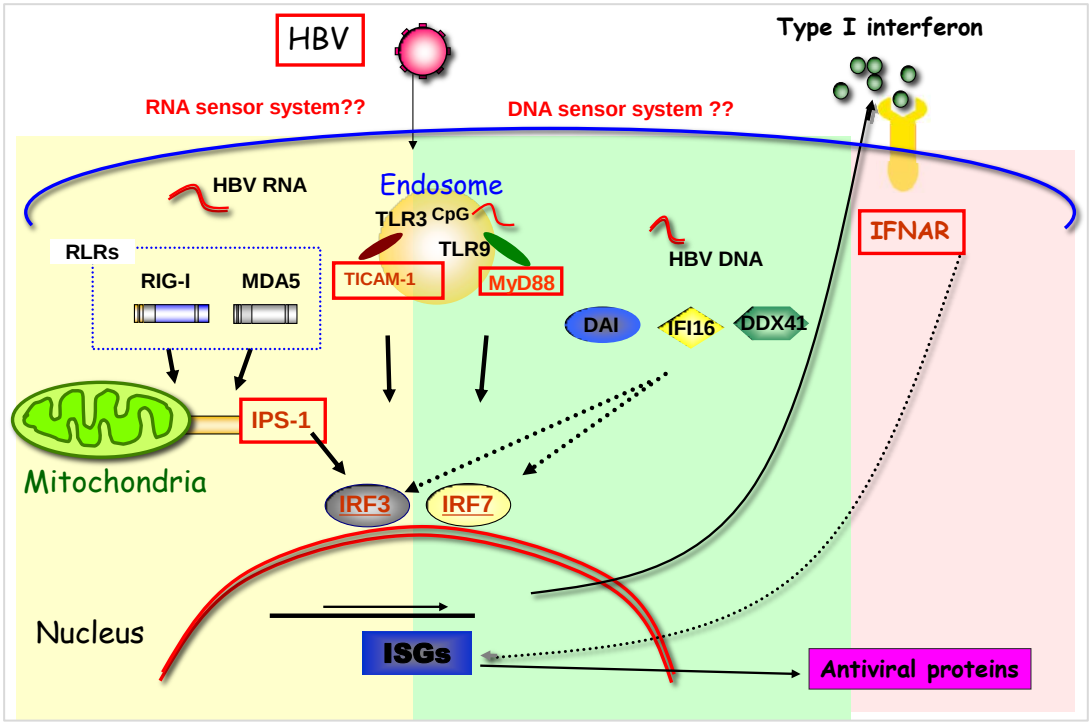


Figure: What is the PAMPs of HBV?

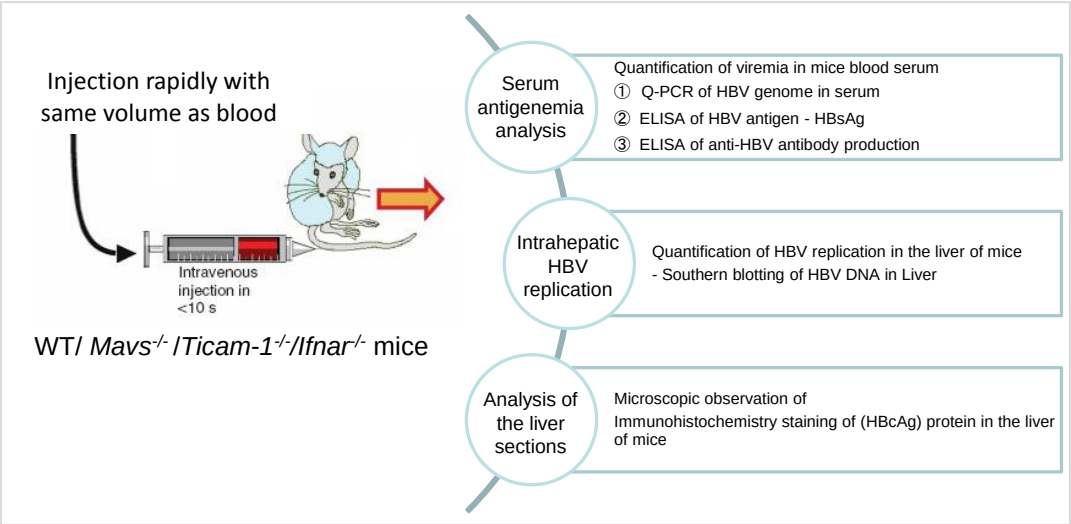


Figure: Flow chart of the experimental procedures

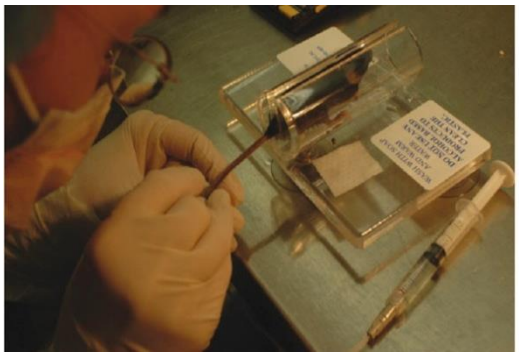
Hydrodynamic injection might be an effective and liver-targeting *in vivo* transfection method to study the innate immune responses against HBV?

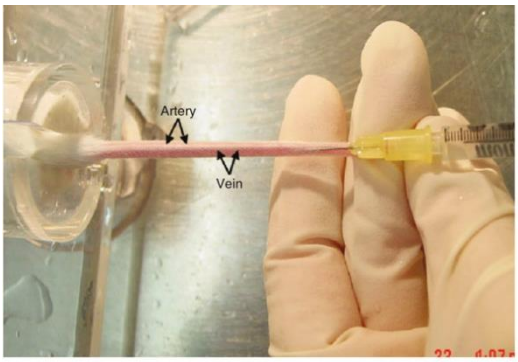
Since the susceptibility to HBV infection is limited to human and primates, we still lack of the appropriate tool to address the immunological events upon the onset of HBV. To meet this challenge, we are using hydrodynamic injection as a method to create a mouse model for the acute hepatitis B infection.

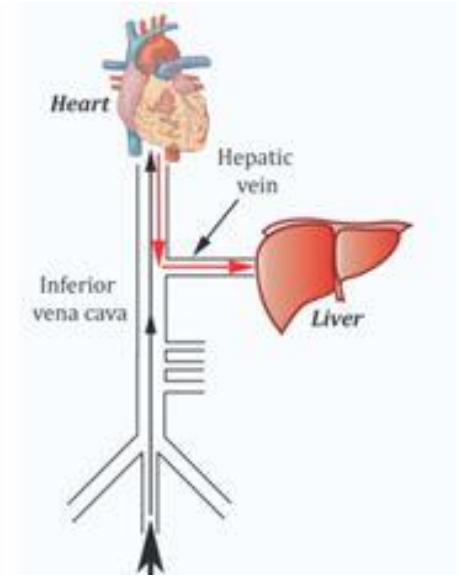
Hydrodynamic delivery, the application of controlled hydrodynamic pressure in capillaries to enhance endothelial and parenchymal cell permeability, had its inception in the late 1990s with investigations into intravascular injection of plasmid DNA solution for gene delivery in whole animals [27]. Budker et.al and Zhang et al has respectively tried out the hydrodynamics based procedure for systemic gene delivery in a mouse model. Since then it has been used as a common method for DNA and RNA delivery in rodents and proven useful for a different range of applications[28] .

Different from the carrier-based strategy, hydrodynamic gene delivery combines "naked" DNA and hydrodynamic pressure generated by a rapid injection of a large volume of fluid into a blood vessel to deliver genetic materials into parenchyma cells. In 2002, *Yang et al* employed the hydrodynamic injection of the viral DNA of HBV to establish a mouse model of acute HBV infection [29]. In their study, after transfection of hepatocytes in vivo with a replication-competent, over length, linear HBV genome, viral antigens and replicative intermediates were synthesized and virus was secreted into the blood. Viral antigens

disappeared from the blood as early as 7 days after transfection that coincident with the appearance of antiviral antibodies.







Intravenous injection of a large volume of solution (about 10% of body weight)

Hydrodynamic injection mechanism

- The large volume exceeds the cardiac output
- Development of a high pressure
- Backflow of the solution towards organs connected to the vena cava
- The liver absorbs most of the injected solution
- 10-40% of hepatocytes are tranfected

Figure: Description of the mechanism leading to the hepatocytes derived expression of foreign proteins following hydrodynamic injection with plasmid DNA.

3. Materials and Methods

Animal studies.

All mice were backcrossed with C57BL/6 mice more than seven times before use. *Ticam-1*$^{-/-}$ and *Mavs*$^{-/-}$ mice were generated in our laboratory as described previously. Whilst *Ticam-1*$^{-/-}$ *Mavs*$^{-/-}$ mice were generated by crossing *Ticam-1*$^{-/-}$ mice with *Mavs*$^{-/-}$ mice. *Irf-3*$^{-/-}$*Irf-7*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). *Myd88*$^{-/-}$ mice were provided by Drs. K. Takeda and S. Akira (Osaka University, Osaka, Japan). *Rag2*$^{-/-}$ mice were kindly provided by Dr N. Ishii (Tohoku University, Sendai, Japan). Female C57BL/6J mice were purchased from Japan Clea (Tokyo) and used at 7–9 weeks of age. All mice were maintained under specific pathogen-free conditions in the Animal Facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan). Animal experiments were performed according to the guidelines set by the Animal Safety Center, Japan.

Hydrodynamic transfection of mice with HBV1.4 plasmid.

The pTER1.4xHBV plasmid containing 1.4 genome length sequences of HBV that was previously shown to produce a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers was used in this study [30]. A total of 50μg of the plasmid was injected into the tail vein of 7-9 week-old mice in a volume of 2.0 ml TransIT-QR hydrodynamic delivery solution (Mirus, USA). The total volume was delivered within 3-8 seconds. Plasmid DNA was prepared by using an EndoFree plasmid system (Qiagen, Germany) according to manufacturer's instructions.

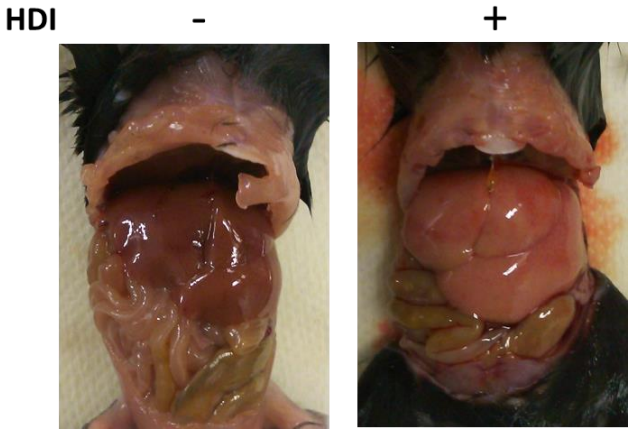


Figure: Physiological changes of the mouse liver after the hydrodynamic injection of a large volume of solution.

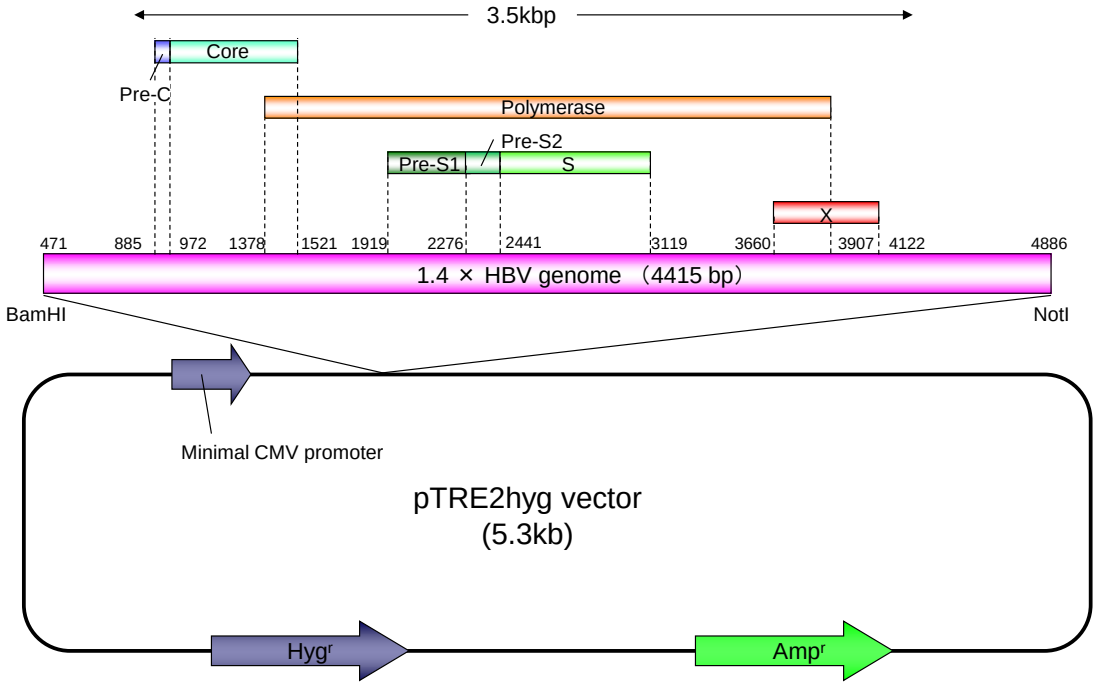


Figure: Plasmid construct of the pTER1.4xHBV that contains the 1.4x full length genomic DNA of HBV

Quantification of HBV DNA by real-time PCR

To determine the HBV DNA in the serum, 30μL of each serum samples was pretreated with 20 units of DNase I (Roche, Germany) at 37° C overnight. The encapsidated viral DNA was extracted with the SMITEST kit (Genome Science Laboratories, Tokyo, Japan) following the manufacturer's instruction and dissolved in 20 μL of TE-buffer. Viral genome thus purified was quantified by real-time PCR using the SYBR green master mix (life technologies, USA) and the HBV-DNA-F/R primer (The primer sequences are described in supporting information 6). Amplification conditions included initial denaturation at 95° C for 10 minutes, followed by 45 cycles of denaturation at 95° C for 15 seconds, annealing at 58° C for 5 seconds and extension at 72°C for 6 seconds. The lower detection limit of this assay is 1000 copies.

Immunohistochemical staining for HBcAg

For immunohistochemical staining of the HBV core antigen, mouse livers were fixed with 4% paraformaldehyde overnight, cryoprotected in 30% sucrose and sectioned at a thickness of 10 μm using Leica cryostat and mounted on Superfrost glass slides. Sections were incubated with the primary antibody (anti-core polyclonal rabbit antibody, DAKO) overnight, followed

by incubation with an immunoperoxidase technique involving avidin-biotin peroxidase complexes (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) according to a method reported previously.

HBV surface antigen antigenemia

Mice were bled on the days mentioned after injection of pTER-1.4xHBV and serum was isolated by centrifugation. Concentration of HBsAg in the serum was quantified by sandwich ELISA in commercial ELISA kits following the manufacturer's protocol. (XpressBio, USA). The reporting unit is Signal /Cut Off ratio of the 1000-fold diluted serum at O.D 450nm.

Southern blotting to detect intracapsid HBV DNA

Viral DNA was isolated from intracellular viral capsids and detected with specific DIG-labeled probe as described previously. In brief, to isolate the viral DNA, mouse livers were homogenized and subjected to an overnight sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. 20μg of the isolated DNA was separated in a 1% agarose gel, transferred onto IMMOBILON NY+ charged nylon membrane (Milipore) and detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer.

Anti-HBs antibody ELISA

IgG antibodies specific for HBsAg were detected by ELISA as described previously with slight modification (Yang et al 2002). A 96-well plate was coated with antigen of HBs in carbonate buffer and followed by blocking of 2%BSA. Plasma samples were diluted 5x and then incubated in the antigen-coated wells for 3h at room temperature. A Horseradish Peroxidase (HRP) conjugated goat anti-mouse IgG (Southern Biotechnology, USA) and TMB were used to develop the signal. Plates were read at 450 nm. Normal mouse plasma was used to generate cutoff values. The antibody titers are reported as the reciprocal of the A450 (sample)/ A450 (normal mouse average). Samples with value > 1 are considered scored positive.

Quantitative HBV or cytokines mRNA in the organs

Each organ was extracted from the mice on the days mentioned after hydrodynamic injection of the HBV plasmid. Total RNA of the organs was isolated with TRIZOL according

to the manufacturer's protocol. Using 0.5-1 μg of total RNA as a template, cDNA was obtained using a high capacity cDNA transcription kit (Applied Biosystem) according to manufacturer's instruction. qPCR was performed using a Step One real time PCR system (Applied Biosystems). The expression of cytokines mRNA was normalized to that of β-actin mRNA in each organ, and the fold increase was determined by dividing the expression in each sample by that of the mice receiving the control plasmid. The primer sequences are described in supporting information.

Quantitative cGAS, STING and MAVS expression in cell lines

Total RNA was isolated from L929 cells, RAW264.7 cells, immortalized mouse hepatocytes, Huh7 cells and HepG2 cells with TRIZOL according to the manufacturer's protocol. Using 0.5-1 μg of total RNA as a template, cDNA was obtained using a high capacity cDNA transcription kit (Applied Biosystem) according to manufacturer's instruction. qPCR was performed using a Step One real time PCR system (Applied Biosystems). The expression of each targeted mRNA was normalized to that of β-actin mRNA in each sample, and shown as relative expression. The primer sequences used are described in supporting information.

Reporter gene assay

To prepare the HBV RNA, immortalized mouse hepatocytes established in our laboratory previously were transfected with either control plasmid or pTER1.4xHBV. Total RNA containing the HBV RNA was isolated after 12h and confirmed with RT-PCR while the one transfected with only control plasmid was used as a control. The isolated RNA was later used as stimuli for the reporter gene assay of IFN-β. Briefly, the immortalized hepatocytes were again transfected with the reporter plasmids. After 16 h, the immortalized hepatocytes were transfected with the stimuli including PIC, a control plasmid, HBV RNA and pTER1.4xHBV using FuGENE HD (Roche). Cells were lysed at the time point mentioned using passive lysis buffer, Firefly and Renilla luciferase activities were determined using a dual-luciferase reporter assay kit. The Firefly luciferase activity was normalized by Renilla luciferase activity and was expressed as the fold stimulation relative to activity in non-stimulated cells.

Statistical analysis

The statistical significance of the obtained data in this study was analyzed using a two tail unpaired t test and $p < 0.05$ was regarded as statistically significant.

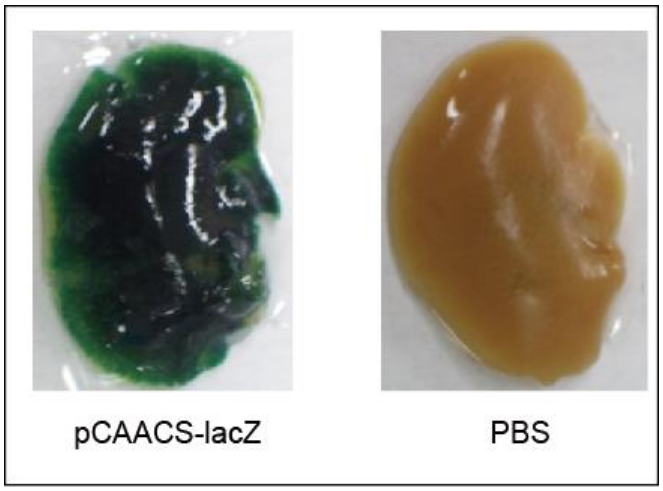
4. Results

MAVS and TICAM-1 are dispensable in suppressing HBV replication

To ensure the efficiency of delivery of the HBV transcriptional template into the mouse liver, a plasmid harboring the lacZ gene was used to transfect the liver cells using the hydrodynamic injection method. X-gal (a substrate for lacZ) staining showed that nearly the entire liver of injected mice has successfully received the injected plasmid (Fig 1). An independent determination of transfection efficiency was carried out using a plasmid harboring the GFP fragment. The comparable transfection efficiencies observed did not differ significantly among the different mouse strains (data no shown). Furthermore, quantification of HBV mRNA in the organs of WT and knockout mice on day 3 after hydrodynamic injection revealed that HBV mRNA was amplified mainly in the liver but not in other organs including kidney, lung, heart, Spleen or thymus (Fig 2). Only weak HBV signals were detected in other organs in some types of knock out mice. These results demonstrated that HBV replication *in vivo* using the hydrodynamic injection method was efficient and liver-specific.

Fig 1.

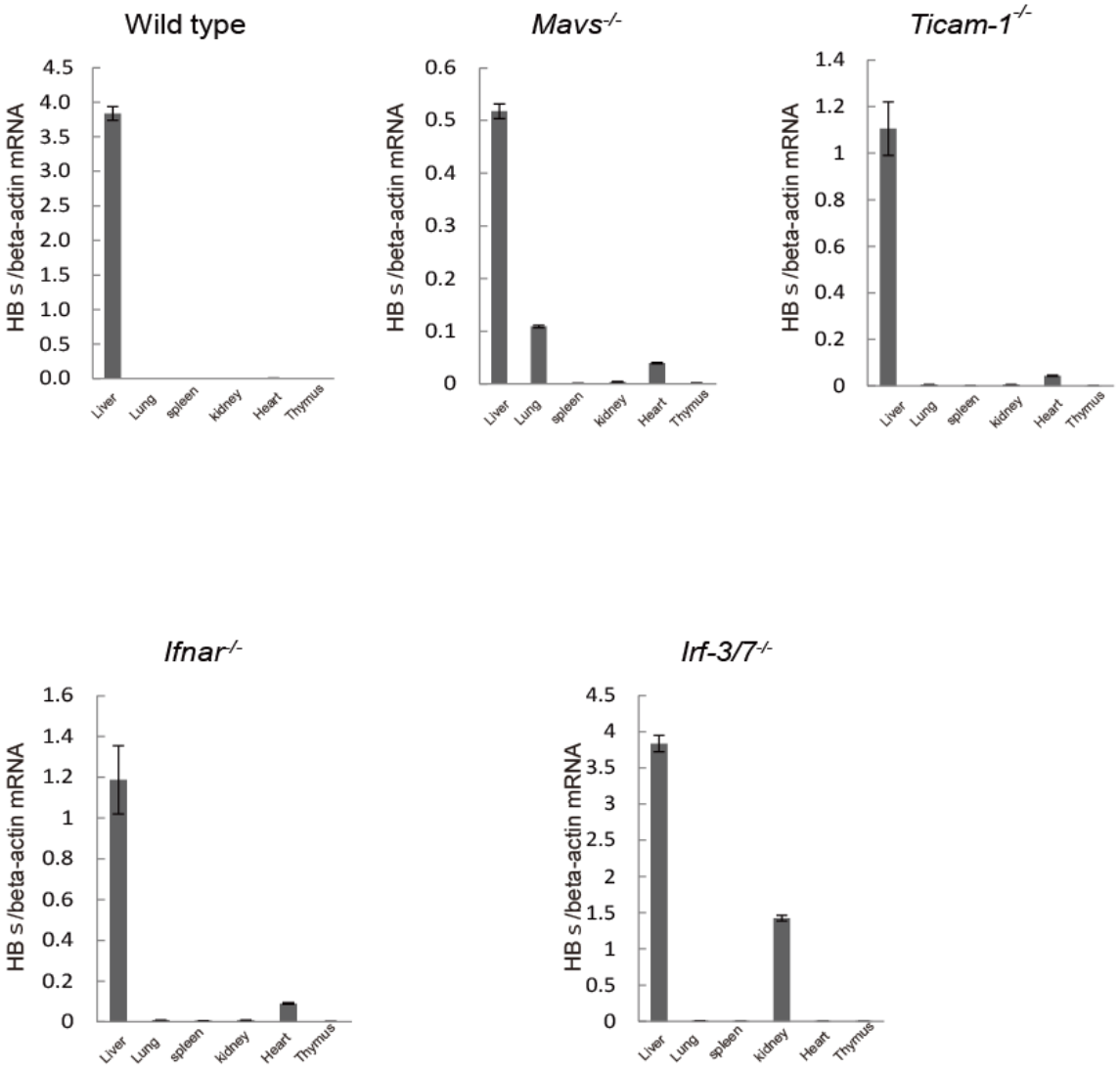
A.



Hydrodynamic injection enables efficient delivery of plasmid into the mice livers. 50μg of a plasmid harboring the lacZ gene was injected into the mouse and the mouse liver was isolated after 24h. The isolated whole livers were stained with X-gal (a substrate for lacZ) overnight at 37ºC. Images shown are the liver of mice injected with pCAACS-*lacZ* (*Left*) and PBS only (*Right*) after the staining.

Fig 2.

B.



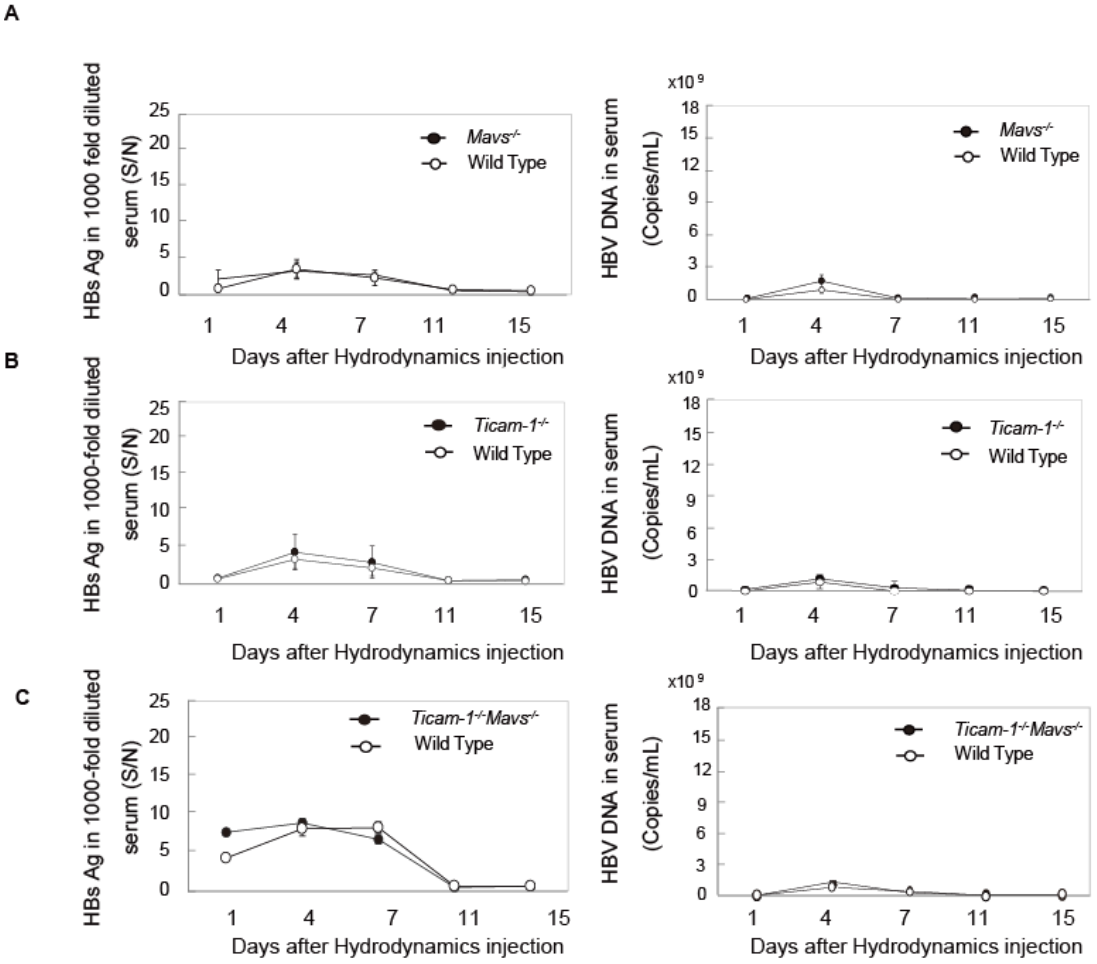
HBV mRNA was mainly detected in the livers of all the mice strains.

WT, *Mavs*$^{-/-}$, *Ticam-1*$^{-/-}$, *Irf-3/7*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were hydrodynamically injected with HBV replicative plasmid and organs including livers, lungs, spleens, Kidney, heart and thymus were isolated on day 3 post injection. The HBV mRNA of the various organs was determined from the total RNA with qPCR. Data shown is the mean of three mice for each mice strain.

Next we hydrodynamically transfected replication-competent HBV DNA into the wild type, *Mavs*$^{-/-}$, or *Ticam-1*$^{-/-}$ and *Mavs*$^{-/-}$/*Ticam-1*$^{-/-}$ mice to access the role of these viral RNA sensing pathways in response to HBV. Serum HBV surface antigen (HBs Ag) and HBV DNA levels were monitored regularly as surrogate markers of HBV replication *in vivo*. WT mice displayed acute, self-limiting hepatitis with peak HBs antigenemia on day 4 after DNA injection. Subsequently, HBs Ag in WT mice sera decreased and terminated by day 11. *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice displayed HBs Ag clearance kinetics that closely paralleled the WT mice response (Fig 3A-B left panels). Serum HBV DNA levels were quantified using real-time PCR. The average titer of serum HBV DNA in 15 WT mice injected with HBV DNA was below 1x 10^4 copies per milliliter at 1 day post-injection (dpi) and reached 2 x 10^9 copies per milliliter at 4 dpi (Fig. 3A-C, right panels). At later time points, most mice showed no detectable virus titer. Similar results were obtained with *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice (Fig. 3A, B). The serum HBV DNA and HBs Ag results showed only a marginal effect for the absence of MAVS or TICAM-1 compared to WT mice. The results suggested that the pathways involving these two adaptor proteins were dispensable for triggering the immune responses that suppressed HBV replication.

To determine whether the RIG-I/MDA5-MAVS and TLR3-TICAM-1 RNA-sensing pathways were dispensable for suppressing the HBV replication, similar studies were performed in mice lacking both the MAVS and TICAM-1 adaptor proteins (Fig 3C). No notable differences were observed between WT and MAVS/TICAM-1 double knockout mice in serum HBs Ag and HBV DNA levels, consistent with other data obtained. In addition, similar kinetic of intrahepatic clearance of the HBV template as well as HBV replication was observed in WT, *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice as revealed by southern blotting using HBV specific probes (Fig 4).

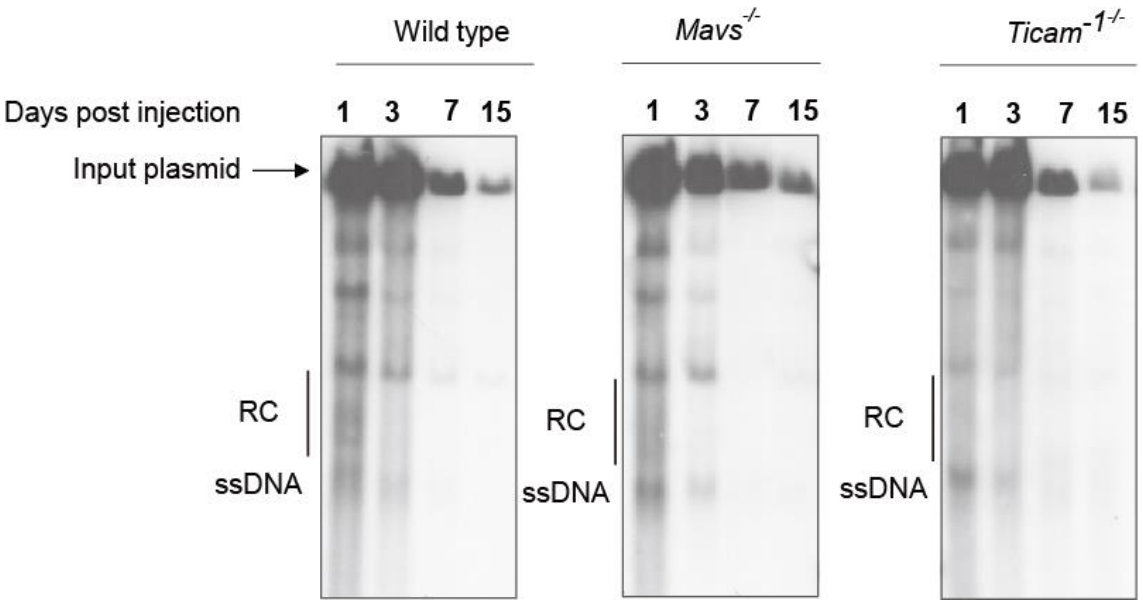
Fig.3



MAVS and/or TICAM-1 are dispensable in the regulation of HBV propagation.

HBsAg or HBV DNA were measured with sera from mice of *Mavs*$^{-/-}$ (A), *Ticam-1*$^{-/-}$ (B), *Ticam-1*$^{-/-}$*/Mavs*$^{-/-}$ (C), compared to the wild type mice. These mice were hydrodynamically injected with 50 μg of the pTER-1.4xHBV plasmid that contains full genome DNA of HBV. Mouse sera were isolated at the time points indicated. The HBsAg titers in the 1000-fold diluted serum (Left) and HBV DNA (Right) in the knockout mice (●) were compared to the wild type mice (○).Wild type mice (n=15), *Mavs*$^{-/-}$ (n=13), *Ticam1*$^{-/-}$ (n=10), *Ticam-1*$^{-/-}$*/Mavs*$^{-/-}$ (n=6). The serum HBsAg titers were determined with an enzyme immunoassay at O.D 450nm [calculated as signal over noise ratios (S/N)]. Sera HBV DNA were determined by Q-PCR and indicated as copies per milliliter. Error bars indicate SD here and in the other figures. The statistical P values were analyzed and no significant differences were observed in the (A), (B) and (C), between the wild type and these transgenic mice.

Fig.4



Southern blot analyses for detection of the HBV replicative intermediates.

No significant difference was detected in the HBV replication intermediates in the liver of the WT and *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice injected with HBV plasmid. The WT and *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice were hydrodynamically injected with 50 μg of the pTER1.4xHBV plasmid. At the indicated time point, the mice were sacrificed and liver genome was extracted. The extracted genome was analyzed with southern blot using an HBV-specific probe. The sizes corresponding to the injected DNA and the replicative intermediates are indicated on the left and right.

IRF-3/7 and IFNAR are critical factors for HBV replication regulation

To investigate the mechanisms underlying the rapid termination of HBV replication in WT mice, we examined HBV clearance in IRF-3/7-deficient mice. Activation of transcription factors including IRF-3 or IRF-7 is essential for raising immune responses including IFN production. Unlike *Mavs*$^{-/-}$, *Ticam-1*$^{-/-}$ or WT mice, mice lacking the transcription factors, IRF-3/7 had markedly high amounts of HBsAg and HBV DNA in sera (Fig. 5A). A sharp peak of HBs Ag in sera occurred in *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice on day 4 post injection. However, in spite of the high virus titer at the early stage, HBs Ag and DNA in sera were cleared with kinetics that paralleled the WT mice response and viremia was eliminated by day 11. Hence, the substantial differences in the serum viremia between WT and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice at the early stage after transfection presumably reflects the importance of the genes being expressed with these transcription factors in the suppression of the HBV replication. IRF-3 and IRF-7 are the key molecules in the suppression of HBV viremia at the early stage of post HBV injection.

Since type I IFN stimulates the IFNAR pathway to amplify type I IFN production, we hydrodynamically transfected HBV plasmid into mice lacking the gene of the type I IFN receptor (*Ifnar*$^{-/-}$) and assessed the suppression of HBV replication. *Ifnar*$^{-/-}$ mice showed markedly high titers of viral DNA and antigens in sera (Fig. 5B) similar to *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice. In addition, southern blot analysis shown that higher viral intrahepatic replication was detected in the liver of *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ and *Ifnar*$^{-/-}$mice compared to the WT mice that received the HBV full genome (Fig 6).

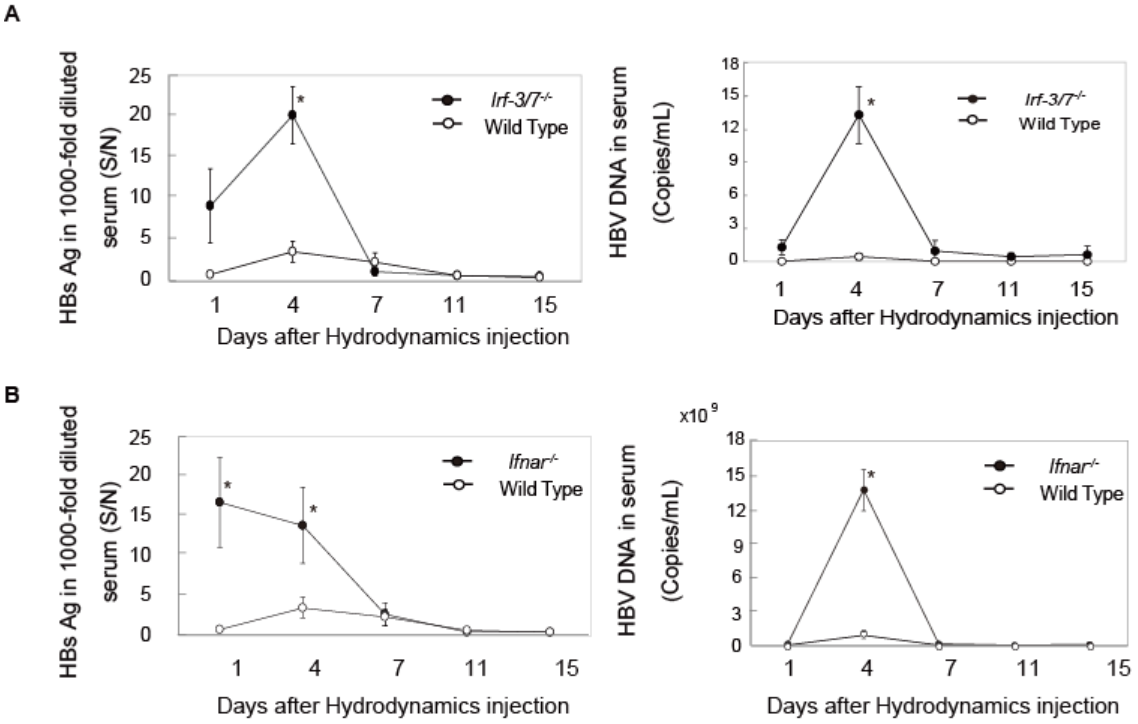
Presence of HBc Ag-positive hepatocytes was also monitored by immunohistochemical staining of liver sections from mice of each strain at day 4 post injections (Fig. 7). Data of observed HBc Ag-positive hepatocytes were in good agreement with the results on sera HBs Ag and HBV DNA: only deficiency of IRF3/7 and IFNAR resulted in a sharp increase of viremia in mice at the early stage (<day 4). Fewer HBc Ag-positive hepatocytes were observed in *Mavs*$^{-/-}$ and *Ticam1*$^{-/-}$ as well as WT mice at day 4 post injection than in *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ or *Ifnar*$^{-/-}$ mice (Fig. 7).

To gain an insight into the cytokine production in the liver in response to the HBV genome and its replication, we quantified the expression of type I IFN, IFN-ɤ, IL-7, IL-12p40, and chemokines including CXCL9, CXCL10 and CXCL11 mRNA in the livers of wild type mice receiving either the control plasmid or plasmid carrying the HBV full genome on days 1, 3, 7 and 10 after hydrodynamic injection. Replication of HBV in the liver did not cause any

significant changes in the expression of the cytokines or chemokines except the IFNs and CXCL-10 (Figure 8A-H). Similar study was carried out in wild type and *Ifnar*$^{-/-}$ mice in order to further elaborate the type I IFN production. The IFNs increased in WT mice livers receiving the HBV full genome compared to the mouse livers receiving the control plasmid (Fig 8I-K). This increase was not observed in *Ifnar*$^{-/-}$ mice lacking the interferon receptor. Although there appeared to be slight individual-to-individual differences in the apparent peaks of IFN-α induction, the result indicated that IFN-β was responsible for early suppressing HBV replication. However, the reason of the lag in the induction of IFN-γ between the WT and *Ifnar*$^{-/-}$ mice remains unclear.

Taken together, these results suggested that type I IFN was indispensable for suppressing HBV replication at the early stage after viral genome entry. Type I IFN binds to its receptor to induce intracellular antiviral proteins to disrupt HBV replication. The results however infer that intrahepatic HBV clearance at the later stage is independent of IFN.

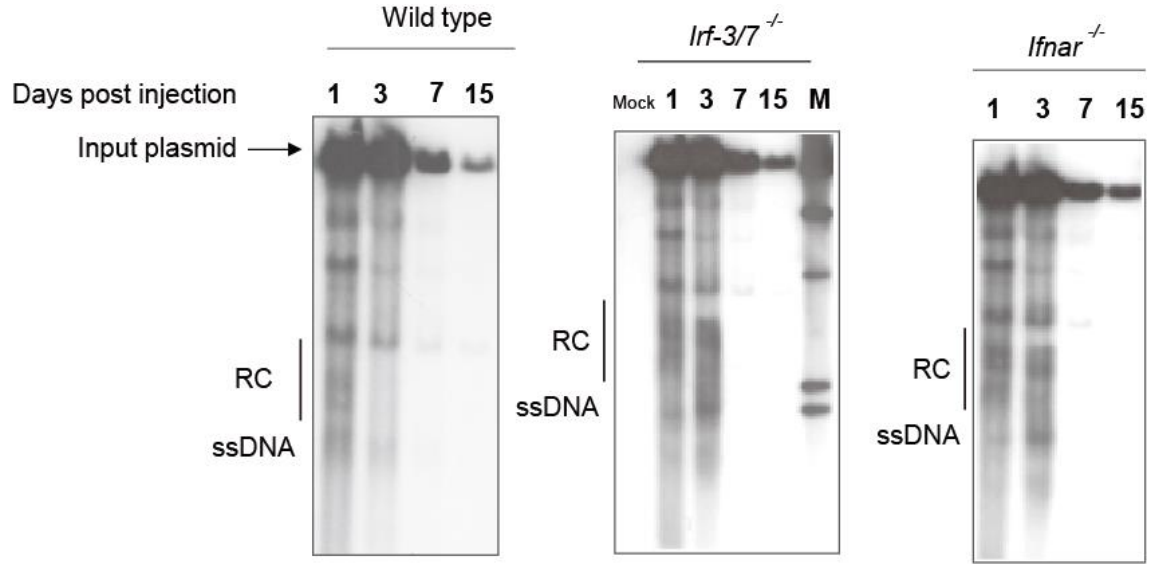
Fig.5



IFNAR and IRF-3/7 are critically associated with regulation of HBV propagation in mice livers.

HBsAg or HBV DNA were measured with sera from mice of *Irf-3*$^{-/-}$*/Irf-7*$^{-/-}$ (A) and *Ifnar*$^{-/-}$ mice (B) compared to the wild type mice. These mice were hydrodynamically injected with 50 μg of the pTER-1.4xHBV plasmid that contains full genome DNA of HBV. Mouse sera were isolated at the time points indicated. The HBsAg titers in the 1000-fold diluted serum (Left) and HBV DNA (Right) in the knockout mice (●) were compared to the wild type mice (○).Wild type mice (n=15), *Irf-3*$^{-/-}$*/Irf-7*$^{-/-}$ (n=12) and *Ifnar*$^{-/-}$ (n=13). The serum HBsAg titers were determined with an enzyme immunoassay at O.D 450nm [calculated as signal over noise ratios (S/N)]. Sera HBV DNA were determined by Q-PCR and indicated as copies per milliliter. Error bars indicate SD here and in the other figures. The statistical P values were analyzed and * $P<0.01$ in (A) and (B) are time points statistically different between WT and transgenic mice.

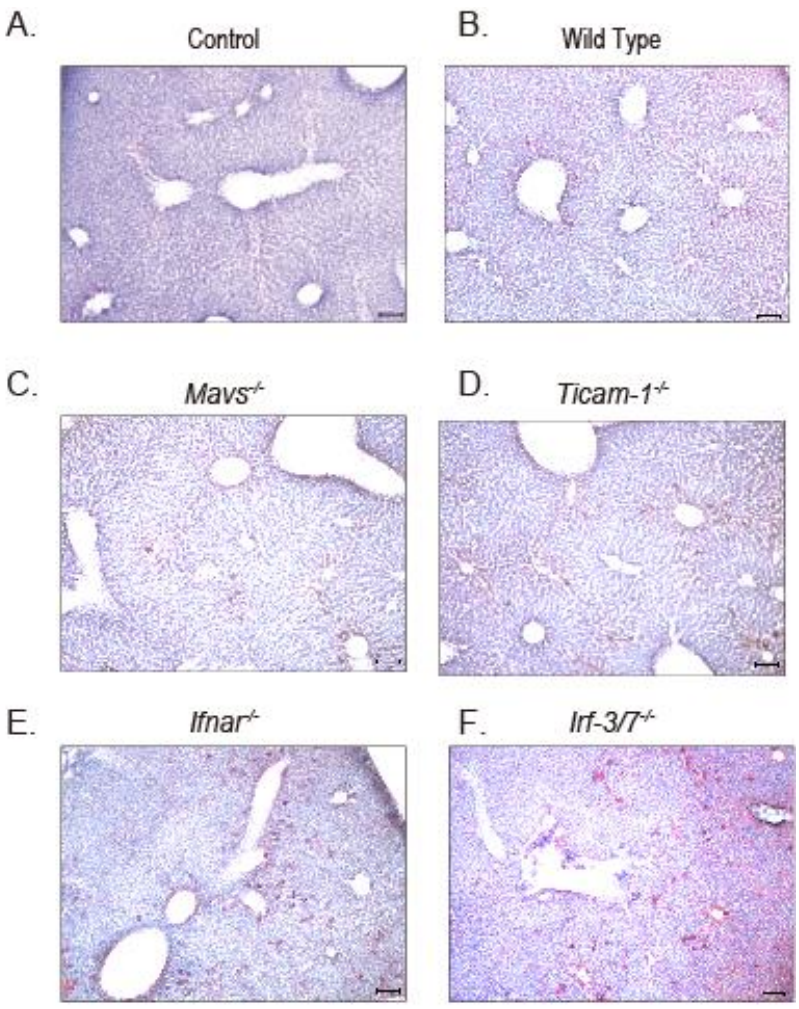
Fig.6

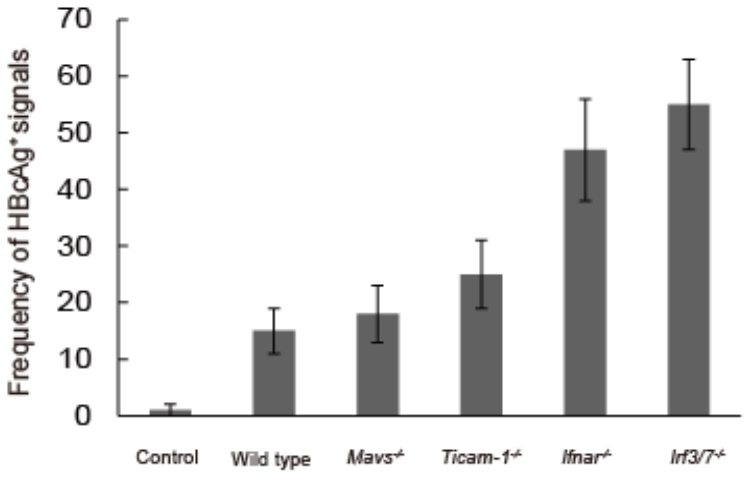


Southern blot analyses for detection of the HBV replicative intermediates.

HBV replicative intermediates were increased in *Irf-3/7*$^{-/-}$ and *Ifnar*$^{-/-}$ mice injected with HBV plasmid. The WT, *Irf-3/7*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were hydrodynamically injected with 50 μg of the pTER1.4xHBV plasmid. At the indicated time point, the mice were sacrificed and liver genome was extracted. The extracted genome was analyzed with southern blot using an HBV-specific probe. The sizes corresponding to the injected DNA and the replicative intermediates are indicated on the left and right

Fig.7

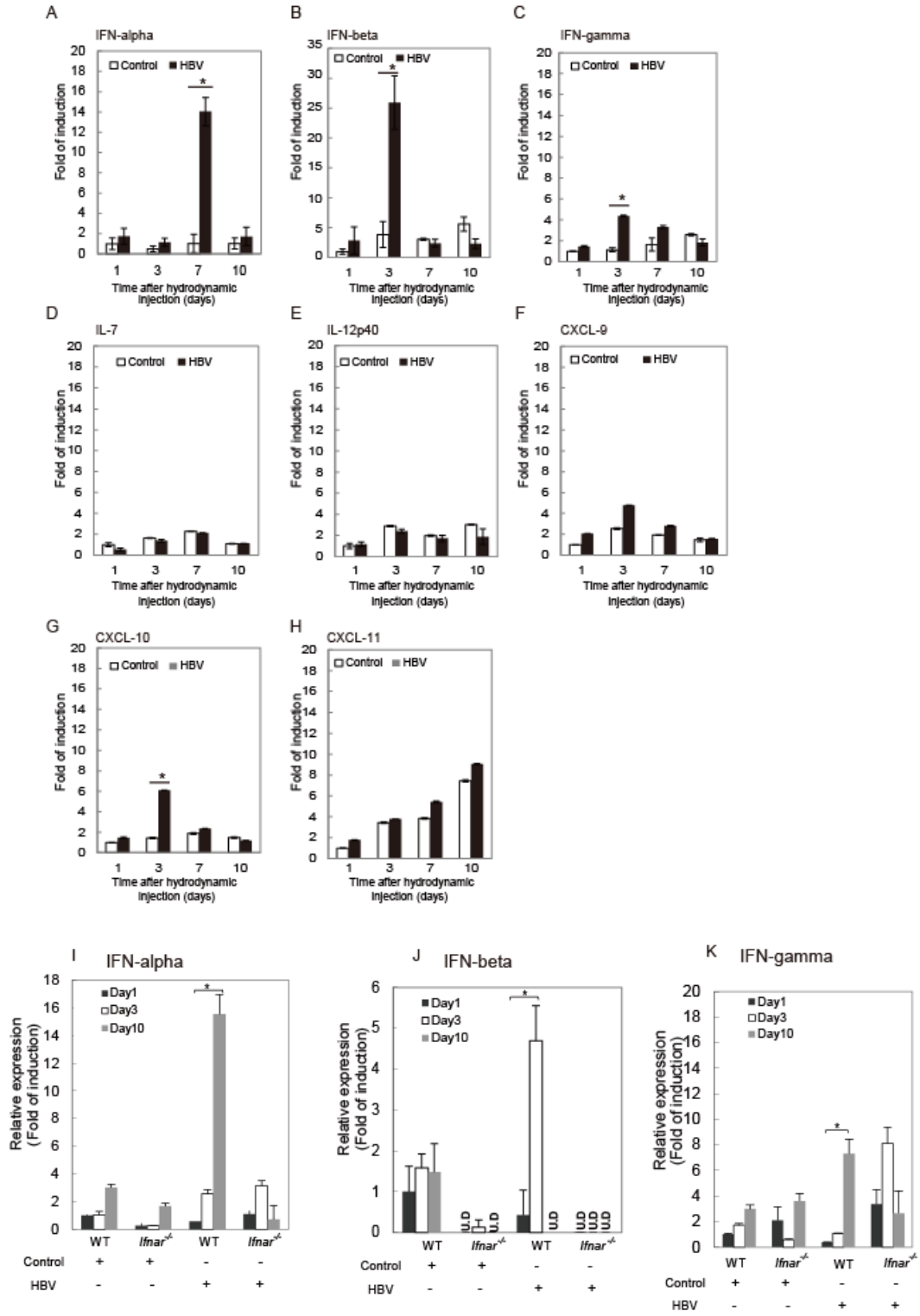




Lacking IFNAR and IRF-3/7 causes an increase of HBc antigen in the mice liver injected with the HBV replicative plasmid.

The HBc protein in the livers on day 3 post injection were visualized with immunohistochemical staining of the mice liver sections embedded in OCT using an anti-HBc antibody for HBcAg. Representative sections are shown. HBcAg-positive cells were absent in the WT mice that received only the control plasmid (A). Only marginal differences were observed in the frequency of HBcAg-positive cells between WT (B), *Mavs*$^{-/-}$ (C) and *TICAM-1*$^{-/-}$ (D) mice. Frequency of HBcAg-positive cells in the livers of the *Ifnar*$^{-/-}$ (E) and *Irf-3/7*$^{-/-}$ (F) mice are more prevalent compared to the WT. The scale bars represent 10 μm. The images are displayed at 200x magnification. Frequency of HBcAg-positive signals between the different mouse strains shown is based on the 3 images of each.

Fig.8



Type I and II IFNs expression is induced by HBV replication and lacking the type I IFN receptor (IFNAR) causes failure of these inductions.

Wild type mice were hydrodynamically injected with 50 μg of the pTER-1.4xHBV or control plasmid as described and livers were isolated on day 1, 3, 7 and 10 post injection. The expression of IFN-α (A),

IFN-β (B), IFN-γ (C), IL-7 (D), IL-12p40 (E), CXCL-9 (F), CXCL-10 (G) and CXCL-11 (H) mRNA was determined by reverse transcription followed by real-time PCR and was expressed as the fold of induction relative to the WT mice receiving the control plasmid. Induction of IFNs and CXCL-10 was observed in the mice receiving the HBV plasmid. Similar studies were conducted in the WT and *Ifnar*$^{-/-}$ mice. *Ifnar*$^{-/-}$ mice shows reduced expression of the IFNs compared to the WT. Data represent the mean of 3 mice on each strain and time point mentioned.*P<0.05

HBV clearance in a later stage by acquired immunity

Previous studies by Yang et al and other groups showed that HBV replication persists indefinitely in globally immunodeficient mice such as NOD/Scid mice hydrodynamically injected with the replication competent plasmid carrying the full genome of HBV. To investigate if the elevated viral titer in *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice on day 4 post hydrodynamic injection and intrahepatic HBV clearance were related to immune effectors including T and B cells, HBV clearance was examined in *Rag-2*$^{-/-}$ mice. The lack of V(D)J recombination in this strain results in failure to produce mature B or T lymphocytes. As shown in Fig. 9, the absence of mature T cells and B cells in the *Rag-2*$^{-/-}$ mice did not results in elevated viral titer immediately after transfection, unlike in *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice. However, *Rag-2*$^{-/-}$ mice failed to clear the input plasmid and HBV products, as sera HBs antigen and HBV DNA were detected up to day15 (Fig. 9A), by the time viral replication was terminated in all the other strains tested (Fig 9C-D). In other words, activation of the immune effectors such as B and T cell is responsible for the intrahepatic HBV clearance, their activation being independent of IFN and IRF-3/7.

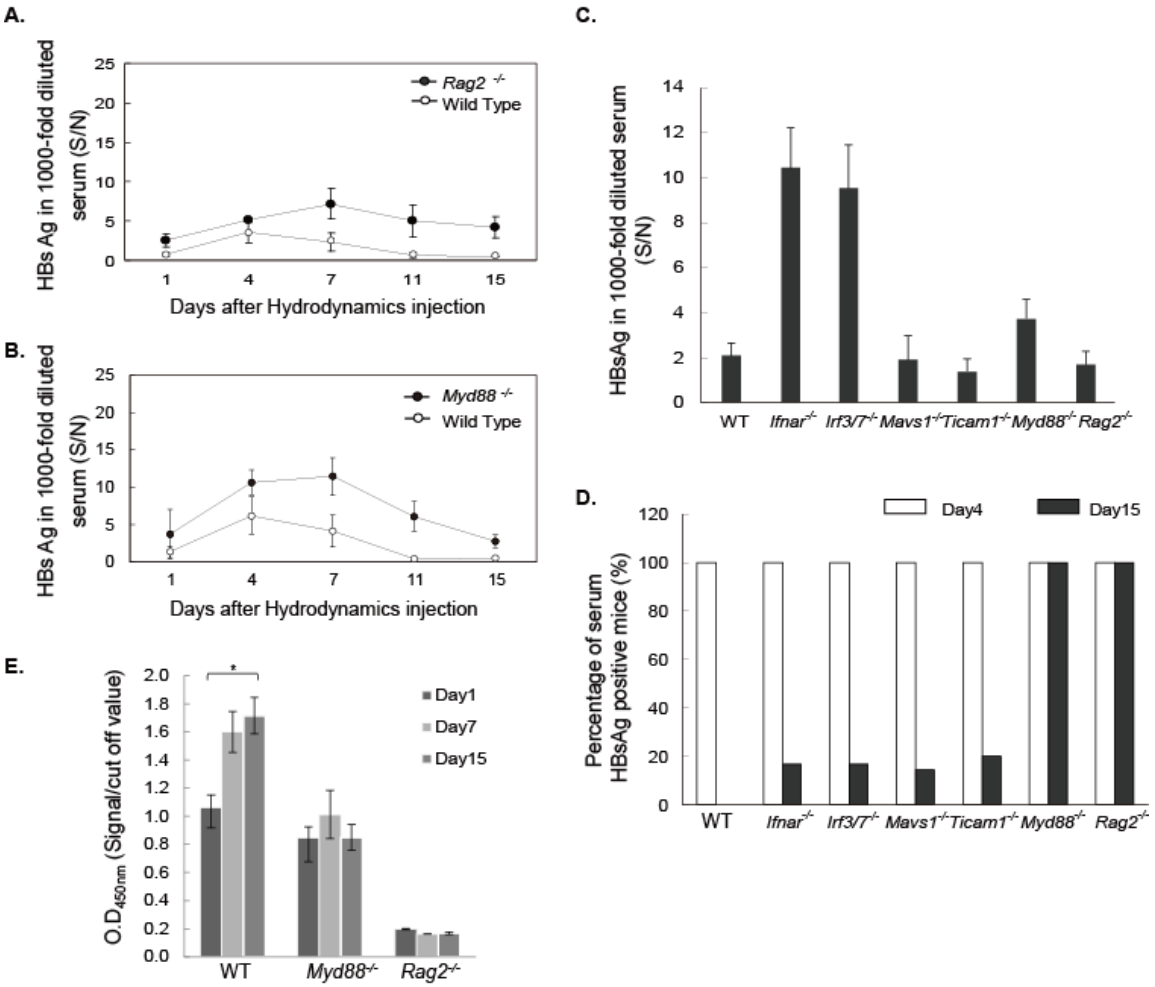
MyD88 deficiency leads to slower HBV clearance

MyD88-dependent pathway has been known leading to the production of inflammatory cytokines and is common to all TLRs, except TLR3. To examine whether a MyD88-dependent pathway is required in the intrahepatic clearance of the HBV, we monitored the serum HBs Ag in MyD88-deficient mice. As shown in Fig. 9B, an increase in sera HBs Ag in *Myd88*$^{-/-}$ mice was observed, although without particular antigenemia peaks at the early stage of transfection in *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice (Fig. 9B, C). Instead, a delay in the elimination of the HBV was observed (Fig. 9B, D). Typically, WT mice or other mouse strains lose serum HBs antigens from day 11 post injection. However, serum antigen was detectable

on day 15 in *Myd88*$^{-/-}$ mice. Delayed elimination of HBV plasmid and ssDNA in the liver was observed in Southern analysis of the liver from *Myd88*$^{-/-}$ mice compared with WT, Mavs-/-, and Ticam-1-/- mice (Fig.10).

Additionally, ELISA to determine anti-HBs Ag antibody production in mouse sera post hydrodynamic injection revealed that anti-HBs antibody was produced in WT mice from day 7 and peaked at day 15 (Fig. 9E). RAG2-deficient mice lacking mature T and B cell failed to produce any antibody and *Myd88*$^{-/-}$ mice as well had lower or nearly undetectable anti-HBs antibody in serum in comparison to the typical response of WT mice at later transfection stages. These results suggested that MyD88 and RAG2 were crucial for triggering acquired immunity against HBV in vivo.

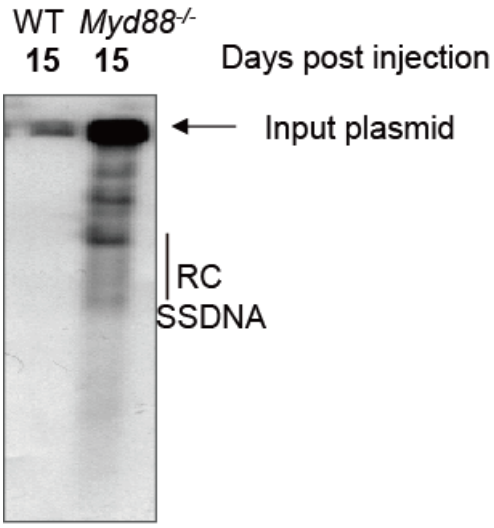
Fig 9.



Mice lacking RAG2 and MyD88 show insufficient clearance of HBV

(A-B) The Rag2-/-, Myd88-/- or wild type mice were hydrodynamically injected with 50 μg of pTER-1.4xHBV and HBsAg in the mouse sera at the time points indicated were analyzed with ELISA as described. (C) HBsAg in 1000-fold diluted serum from all the mice strains including WT, Ifnar-/-, Irf-3/7-/-, Mavs-/-, Ticam-1-/-, Myd88-/- and Rag2-/-, at day 4 after the hydrodynamics injections. Only Ifnar-/- and Irf-3/7-/- mice show a remarkable increase while moderate increase of sera HBsAg in Myd88-/- mice. (D) HBsAg persistence rates in all the mice strains receiving pTER1.4HBV were determined by the percentage of serum HBsAg positive mice on day4 (■) and day 15 (□) after the hydrodynamics injections. Serum HBsAg are found persistent only in mice deficient in MyD88 and RAG2 on day 15 as 100% of the mice from these two strains are HBsAg positive. (n=8 for each mice strain). (E) Lacking of MyD88 and RAG2 leads to the failure of the knockout mice to produce anti-HBs IgG compared to the WT mice on day15 post injection as determined by the ELISA using antigen of HBs. (n=3 for each mice strain) *p <0.05

Fig.10



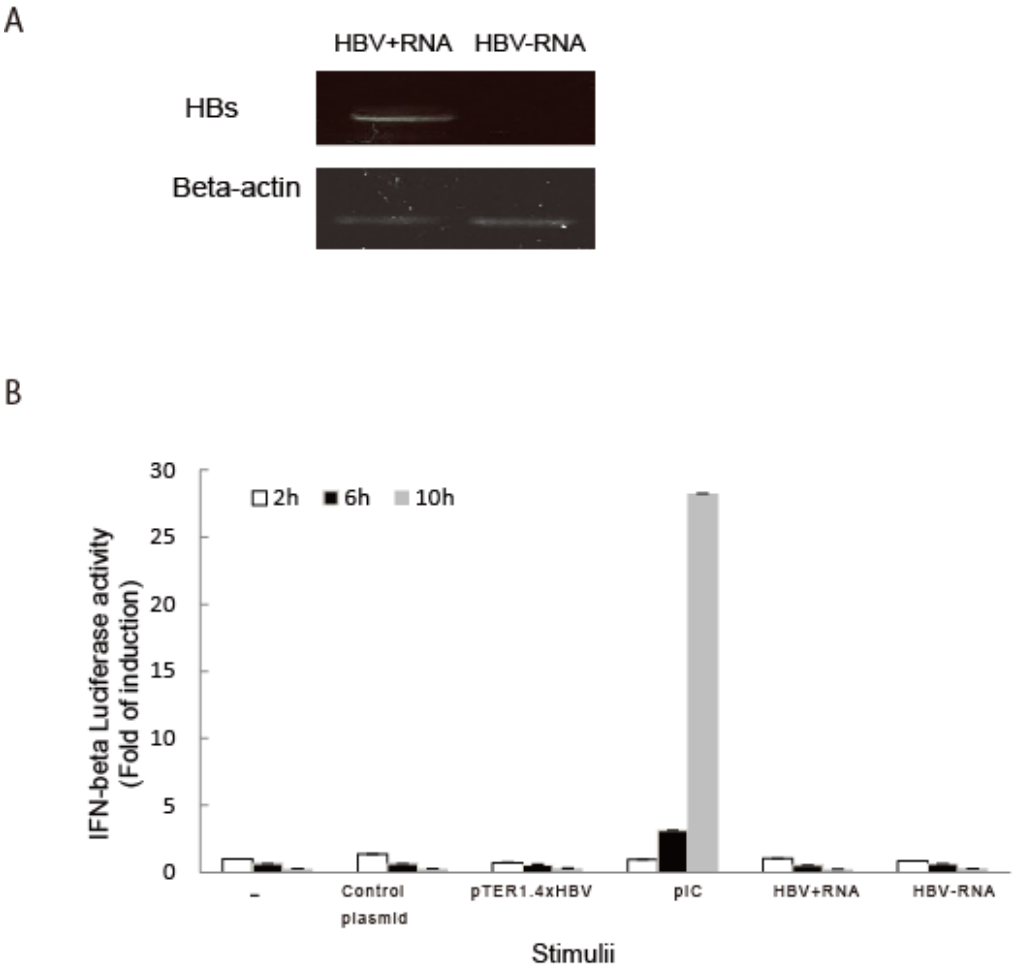
Southern blot analyses for detection of the HBV replicative intermediates.
Prolong persistency of the replicative intermediate was detected in the *Myd88*$^{-/-}$ mice but not WT. The mice livers of *Myd88*$^{-/-}$ and WT mice were isolated on day 15 after hydrodynamically injected with the HBV plasmid. Genome extracted from the livers was used for southern blot analysis with HBV-specific probe. Replicative intermediates of HBV was detected in the liver of the *Myd88*$^{-/-}$ but not WT on day 15 post injection.

A MAVS/TICAM-1-independent IFN-inducing pathway may contribute to regulation of HBV replication in the mouse hydrodynamic injection model

To further asses the possibility of HBV RNA acting as PAMPs to trigger the induction of type I IFN in hepatocytes, we transfected the immortalized hepatocytes with a plasmid containing the full genome of HBV as well as RNA containing the HBV mRNA. Along with synthetic analog of dsRNA, Poly(I:C) as a control, we determined the activity of the IFN-β promoter upon the stimulation using reporter gene assay (Fig 11). Unlike Poly(I:C), neither the full genome of HBV nor RNA induce any activity of the type I IFN promoter in the immortalized hepatocytes. Furthermore, we quantified the endogenous expression of genes including cGas, Sting and Mavs in the hepatocytes cell lines in order to access the intrinsic RNA or DNA-

sensing pathways (Fig 12). We found that the hepatocytes cell lines including those of mouse or human originated expressed extremely low amount of Sting compared to the intrinsic Mavs. However, other cell lines including the RAW 264.7 and L929 which is a murine macrophage and fibrosarcoma cell line respectively has higher endogenous expression of Sting rather than Mavs. Taken together, we hypothesized that HBV RNA is unlike to be the viral component that are recognized by the pattern recognition receptors that trigger the antiviral response. The answer will be most likely found among the growing family of PRR able to discriminate intracellular pathogen DNA, for instance cGAS or STING. However, it is clear that much additional work will be required before a complete understanding of how the intracellular pathogen of HBV DNA are sensed to trigger the innate immune responses.

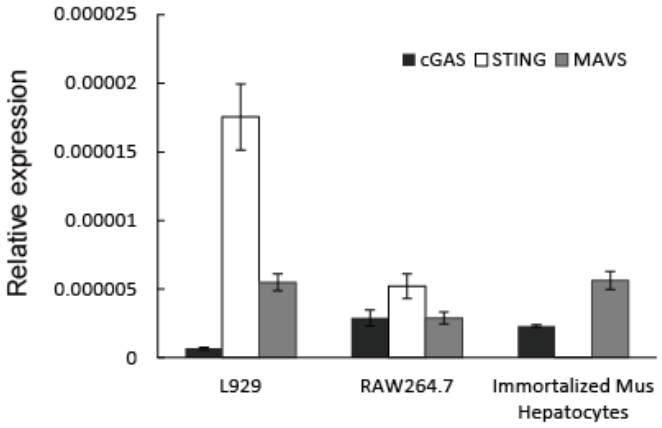
Fig.11

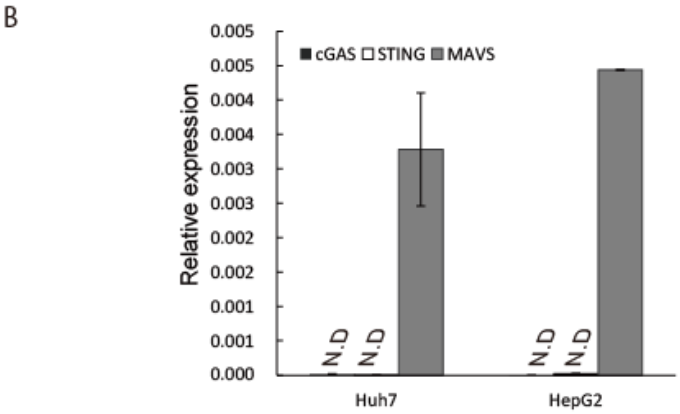


HBV RNA containing stimuli and HBV full genome failed to induce IFN-β in the immortalized mouse hepatocytes.

(A) RT-PCR analysis for detection of HBV RNA. RNA containing the HBV RNA (HBV+RNA) was generated by isolating the total RNA from the immortalized mouse hepatocytes after 12h transfected with pTER1.4xHBV. Total RNA isolated from the immortalized mouse hepatocytes transfected with only the control plasmid was used as control (HBV-RNA). The HBV RNA was confirmed with RT-PCR using a primer set for the HBs mRNA and mouse β-actin respectively.(B) Reporter assay for detection of IFN-β in mouse hepatocytes. The control plasmid, pTER1.4xHBV, HBV+RNA, HBV-RNA and PIC were transfected into the immortalized mouse hepatocytes as stimuli. The IFN-β promoter activities as determined at 2h, 6h and 10h post transfection by a dual-luciferase reporter assay kit shows immortalized mouse hepatocytes failed to response to these stimuli except PIC. Data represent 3 independent experiments.

Fig.12





Immortalized hepatocytes have lower endogenous expression of the adaptor molecules for DNA-sensing pathway than the RNA sensing pathways.

(A) Endogenous mRNA levels of cGAS, STING and MAVS in mouse cells. Total RNA was isolated from the mouse cell lines including RAW 264.7 (murine macrophage cell line), L929 (murine fibrosarcoma cell line) and immortalized mouse hepatocytes. Endogenous expression of cGAS, STING and MAVS in these cell lines was quantified with qPCR using the specific primer sets. Data shown is the relative expression of these genes to mouse β-actin that was used an internal control. The immortalized mouse hepatocytes are lacking the endogenous expression of STING compared to MAVS. (B) Endogenous mRNA levels of cGAS, STING and MAVS in Huh7 and HepG2 hepatocarcinoma cell lines. The mRNA expression was quantified using the total RNA isolated. Huh7 and HepG hepatocarcinoma as well show lower endogenous expression of STING and cGAS than MAVS. Data represent 3 independent experiments. ND; not detected.

5. Discussion

In the present study, several different knockout mice were analyzed in an attempt to define the mechanism of innate immunity against HBV in vivo. The evidence we obtained indicated that viral replication was not affected by MAVS or TICAM-1 knockout, but absence of IRF3 or IRF7 transcription factors, as well as the IFN receptor, had an adverse effect on the inhibition of HBV replication. The results herein demonstrated that TICAM-1 and MAVS pathways were not required in either suppressing the virus replication or intrahepatic clearance of HBV replicative plasmid *in vivo*.

Although a DNA virus, HBV has the unique feature of replicating via an RNA proviral intermediate that is copied into DNA. Thus, defining the virus component, either HBV DNA or RNA that triggers the antiviral response is crucial to understand the immune mechanisms that are responsible for eliminating HBV during infection. HBV RNA has been suggested as the putative pathogen-associated molecular pattern (PAMP) of HBV in a few reports previously [25,26,31]. HBx or HBs inhibit IFN-β induction followed by activation of TLR3 or RIG-I pathways with polyI:C or SeV, respectively. However, these findings must be interpreted with caution, as polyI:C and SeV are heterologous inducers for evaluating either the TLR3 or RIG-I pathway[25,26]. No definitive conclusion on activation of the TLR3 or RIG-I pathway by HBV RNA in vivo has been reported yet.

Viral RNA is recognized largely by RIG-I or MDA5 in cytosol of infected cells and by TLR3 or TLR7/8 in endosome of other non-infected cells. These RNA sensors require MAVS, TICAM-1 or MyD88 as adaptor proteins to induce type I IFN. On the other hands, cytoplasmic DNA is recognized by DNA sensors including DAI, IFI16, RIG-I, DHX9 (helicase) and cGAS. STING is the only adaptor for all IFN-inducing DNA sensors in mouse cells reported so far, although some of these sensors are reported to induce type I IFN via MAVS in human cells. These adaptors; TICAM-1, MAVS and STING, all link to activation of IRF-3/7 which act as transcription factors that induce activation of the type I IFN promoter during viral infections. Involvement of different pathways in the induction of type I IFN is critically depends on the virus species and cell type. Cell-type specific contribution of other sensors, including DEAD box helicases might occur in some cases of infection. However, in hepatocytes no contribution of other sensors except RIG-I/MDA5 and TLR3 has been reported so far.

Using the murine hydrodynamic injection model, we found that mice deficient in IRF-3 and IRF-7 or IFNAR do not inhibit HBV replication as effectively as their wild type

counterparts and result in elevated HBV titers in the mice sera and livers. These findings imply that type I IFN acting on IFNAR was indispensable for evoking anti-HBV protective responses although such hypothesis is in disagreement with previous findings that HBV does not induce detectable changes in type I IFN expression during the early weeks of infection[32]. There are a few possibilities of how type I IFN is produced in the mouse receiving HBV template plasmid. One of them is that HBV could be recognized by pathways that do not link to MAVS or TICAM-1 and facilitate IFN production in the cytoplasm. For instance, STING-dependent signaling lead to type I IFN induction and it has been shown that this can be MAVS and TICAM-1 independent. Notably, STING-dependent signaling is especially associated with DNA-mediated induction of type I IFN via IRF-3/7 and genomic DNA is an important part of HBV replication. It would be interesting to clarify such hypothesis using Sting-/- mice in the near future.

To elucidate the molecular pattern which triggers type I IFN induction, we transfected either HBV DNA or RNA into the immortalized hepatocytes. To our surprise, we are unable to detect significant IFN-β induction with neither HBV replicative DNA nor HBV RNA. As we looked into the possible reasons accounted for the lack of innate immune responses against HBV in hepatocytes, we found that the endogenous expression of STING in hepatocytes cell lines including HepG2 and immortalized mouse hepatocytes is extremely low compared to other cell lines like macrophages or dendritic cells, hence, suggesting that STING-dependent signaling might play a crucial role in inducing type I IFN in response to HBV. The produced IFN in turn activates the IFNAR pathway. Since there are various cells populations in the liver that express IFNAR and therefore subsequently initiate a natural signaling cascade for amplification of IFN production via the Jak-STAT pathway.

Another possible way for HBV to induce IFN is via the HBV-stimulated nonparenchymal or resident myeloid cells. Even though there has been no report suggesting that HBV substantially infects pDC. *Isogawa et al.* demonstrated that freshly isolated CD11c+ cells of intrahepatic myeloid cells rather than the hepatocytes expressed TLRs including TLR2, 3 and 9 [33]. Therefore, resident myeloid cells might induce IFN to further prevent the spread of HBV by activating the IFNAR pathway in bystander cells or hepatocytes.

Although *Myd88*$^{-/-}$ mice receiving HBV DNA injection did not exhibit significantly high virus titers in the early phase unlike those observed in *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$*Irf-7*$^{-/-}$ mice, interestingly MyD88 is required for the intrahepatic clearance of the HBV replicative template. The fact that the transcriptional template persists in the absence of MyD88 suggests that MyD88 may play a pivotal role in intrahepatic HBV clearance in the mice model.

Notably, MyD88 is the adaptor molecule for TLR7 and 9 in pDCs [18]. Deficiency of MyD88 in pDC may result in failure in inducing acquired immunity for HBV. Our findings show that HBV-specific antibodies are being efficiently produced in WT but not *Myd88*$^{-/-}$ mice. In addition, the number of pDC has been previously reported to be reduced in vivo during several systemic viral infections including HBV [34]. In one of the most recent reports, Shunjuan et al. showed that HBV-derived CpG induces potent IFN-α production by human pDCs, which may partially explain how pDC interact with HBV in infection [35]. However, the cause of weak participation in the early response of IFN induction in *Myd88*$^{-/-}$ mice remains to be determined.

Recombinant IFN-α is a standard treatment for chronic HBV patients. Nevertheless, direct treatment with IFN yields only about 30% of improvement in HBV patients and little are known about why most chronic HBV patients do not respond to IFN therapy [36]. As demonstrated in our study, virus persistency can be independent of the type I IFN-inducing system. This observation leads to the suggestion that type I IFN is indispensable for inducing antiviral molecules to control viral replication and spread before the onset of more specific and powerful adaptive immune responses. This appeared to be factual at least in our knockout mouse models as virus titers were highly elevated in *Ifnar*$^{-/-}$ mice at the initial few days post injection, conversely, type I IFN did not have any influential effects on clearance of the HBV template in the later stages. Such observation coincide with the latest study conducted in patients of chronic HBV infection by Tan et.al whereby IFN-α treatment were shown to modulates innate immune parameters in the patients but without any detectable effect on the HBV specific adaptive immunity [37]. The missing link between induction of type I IFN and anti-HBV cellular effectors needs to be further investigated in the mouse models, including the mechanism of MyD88 participation in activation of the cellular immune response during infection. Elucidating molecular mechanisms connecting between innate pattern sensing and evoking cellular effectors may provide a reasonable explanation on the failure of IFN-treatment in HBV infection.

Collectively, our study validates the use of hydrodynamic transfection method in mimicking the acute HBV infection in the mouse models and demonstrated the host–virus relationship during HBV infection in many aspects. Since HBV infectious models with immunologically well-defined laboratory animals do not exist, the result presented in this study herein provides an insight into dispensability of RNA sensors for induction of IFN by HBV RNA and the complexity of innate and adaptive immunity during HBV clearance.

Chapter 2

ISG20: IFN inducible effector that suppresses HBV replication

1. Introduction

Interaction between viral replication and host immunity

The outcome and progression of HBV infection are determined by the interaction between viruses and host immune system. As we have demonstrated in our study earlier that mature T and B cells as well as the adaptor molecule MyD88 are required for achieving successful clearance of HBV DNA template, whilst type I IFN plays an indispensable role in suppressing the propagation of the viral replication in the liver. It is also clear that the balance between viral replication and host immunity determines the course of viral infection. T lymphocytes and humoral responses specific of HBV are indispensable to clear the virus, lack of these effectors of the adaptive immune response might cause chronic liver disease as the host fail to eliminate the virus DNA template. However, it is crucial that before the onset of the adaptive immune response, the host innate immunity also plays a critical role in the control of the viral replication and central to this cellular response is the secretion of interferons (IFN-α/β).

Interferon and HBV

The interferon (IFN) system that being regulated tightly in a constrict fashion but activated rapidly is a major innate immune response towards viral infection. The secreted IFNs engaged through cell surface IFN receptor, leading to the induction of hundreds of IFN stimulated genes (ISGs) via the JAK-STAT pathway. Although substantial evidence revealed that HBV has developed strategies in antagonizing the host innate defenses, treatment of HBV with IFN to a certain extent has been shown to suppress viral replication in laboratory studies as well as clinical treatments.

The hall mark of the innate immune response is production of IFNs while it was generally accepted that IFNs can potentially inhibit HBV replication. Some investigations have postulated that the IFNs produced by Poly I:C exerted anti-HBV effects at the level of pg-RNA containing capsids[38]. The result obtained is compatible with the duck IFN-α selectively eliminates pgRNA-containing capsids from HBV-infected primary duck hepatocytes.

A few studies in the recent years also revealed a few host molecules that might play a role in suppressing the HBV viral replication, namely the human MxA[39], IDO, TRIM22 and APOBEC3 (A3) cytidine deaminases[40]. These molecules are generally up-regulated with the

interferon induction and shown to inhibit HBV replication in somewhat different manners. Human MxA, an interferon-inducible cytoplasmic dynamin-like GTPase, possesses antiviral activity against multiple RNA viruses. It has been demonstrated that in HepG2.2.15 cells, MxA GTPase independently suppressed the production of hepatitis B surface antigen and HBV DNA without changing the level of hepatitis B core antigen (HBcAg) and the distribution of HBV mRNA. While APOBEC3 exhibit the ability to hyperedit HBV DNA and inhibit HBV replication. It was suggested that A3 deaminases are incorporated into nascent HBV capsids where they cleave amino groups from cytidine bases converting them to uracil in newly synthesized DNA following reverse transcription of pregenomic RNA[40].

Despite IFN may prompt intracellular antiviral responses by targeting multistep in the HBV life cycle; the mechanisms of the anti-HBV effect of the type I IFN are not completely explored. Thus it has been the interest many to identify the host proteins that are able to hamper HBV replication under the induction of IFNs.

	cccDNA minichromosome	Promoter and enhancer	Posttranscriptional control	Nucleocapsid formation	others
IFNs	Modyfying the composition of HBV minichromosome [41,42] Suppressing cccDNA transription[41,42] Acceleraating cccDNA decay[42]	Suppressing HBV ENI and ENII activity [43,44]	Stimulating HBV RNA degradation[45,46]	Inhibiting nucleocapsid formation[47] Accelerating nucleocapsid degradation[48]	
APOBEC	Blocking nucleocapsid maturation	Suppressing HBV S promoter activity[49]			Editing HBV genome[50]
MxA			Inhibiting the nuclear export of HBV RNAs[40]	Blocking HBV nucleocapsid formation[39]	
MyD88			Accelerating the decay of HBV pgRNA[51] Inhibiting the nuclear export of the HBV pre-S/S RNAs[52]		
IDO			Inhibiting translation through tryptophan depletion[53]		
TRIM22		Inhibiting HBV core promoter activity[54]			
IFITs		Inhbiting HBV S promoter activity[55]	Inhibiting HBV replication at posttranscriptional steps		
ZAPs			Inhibiting HBV replication at posttranscriptional steps[56]		

Table: summary of the anti-hepatitis B effects of interferon and interferon stimulated genes

Interferon-inducible-gene-20kb (ISG20)

ISG20 is a 3'-5' exonuclease whose gene is transcriptionally induced by both type I and type II and III IFN (unpublished data). Its induction by IFN is strictly dependent upon the activation and binding of IRF1 to a specific ISRE on the ISG20 promoter. It has recently emerged as a second IFN-regulated RNase that inhibits RNA virus replication. Numerous studies using microarrays approaches strongly proposed that ISG20 may be a major effector of the innate immune response. In the absence of IFN treatment, ISG20 overexpressing HeLa cells showed resistance to infections by vesicular stomatitis virus (VSV), influenza virus and encephalomyocarditis virus as well as the hepatitis C virus (HCV), which is all RNA viruses [57]. It has been clearly demonstrated that ISG20 might represent a novel antiviral pathway in the mechanism of IFN action.

Interferon-inducible-gene-20kb (ISG20) and HBV

The various experiments have suggested that the antiviral activity of ISG20 is mediated by its exonuclease activity. It is conceivable that ISG20 might affect viral development by degrading the viral RNA. However, surprisingly little is known about the contribution of ISG20 in the antiviral activity against DNA viruses.

In 2004, *Wieland et al* conducted a gene chip analysis on the Chimpanzees acutely infected with HBV. In this study, they have identified ISG20 as one of the viral clearance associated genes for HBV[58]. Furthermore, *Hao et al* (2008) has published some preliminary results of the effect of human ISG20 on HBV[54]. Later on, *Lu et al* (2013) published their work on the differential expression of ISG20 in chronic hepatitis B patients and relation to interferon-α therapy response[59]. These studies greatly strengthen the hypothesis that ISG20 might play a role in the HBV antiviral pathway. However, additional studies are definitely needed to clarify the biological contribution of ISG20 in the antiviral processes.

2. Aim of the current study

Interferon is an approved medication for chronic hepatitis B virus patients. The current study was set out for the screening of interferon stimulated genes for their potential antiviral activity against HBV replication.

ISG20 is an IFN-induced antiviral exoribonuleause that acts on single-stranded RNA

and also with minor activity towards DNA. Although numerous reports reveal the antiviral activity of ISG2 against the RNA virus, little has been reported on the DNA viruses. Since mRNA intermediate is an essential component for DNA viruses to fulfill their life cycle, we could relate the possibility that ISG20 might specifically target certain DNA viruses encoded mRNA for degradation to inhibit virus replication.

Herein, we would like to further investigate the role of ISG20 in suppressing the HBV replication as one of the prominent IFN-inducible molecule. Elucidation of the molecular mechanisms of the intricate cellular antiviral response mediated by ISG20 will shed light on cell biology and virus-host interaction during viral pathogenesis, and potentially lead to the development of novel therapeutic that exploit the host molecules to control the viral infection.

3. Materials and Methods

Animal studies and hydrodynamic injection of HBV full genome plasmid

All mice were backcrossed with C57BL/6 mice more than seven times before use. *Ifnar*$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). Female C57BL/6J mice were purchased from Japan Clea (Tokyo) and used at 7–9 weeks of age. All mice were maintained under specific pathogen-free conditions in the Animal Facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan). A total of 50μg of the plasmid containing the HBV full genome was injected into the tail vein of 7-9 week-old mice in a volume of 2.0 ml TransIT-QR hydrodynamic delivery solution (Mirus, USA). The total volume was delivered within 3-8 seconds. Plasmid DNA was prepared by using an EndoFree plasmid system (Qiagen, Germany) according to manufacturer's instructions.

Cell cultures and plasmid

Human hepatocytes-derived HepG2 and Huh7 cells were obtained from the ATCC and maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The immortalized mouse hepatocyte cells from the wild type and *Ifnar*$^{-/-}$ mice were established in our laboratory as reported previously. The HBV-T23 cells, human hepatocytes derived cell line that constitutively producing the HBV virions were cultured in the DMEM with the addition of hygromycin B to maintain the genomic incorporated HBV plasmid. Recombinant mouse IFN-α was purchased from Sigma-Aldrich. The pTER1.4xHBV plasmid containing 1.4 genome length sequences of HBV that previously shown to produce a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers was used in this study. The human

Isg20 was cloned into the plasmid with a CMV-IE promoter using the specific primers. Disruption of the individual Exonuclease domain in the *Isg20* was carried out using QuickChange II site-Directed mutagenesis kit (Agilent). All of the above wild type and mutant Isg20 mutant genes have an N-terminal Flag-tag sequence in the expression vectors.

Cell transfection

Cells in each well of the 24-well-plate were transfected with 1 μg of plasmid using Lipofectamine 2000 (Life technologies) by following the manufacturer's directions. Transfected cells were harvested at the indicated time points.

Northern blot assay

Viral RNA was isolated and detected with specific DIG-labeled probe as described previously. In brief, the total cellular RNA was isolated with TRIZOL and 20μg of the isolated total cellular RNA was resolved in a 1.5% agarose gel containing 2.2 M formaldehyde and transferred onto IMMOBILON NY+ charged nylon membrane (Milipore) and detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer.

Southern blot assay

Viral DNA was isolated from intracellular viral capsids and detected with specific DIG-labeled probe as described previously. In brief, to isolate the viral DNA, homogenized mouse livers or cell transfected with the HBV plasmid were subjected to an overnight sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. 20μg of the isolated DNA was separated in a 1% agarose gel, transferred onto IMMOBILON NY+ charged nylon membrane (Milipore) and detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit.

Western blot assay

Cells were lysed with RIPA buffer and separated on a 10% SDS-PAGE. Proteins were transferred onto Immobilon PVDF-FL membrane (Milipore). The membranes were blocked with 3% skim milk and probled with antibodies against FLAG-tag, HBc antigen, or β-actin. Bound antibodies were revealed with HRP-bound secondary antibodies. The immunoblotting signals were visualized and quantified.

Immunofluorescence

The Huh7 cells were transfected with the plasmid containing ISG20 and HBV sequences for 48 h and followed by fixation with 2% paraformaldehyde and permeablization of the cell membrane with 0.1% Triton X-100. Cells were then immunostained with anti-FLAG or anti-HBc antibodies and the bound antibodies were visualized by Alexa Fluor 488 goat anti-mouse IgG. Nucleic were counterstained with DAPI. Cell were imagined with a Nikon fluroscent microscope and photographed with a charge-coupled device camera.

ELISA of HBs antigen

Concentration of HBsAg in the mice serum or culture supernatant was quantified by sandwich ELISA in commercial ELISA kits following the manufacturer's protocol. (XpressBio, USA) and the reporting unit is ng/mL at O.D 450nm.

Quantitative RT-PCR

Dnase-I treated total cellular RNA was used to generate cDNA by SuperScript III Reverse Transcriptase (Life Technologies). Real-time PCR was performed with SYBR Green Master (Roche) and the LightCycler 480 System (Roche) by using HBV and Isg20 specific primers. The gene expression data were normalized to β-acting from the same samples.

4. Results

Identification of novel ISG as new antiviral

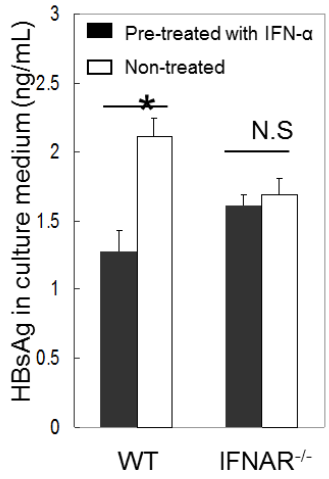
We examined the effectiveness of IFN to control the HBV replication by pre-treating the immortalized hepatocytes derived from the wild type and *Ifnar*$^{-/-}$ mice with 1000 unit of recombinant IFN-α before introducing the plasmid containing the replicative full genome of HBV. HBs antigen secreted into the supernatant was analyzed with ELISA. As shown in Fig.1, the intracellular antiviral response on HBV elicited by the IFN-α pre-treatment was observed in the WT hepatocytes but not the immortalized hepatocytes lacking the IFN receptor (Fig.1a).

Since type I IFNs act on target cells by inducing an array of interferon stimulated genes (ISG) that limit viral infection, we attempted at screening the ISGs for their potential antiviral activity against HBV replication. Among the 6 candidates we picked up from the gene array, we found that overexpression of *ISG20* led to a significant reduction of HBs antigen secreted from the immortalized hepatocytes (Fig. 1b). In addition, suppression of the

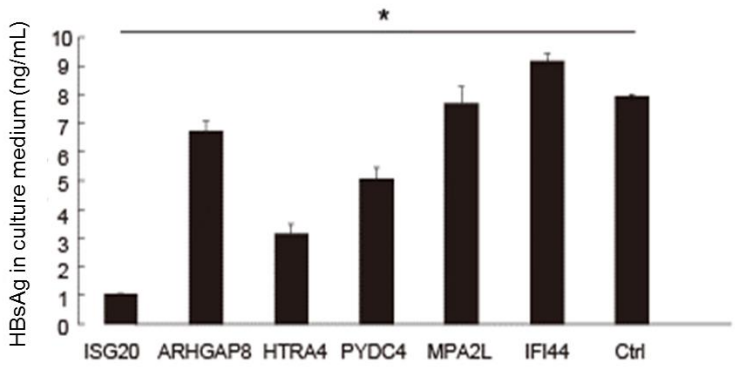
intracellular HBV DNA replication was also observed. This was achieved primarily through reducing the steady state levels of viral pg-RNA that acts as the template for the HBV DNA synthesis.

Figure 1

A.



B.



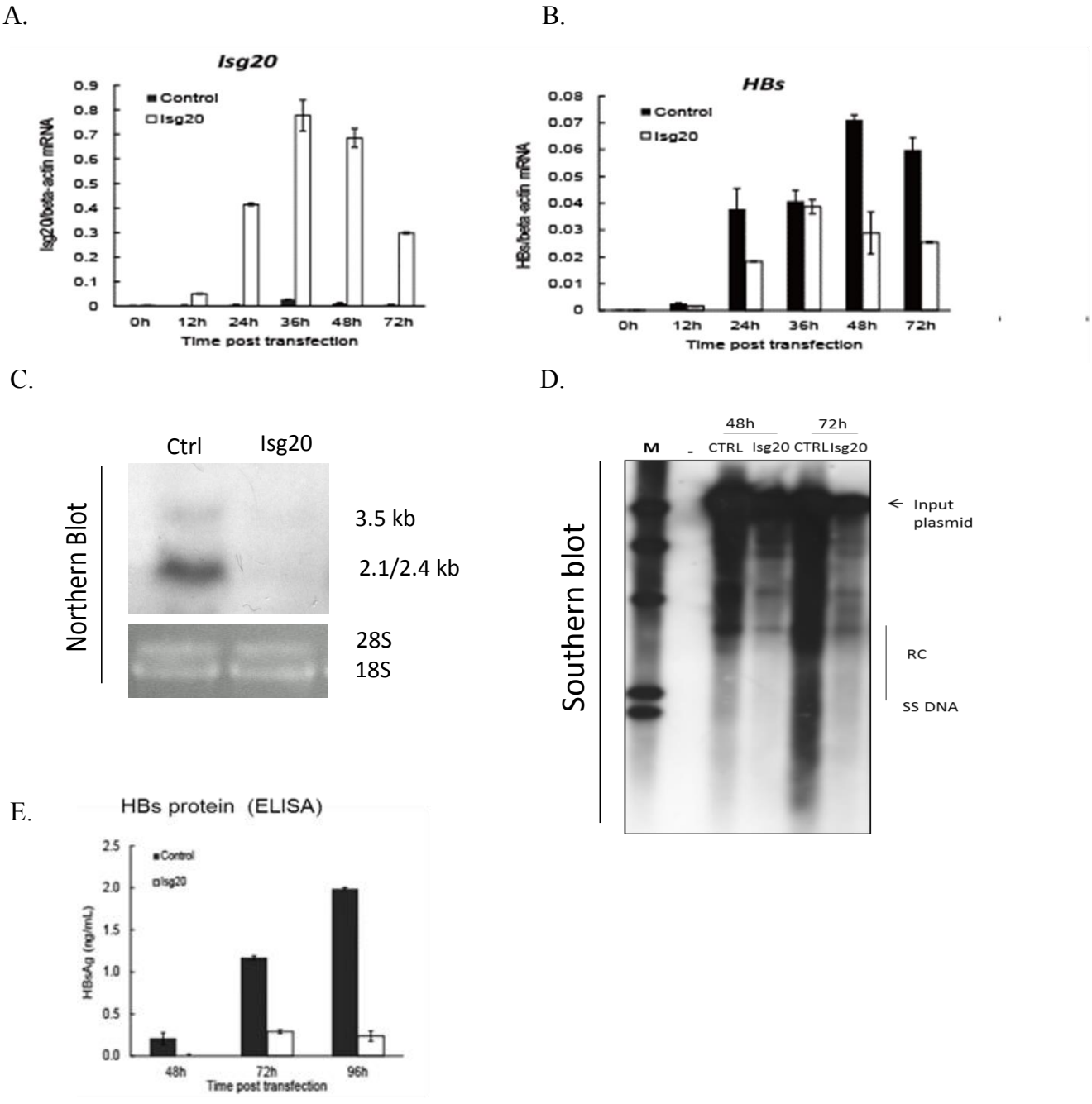
Pre-treatment of IFN-α is crucial in suppressing HBV replication and ISG20 is the novel IFN-inducible molecule in inhibiting the HBV. (A)The immortalized hepatocytes derived from the wild type and *Ifnar*$^{-/-}$ mice were pre-treated with 1000 unit of recombinant mus-IFN-α for 12 hours before transfected with the pTER1.4xHBV. HBs antigen in the culture supernatant after 72h post transfection was analyzed with ELISA. (B) The 6 candidate interferon-stimulated genes were cloned into the expression vector respectively and co-transfected with the pTER1.4xHBV into the hepatocytes derived cells, Huh7 to screen for the anti-HBV activity. HBs antigen in the culture supernatant after 72h post transfection was analyzed with ELISA. Data represent 3 independent experiments. *p <0.05

ISG20 suppressed HBV replication in the hepatocyte derived cell lines

To investigate whether the inhibitory effect of ISG20 on HBV is depending on a cell-type specific host factor, ISG20 was ectopically expressed in the human hepatoma Huh7. Overexpression of ISG20 also efficiently reduced the HBV viral replication in hepatoma Huh7 cells at the viral nucleic acid level including the RNA and DNA as shown in figure 2 (A-D), suggesting that the cell-type specific host factor is not required. This was achieved primarily through reducing the steady state levels of viral pgRNA (Figue 2C), which is the template for HBV DNA synthesis. In addition, the level of the 2.4kb and 2.1kb HBV mRNA that share 100% sequence identity of 3' portion of pg-RNA were also decrease upon ISG20 expression, along with the pgRNA reduction. Since ISG20 has been demonstrated to be a 3' to 5' exonuclease in vitro with specificity for single stranded RNA and to a lesser extent for DNA, we need to rule out the possibility that ISG20 may degrade the transfected HBV plasmid. As shown in the Fig 2D, while ISG20 expression reduced the levels of HBV DNA replication, the co-transfected HBV plasmid signal revealed by PstI digestion and DNA hybridization was almost similar to the control. In addition, overexpression of *Isg20* reduced viral RNA and DNA in the HBV stable cell line HBV-T23, in which HBV RNA is transcribed from an integrated transgene. Collectively, these observations suggested that ISG20 expression does not alter the amount or stability of HBV RNA transcription templates. Consequentially, the down-regulated of HBV RNA and DNA mediated by the ISG20 during the viral replication leads to a great extent of reduction in the HBs antigen detected in the culture supernatant (Fig. 2E). These results suggested that ISG20 antiviral function is a crucial host factor in controlling the intracellular HBV life cycles.

We also observed the antiviral effect of ISG20 against HBV by confocal immunofluorescence microscopy. Cellular distribution of the HBV core antigen was stained with the anti-HBc antibodies while the FLAG-tagged ISG20 was monitored with the corresponding antibody 48h post transfection. As shown in Fig.3 when the Huh7 cells were transfected with the ISG20 expression plasmid, the FLAG-positive cells that expressed the FLAG-tagged ISG20 did not express detectable HBc antigen, whereas the FLAG-negative cells exhibited a higher level of HBc antigen. These data also clearly demonstrated the function of ISG20 in suppressing HBV replication.

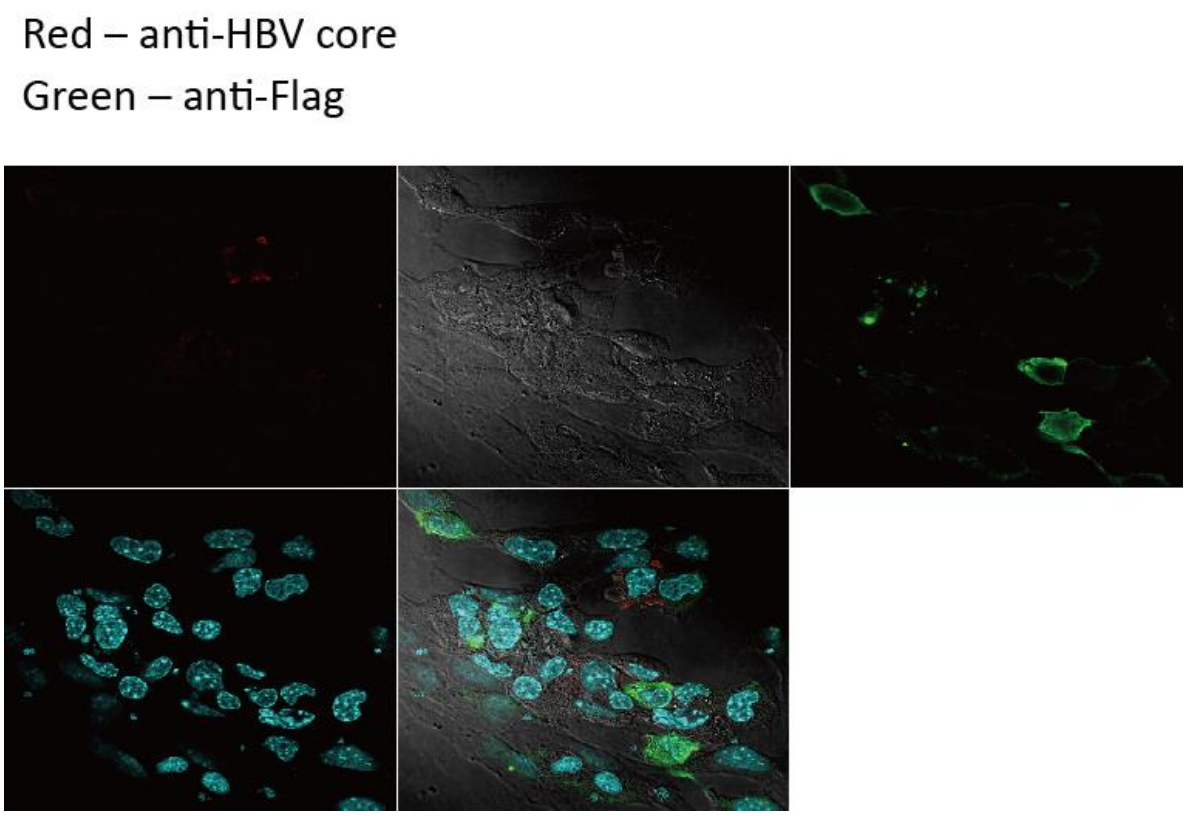
Figure 2



Isg20 is crucial in regulating HBV replication in the hepatocytes cell lines. Huh7 cells in the 24-well plates were co-transfected with 0.5µg of Isg20 or control plasmid and pTER1.4xHBV respectively. Total RNA were isolated at the indicated time point post transfection and the expression of (A) Isg20 and (B) HBs mRNA were quantified with real-time RT-PCR. (C) 25 µg of the total cellular RNA was subjected to Northern blot analysis using probe specifically for HBV. (D) The total cellular DNA was isolated after 48h and 72h after transfection and 500ng of the isolated DNA was subjected to southern blot analysis using DIG-labelled probe specific for HBV. Cells that were not transfected with the pTER1.4xHBV were used as a negative control. Data represent 3 independent

experiments. (E) The HBs antigen in the culture supernatant of those transfected with Isg20 or control plasmid was isolated at the indicated time point was analyzed with ELISA.

Figure 3



ISG20 suppressed HBV in the hepatocytes-derived cell lines.

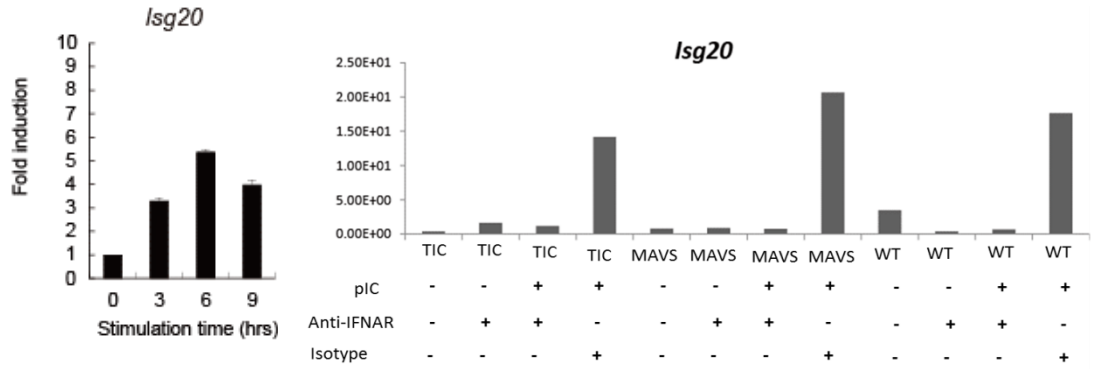
Huh7 cells were transfected with the FLAG-tagged ISG20 expression plasmid and pTER1.4xHBV. Cellular distribution of ISG20 was stained with FLAG antibodies while the HBc antigen of the viral protein was stained with the anti-HBc antibodies and corresponding fluorescence-labeled secondary antibodies 48h post transfection. DAPI staining of nucleus is shown in the lower left panel. Merged signals of ISG20 and HBc and nucleus are shown in the lower right panel. Signal of HBc antigen was not detected in the cell whereby ISG20 was detected, indicating that ISG20 expression suppress the viral replication.

Inducible expression of ISG20 in hepatocytes and BMDC in an IFN dependent-manner

It has been reported that expression of the Isg20 mRNA occurs in a wide variety of tissues, including peripheral blood leukocytes, thymus, spleen, colon, and lung. To determine the expression profile of ISG20 in hepatocytes-derived cell lines, we tested the Isg20 mRNA expression upon the stimulation of recombinant IFN-α. As shown in Figure 4A, Isg20 mRNA expression was up regulated in the immortalized hepatocytes under recombinant IFN-α treatment. We also examined the expression profile of Isg20 in the bone marrow derived dendritic cells (BMDC) upon the pIC stimulation (Fig 4B). BMDC derived from the WT, MAVS and TICAM-1 knock out mice was stimulated with pIC. The expression of Isg20 was up regulated when the TLR3-MAVS or TLR3-TICAM-1 pathway was activated by pIC but abolished upon the neutralization with the anti-IFNAR antibody. These observations suggest that Isg20 is an interferon stimulated gene (ISG) in response to the activation of cellular innate immunity and play a crucial role in the hepatic immune response.

Figure 4

A. B.

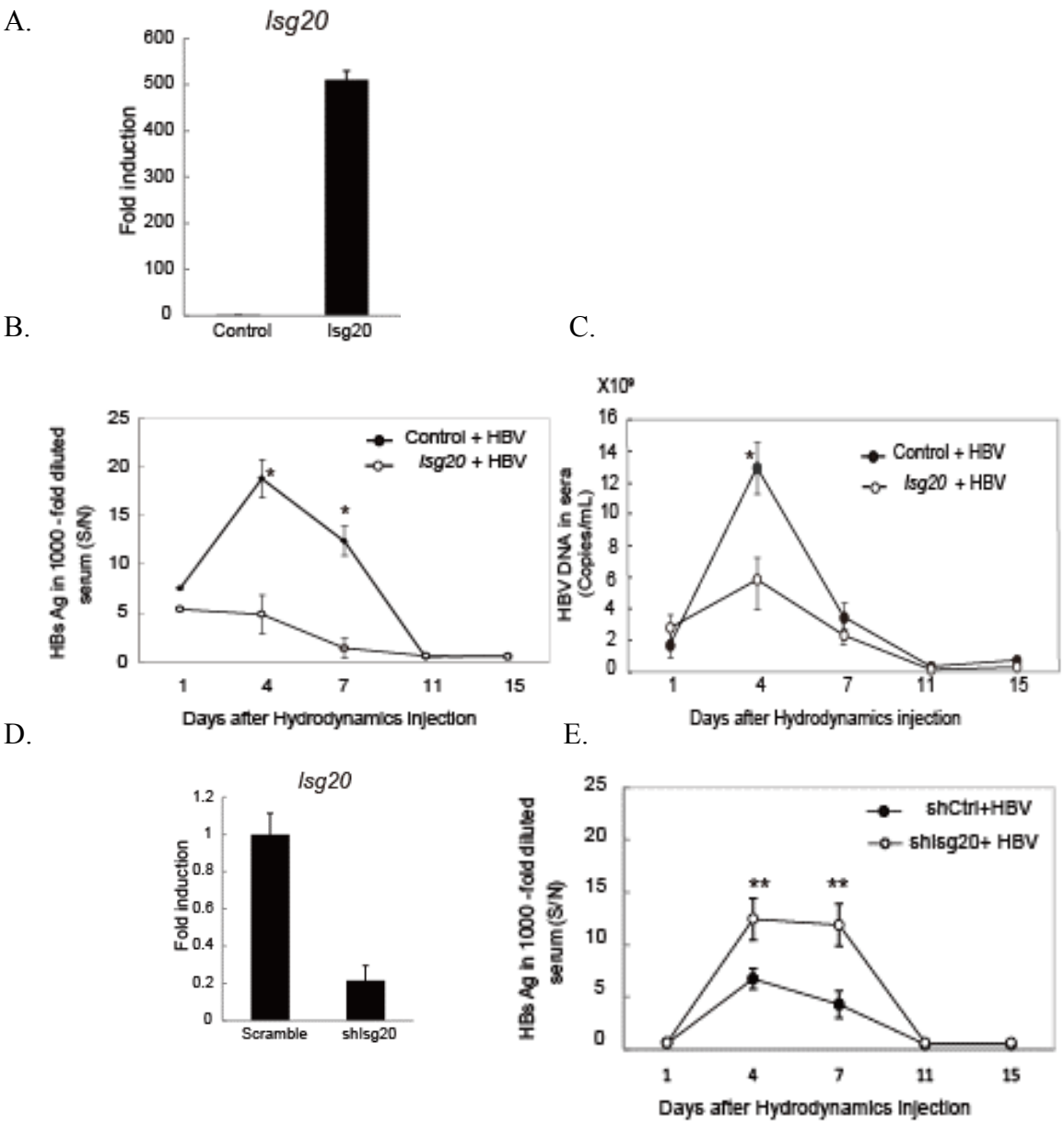


ISG20 expression is induced in a type I IFN dependent manner. (A) The hepatocytes derived cells, Huh7 were stimulated with 1000 unit of recombinant IFN-α and total cellular RNA was isolated at indicated time point and subjected to real-time RT-PCR for the analysis of ISG20 expression.(B) The Bone marrow derived dendritic cells isolated from the wild type, *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice and stimulated with pIC. Anti-IFNAR antibody or the isotype were added to investigate the IFN neutralization effect of the pIC induction on the ISG20 expression which was analyzed with real-time RT-PCR.

ISG20 suppressed HBV replication in *Ifnar*$^{-/-}$ mice liver

Due to the shortcoming of existing animal models of HBV infection currently, we took advantage of the hydrodynamic injection transfection method as a mean to introduce the replication competent HBV full genome into the liver of the genetically modified mice. The results in our previous study shows that the input HBV DNA apparently reached the nucleus of hepatocytes in the mice livers, where it was transcribed to produce the viral transcripts and rapidly translated to produce a high serum titers of HBs antigen, HBc antigen as well as the virions. Furthermore, a robust viral replication was observed in the mice lacking the I IFN receptors at the early time point demonstrated the essential role of IFN in the suppression of the virus. Herein, we examined the effectiveness of ISG20 *in vivo* in the mice lacking the type I IFN receptors by hydrodynamically injected the *Isg20* expression plasmid along with the HBV full genome. As shown in figure 5A-C, HBs antigen and HBV DNA detected in the serum of the *Ifnar*$^{-/-}$ were highly elevated at day 4 and 7 post injection in those receiving the control plasmid. In contrary, such robust viral replication at the early time point was eliminated when the Isg20 was overexpressed in the mice liver. Similar study was conducted in the wild type mice to investigate the effect of silencing the intrahepatic endogenous ISG20 on HBV replication (Fig5D-E). As expected, abolishing the endogenous ISG20 resulted in a higher HBs antigen detected in the mice serum compared to the control. These results indicated ISG20 is one of the crucial molecules of the IFN-stimulated genes mediated suppression on HBV. Interestingly, deficiency of the type I IFN receptor did not seem to affect the complete elimination of the HBV DNA template at the later time point, presumable reflecting the possibility that type I IFN is dispensable in activating the adaptive immune response. In addition, we also examined the intrahepatic viral replication in the mice liver post injection. Southern blot analysis using the total DNA isolated from the mice liver on day 3 post injection (fig 6B) shows that the ISG20 overexpression has slight changes on the input plasmid while a significant impact was observed on the replicative intermediates of HBV. Consistent with the result obtained on intrahepatic viral replication and serum antigenemia , the *Ifnar*$^{-/-}$ mice transfected with the HBV full genome and control or Isg20 plasmid respectively exhibit different expression of HBcAg in the liver. The frequency of the HBcAg-positive hepatocytes decreased greatly in the liver of the mice receiving the ectopic expression of Isg20 (Fig 6A).

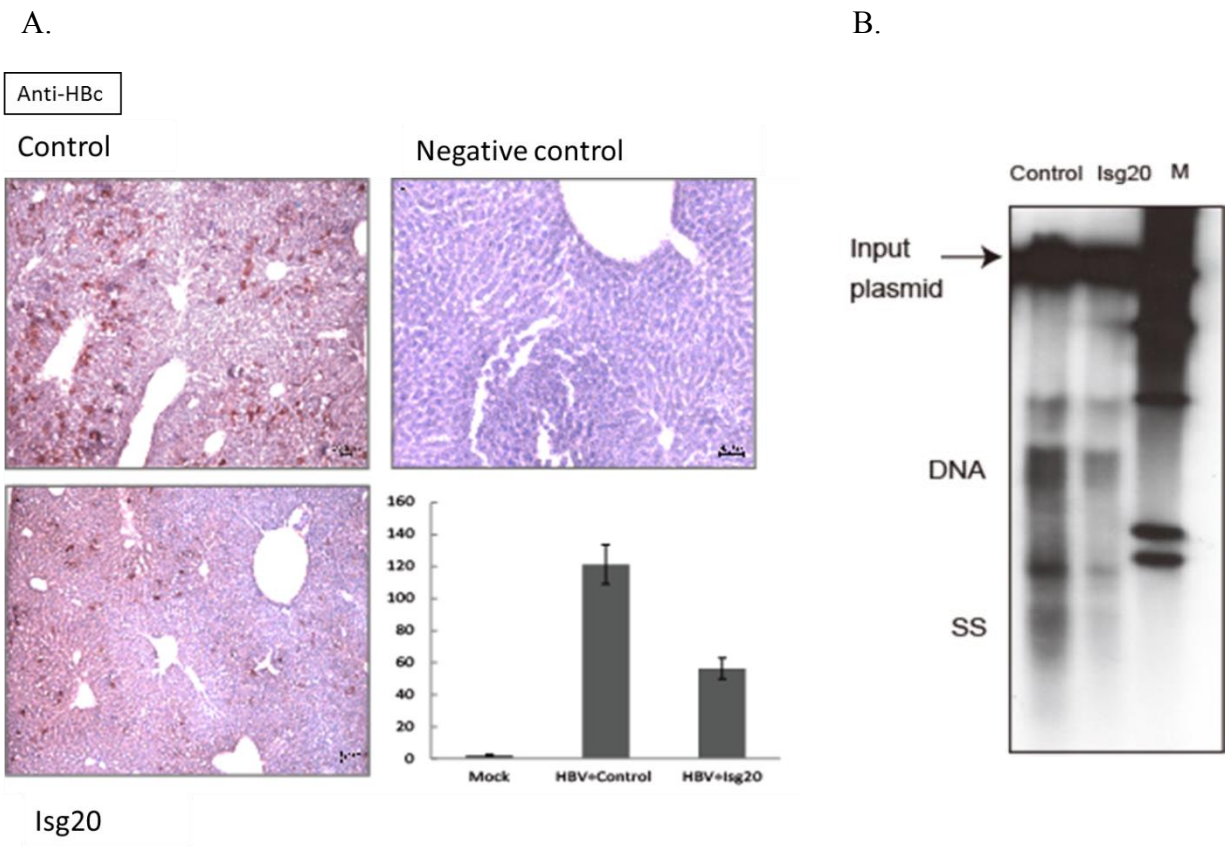
Figure 5



Overexpression of ISG20 suppressed the serum antigemia of HBV in the *Ifnar*$^{-/-}$ while silencing the endogenous ISG20 leads to an up-regulated HBs Ag in the serum of wild type mice receiving the pTER1.4HBV plasmid via the hydrodynamics injection method. The *Ifnar*$^{-/-}$ mice lacking the IFN receptors were hydrodynamically injected with 50 μg of the pTER-1.4xHBV plasmid that contains full genome DNA of HBV together with the control plasmid or ISG20 expression plasmid. (A) The mice liver was dissected on day 3 post injections and total RNA was isolated to confirm the expression of ISG20 in the liver with real-time RT-PCR. (B) HBsAg and (C) HBV DNA were measured with sera isolated at the time points indicated from mice of *Ifnar*$^{-/-}$ mice injected with the plasmid. The HBsAg titers in the 1000-fold diluted serum and HBV DNA in the knockout mice receiving the control plasmid (●) were compared to those receiving the ISG20 expression plasmid (○).The serum HBsAg titers were determined with an enzyme immunoassay at O.D 450nm [calculated

as signal over noise ratios (S/N)]. Sera HBV DNA were determined by Q-PCR and indicated as copies per milliliter. Error bars indicate SD here and in the other figures. (D) and (E) Similar study was conducted in the wild type mice using the plasmid containing the shRNA to silence the endogenous ISG20 and the control shRNA.

Figure 6



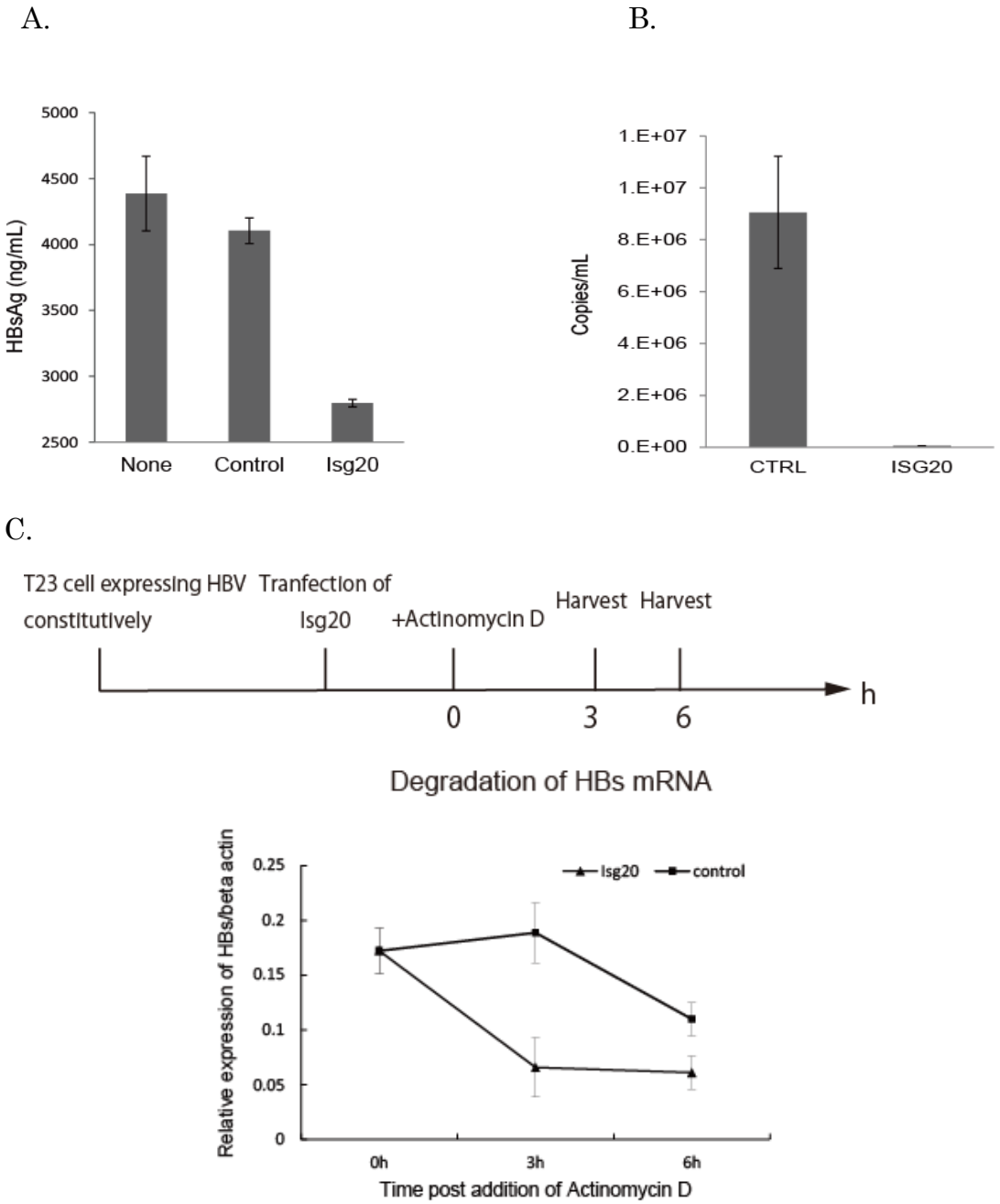
ISG20 regulates HBV replication in the mice liver. (A) The *Ifnar*$^{-/-}$ mice were hydrodynamically injected with 20 μg of the pTER1.4xHBV plasmid together with the control of ISG20 expression plasmid. HBc protein in the livers on day 3 post injection were visualized with immunohistochemical staining of the mice liver sections embedded in OCT using an anti-HBc antibody for HBcAg. Representative sections are shown. HBcAg-positive cells were greatly reduced in the *Ifnar*$^{-/-}$ mice that received the ISG20 expression plasmid compared to those of the control plasmid (lower panel). The scale bars represent 10 μm. The images are displayed at 200x magnification. Frequency of HBcAg-positive signals between the different mouse strains shown is based on the 3 images of each. (B) On day 3 post injection, the mice were sacrificed and liver genome was extracted. The extracted genome was analyzed with southern blot using an HBV-specific probe. HBV replicative intermediates were greatly suppressed with the overexpression of ISG20. The sizes corresponding to the injected DNA and the replicative intermediates are indicated on the left and right.

ISG20 down-regulates HBV RNA via posttranscriptional mechanism

In order to determine whether ISG20-mediated down-regulation of HBV RNA was due to a transcriptional or posttranscriptional mechanism, we overexpressed ISG20 in the HBV stable cell line HBV-T23, in which HBV RNA is transcribed from an integrated transgene. ISG20-mediated suppression has greatly suppressed the HBs antigen and HBV DNA detected in the culture supernatant which indicated the reduction of the HBV virions released from the intracellular (Fig 7A-B). These results demonstrated that ISG20-mediated down regulation of HBV replication was rather a post-transcriptional mechanism.

To further endorse the above hypothesis, we directly measured the decay kinetics of HBV RNA in the absence and presence of ISG20 overexpression. Briefly, the HBV-T23 cells were transfected with control vector or ISG20 expression vector for 24h before the addition of actinomycin D. Actinomycin D is a compound inhibits the proliferation of cells in a nonspecific way by forming a stable complex with double-stranded DNA (via deoxyguanosine residues), thus inhibiting DNA-primed RNA synthesis of HBV RNA. The HBV RNA was quantified with real-time RT-PCR in a time course study. As shown in Fig 7C that HBV RNA degradation was faster in the presence of overexpressed ISG20 than that in the control experiment, suggesting that ISG20 promotes HBV RNA degradation.

Figure 7

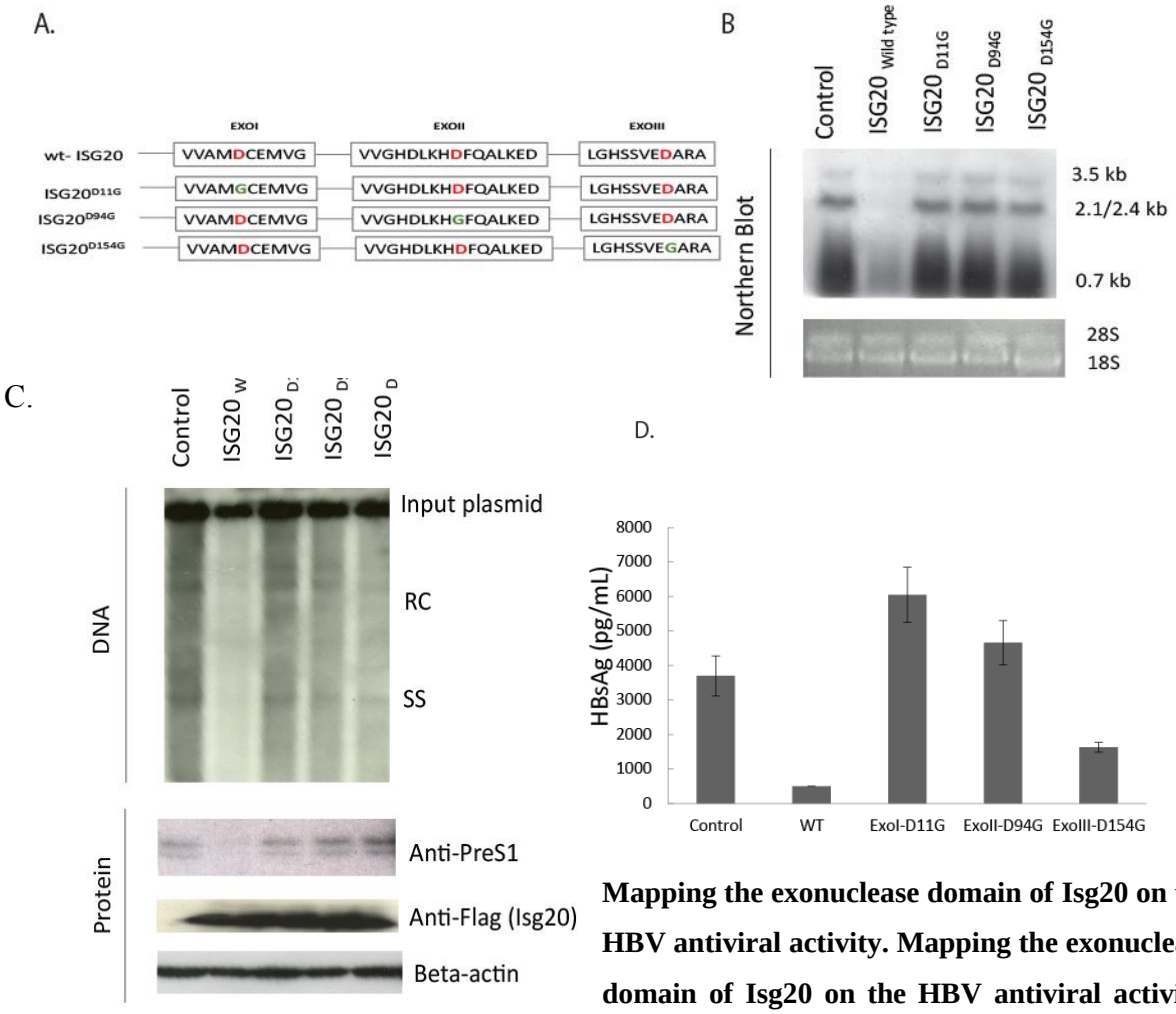


ISG20 down regulates HBV RNA via post-transcriptional mechanism and is independent of the transfected plasmid degradation. HBV-T23 cells constitutively expressing the HBV virion were transfected with the control plasmid or ISG20 expressing plasmid. Culture supernatant was collected after 24h, (A) HBs Ag and (B) HBV DNA was analyzed with ELSIA and qPCR.(C) Actinomycin D, an antineoplastic antibiotic was used as a transcription inhibitor was added and the HBV-T23 cells was transfected the control or ISG20 expression plasmid. Total RNA was isolated at the indicated time point and HBs mRNA was quantified with real-time RT-PCR to examine the effect of ISG20 on the post-transcriptional HBV RNA degradation. The mRNA of HBV was degraded with a higher rate in under the overexpression of ISG20.

Antiviral properties of ISG20 attenuated with substitution in the Exo domains

ISG20 belongs to the DEDD superfamily and contains three separate sequence motifs termed exonuclease domain (EXOI-III), which are highly conserve among rat, mouse and human ISG20. The conformation and spatial disposition of the ISG20 have been recently delineated in the crystal structure of the human ISG20[24]. According to the structure determined, the DEDDH exonuclease has five invariant residues that are crucial for the structure of the active site. These residues include Asp11, Asp94 and Asp 154 that located in the Exo domain I, II and III respectively. To determine the requirement of each exonuclease domain in the ISG20 mediated HBV suppression, amino acid mutation was introduced into each Exo domain of ISG20 to disrupt the active site structure according to the previous studies (Fig 8A). The mutant ISG20 proteins were co-expressed with HBV in Huh7 cells and their effects on viral RNA, DNA as well as protein were analyzed (Fig 8B-D). While the disruption of each individual Exo domain, especially ISG20-D11G (EXO I) and ISG20-D94G (EXOII) particularly has completely abolished the ISG20-mediated HBV RNA decay (Figure 8C) and led to much higher HBs antigen detected in the culture supernatatn. Mutation in the domain EXO III (ISG20-D154G) partially decreased the antiviral activity of ISG20. These observations indicate that each Exonuclease domain contributes to the anti-HBV function of ISG20, and the optimal antiviral activity of IS20 requires especially the Asp in the domain EXO I and II.

Figure 8



Mapping the exonuclease domain of Isg20 on the HBV antiviral activity. Mapping the exonuclease domain of Isg20 on the HBV antiviral activity. (A) Schematic structure of the ISG20 with the three exonuclease domains. (B) Mutations that disrupt each individual exo domain are indicated underneath ISG20 and designated $ISG20_{D11G}$, $ISG20_{D94G}$ and $ISG20_{D154G}$ respectively, according to previous studies. Huh7 cells were co-transfected with pTER1.4HBV and the wild type ISG20 or its mutants. The cellular DNA and protein was isolated at 48h post transfection and subjected southern blot using the HBV specific probe or western blot with the anti-FLAG and anti-PreS1 antibodies. Expression of the wild type ISG20 greatly reduced the HBV replicative intermediates as well as the HBV Pre-S1 protein while disruption of each individual domain almost completely abolished the HBV suppression. Reduction of the input plasmid by the ISG20 expression was barely observed. B-actin served as a protein loading control. (C) Total RNA of Huh 7 cells co-transfected with the pTER1.4xHBV and ISG20 or its mutant(s) expression plasmid was isolated and subjected to northern blot analysis using DIG-labelled probe specific to the HBV. (D) HBs Ag in the culture supernatants was analyzed with ELISA. The anti-HBV activity of ISG20 was attenuated with the disruption of the individual exo domain which leads to the abolishment of the HBV suppression at both the RNA and protein levels.

5. Discussion

IFNs as the crucial effector of the host innate immunity are multifunctional cytokines secreted to interfere with virus infection and replication. Serving as an intrinsic host antiviral factor, ISG20 has been reported to inhibit the infection of a variety of RNA viruses, including EMCV, VSV, influenza virus, HIV and Sindbis virus although the antiviral efficiencies varied. In addition, ISG20 also inhibited the replication of the sub genomic, genotype 1b hepatitis C virus (HCV) RNA replicons in HEK293 cells. Although ISG20 is highly conserved homologues in mouse, human and other primates, ISG20 is not a universal antiviral factor as it was reported that ISG20 does not inhibit SARS-CoV, a highly pathogenic novel human coronavirus in Huh7.5 cells. In our current study, we demonstrated that ISG20 inhibits the replication of HBV, a partially ds-DNA virus belonging to *Hepadnavaridae* family predominantly through the suppression of viral mRNA.

ISG20 belongs to the subfamily within the DEDDH exonuclease family which has both RNAse and DNase activities. Studies with the biochemistry approaches have indicated that ISG20 is a 3' to 5' exonuclease that has a preference for ss-RNA over dsDNA and *Espert et al* as well claimed that ISG20 did not seem to interfere with the replication of a derivative adenovirus strain bearing a β-galactosidase-encoding reporter gene. This demonstrated that single-stranded RNA genomic viruses might be preferential targets for ISG20 [57]. Further investigation with a wider range of RNA and DNA viruses are essential to precisely clarify the viral specification of ISG20.

The homology within the 3' to 5' exonuclease superfamily is concentrated at three conserved exonuclease motifs termed ExoI, ExoII and Exo III. In accordance with the resolved crystal structure of ISG20, we altered the aspartate to glycine residue in the individual Exo domain and demonstrated that the ISG20-mediated down regulation of HBV requires the exonuclease activity. The amino acid substitution in the respective Exo domain has caused the abolishment of ISG20 in suppressing the HBV replication. The currently proposed effector mechanism of ISG20 comprises of the 3' to 5' degradation of HBV RNA, even though, it remains unclear of how ISG20 can specifically degrade particular viral or cellular RNAs. Notably, unlike the RNase L system that implies degradation of viral RNAs and cleavage of cellular 18 S and 28 S RNAs in order to inhibit cellular protein synthesis which may even leads to apoptosis cell death of the host cell to prevent viral propagation, ISG20 have yet been reported to induce apoptotic cell death of the host cell. Therefore, this would be suggesting that another presently unknown mechanism contributes to the IFN-

dependent antiviral response.

The *in vivo* study has also provided some rather interesting results in which allow us to understand better of the virus-host interaction, host antiviral immunity and viral pathogenesis in a bigger picture. Our previous study with the hydrodynamic injection mouse model permitted us to examine the immunological events during the acute HBV infection utilizing the different strains of knock out mice. Our attention has been called to the fact that the *Ifnar*$^{-/-}$ mice exhibit a robust elevation of serum virus titer than the wild type that received the intrahepatic transfection of the HBV full genome. However, such robust elevation of the virus titer was only detected at the early time point and lacking the IFN receptor did not seem to impact the ability of the mice to entirely eliminate the template plasmid. It is apparent that the IFN-induction is absolutely essential in preventing the propagation of the virus in the liver and the current study has demonstrated that ISG20 might be the key molecule in suppressing the viral replication during HBV infection. Although it is clear that much additional work will be required before a complete understanding of how ISG20 specifically degrade the viral nucleic acids.

Summary and Conclusion

Toll-Like Receptors (TLRs) and cytoplasmic RNA sensors have been reported to be involved in the regulation of Hepatitis B virus (HBV) replication but remain controversial due to the lack of a natural infectious model. Our current study sets out to characterize aspects of the role of the innate immune system in eliminating HBV using hydrodynamic based injection of HBV replicative plasmid and knockout mice deficient in specific pathways of the innate system. The evidence indicated that viral replication was not affected by MAVS or TICAM-1 knockout, but absence of IRF3 and IRF7 transcription factors, as well as the interferon (IFN) receptor, had an adverse effect on the inhibition of HBV replication, demonstrated the dispensability of MAVS and TICAM-1 pathways in the early innate response against HBV. *Myd88*$^{-/-}$ mice did not induce significant increase in the initial viremia but substantial viral antigen persisted in the mice sera, a response similar to *Rag2*$^{-/-}$ mice, suggesting that MyD88-dependent pathway participated in evoking an adaptive immune response against the clearance of intrahepatic HBV. Taken together, we show that the RNA-sensing pathways do not participate in the regulation of HBV replication in a mouse model; meanwhile MyD88 is implicated in the HBV clearance.

Taken together, the work we reported herein has demonstrated that ISG20 as a crucial host restriction factor that limits HBV replication and propagation. Mechanistic studies of the inhibitory effect of ISG20 on HBV replication have crucial implications on the better understanding of virus-host interaction as well as the viral pathogenesis during HBV infection. On the ground that HBV RNA is a crucial component in the HBV life cycle, dramatic suppression of viral DNA replication and antigen production would be possible through the ISG20-mediated degradation of viral RNA. Thus, provoking the antiviral activity of ISG20 with small molecule to achieve a beneficial therapeutic value for the management of chronic hepatitis B will be of great theoretical and practical importance.

Acknowledgement

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Supplementary information

Primers used:

HBV

F 5’- TTCCTCTTCATCCTGCTGCT -3’

R 5’- GTCCCGTGCTGGTAGTTGAT -3’

Mouse IFN-β

F 5’ -CCAGCTCCAAGAAAGGACGA -3’

R 5’- CGCCCTGTAGGTGAGGTTAT-3’

Mouse IFN-α2

F 5’- TACTCAGCAGACCTTGAACC-3’

R 5’- GGTACACAGTGATCCTGTGG-3’

Mouse IFN-ɤ

F 5’-GATATCTGGAAGGAACTGGCAAAAG -3’

R 5’- AGAGATAATCTGGCTCGGCTCTGCAGGAT -3’

Mouse β-actin

F 5’- TTTGCAGCTCCTTCGTTGC-3’

R 5’- TCGTCATCCATGGCGAACT-3’

Mouse IL-7

F 5’- CTGCAGTCCCAGTCATCAGTA-3’

R 5’- GTGGCACTCAGATGATGTGACA -3’

Mouse IL-12p40

F 5’-AATGTCTGCGTGCAAGCTA -3’

R 5’-ATGCCCACTTGCTGCATGA -3’

Mouse CXCL-9

F 5’-GATAAGGAATGCACGATGCTC -3’

R 5’- TCTCCGTTCTTCAGTGTAGCAA-3’

Mouse CXCL-10

F 5’-GTGTTGAGATCATTGCCACGA -3’

R 5’-GCGTGGCTTCACTCCAGTTAA -3’

Mouse CXCL-11

F 5’-GGCTGCGACAAAGTTGAAGTGA-3’

R 5’-TCCTGGCACAGAGTTCTTATTGGAG -3’

Human cGas

F 5’- GGGAGCCCTGCTGTAACACTTCTTAT-3’

R 5’- CCTTTGCATGCTTGGGTACAAGGT -3’

Mouse cGas

F 5’- ACCGGACAAGCTAAAGAAGGTGCT-3’

R 5’- GCAGCAGGCGTTCCACAACTTTAT -3’

Human STING

F 5’- GAGAGCCACCAGAGCACAC-3’

R 5’- CGCACAGTCCTCCAGTAGC -3’

Mouse STING

F 5’- CCTAGCCTCGCACGAACTGG-3’

R 5’- CGCACAGCCTTCCAGTAGC -3’