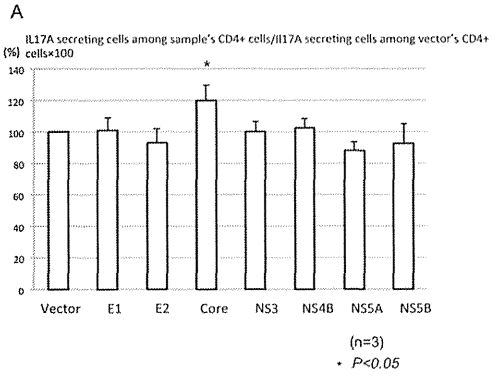

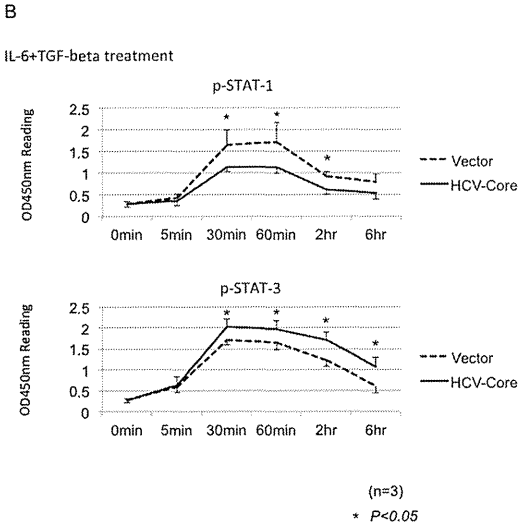

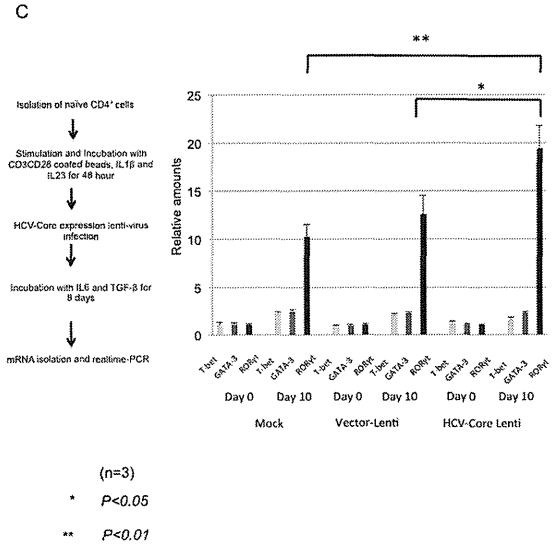

Figure 4. The identification of proteins responsible for enhancing the Th17 development (A). The transfection of various kinds of plasmids expressing HCV-individual proteins (E1, E2, Core, NS3, NS4B, NS5A, NS5B and vector) was carried out by nucleofector. The cells were analyzed at 72 hours post-transfection. The bar graph indicates the IL17A-secreting cells among the sample's $CD4^+$ cells/IL17A secreting cells and the vector's $CD4^+$ cells×100. The obtained data were analyzed by Mann-Whitney U test. Three independent experiments were carried out. **The analysis of STAT-1 and STAT-3 signaling (B).** We used a pathscan to quantify sequentially the phosphor-STAT-1 and STAT-3. The dotted lines indicate data of the vector control. Three independent experiments were carried out. **Long-term culture affected the commitment of naïve T lymphocytes with HCV-core expressing Lenti-virus (C).** The gene expressions of T-bet, GATA-3 and ROR-γt were analyzed by real-time PCR. The relative amounts of mRNA were calculated by ΔΔCT methods. The target gene expressions were analyzed at pre-inoculation of Lent-virus and 10 days after the inoculation of lenti-virus. Three independent experiments were carried out.

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in IL2, IL6, or TGF-β cytokine only conditions with low dose IL1β, IL23 and CD3CD28 coated beads.

The effect of lymphotropic HCV on the Th17 development

The addition of both IL6 and TGF-β1 could significantly induce IL17-secreting T cells (Th17) in comparison to IL6 or TGF-β1 alone (Fig 3A). Both lymphotropic HCV strains (SB-HCV and Ly-HCV) could significantly up-regulate the Th17 development in comparison to Mock and these strains that had been UV-irradiated. Then, we used a co-culture system to analyze the blocking of IL6 and TGF-β1 effects since the expressions of IL6 and TGF-β1 mRNA in PBMCs of double high patients were significantly higher than those in other CH-C patients (Fig. 2D and Fig. 3B). The IL-6 was produced from B lymphocytes. Moreover, the major TGF-β1 producing cells were monocytes in double high patients (Data not shown). The soluble factors produced from PBMCs of Ly-HCV-patient could significantly induce Th17 master gene RORγt in comparison to mock and PBMCs of HCV-1T patient (Fig. 3C). The addition of IL6 and TGF-β1 neutralizing antibody significantly reduced the expression of RORγt, especially IL6 neutralizing antibody (Fig. 3C).

The identification of HCV proteins and signal transduction responsible for the production of IL17A

We used E1, E2, Core, NS3, NS4B, NS5A and NS5B expressing plasmids to transiently express these proteins in naïve T cells. The transfection efficiencies were 45.4±4.96% (average±standard deviation). Among these proteins, only HCV-Core protein could significantly enhance the production of IL17A cells (Fig. 4A)($p<0.05$). In addition to in vitro circumstance, we used NOD/scid/γc^{null} (NOG) mice that are super-immunodeficiency mice[33]. The transfusions of HCV-core expressing human primary lymphoid cells were carried out (*ongoing Kondo Y et al.*). The higher amount of IL17A and RORγt mRNA were detected in the HCV-Core expressing $CD4^+$ cells in comparison to the control groups (data not shown). Then, we sequentially analyzed the STAT-1 and STAT-3 activation by IL6 and TGF-β1 stimulation in the HCV-Core expressing T cells. The results indicated that STAT-3 signaling was significantly enhanced in comparison to mock-transfected T cells (Fig. 4B)($p<0.05$). These data indicated that HCV-Core protein enhanced the STAT-3 signaling following the induction of the Th17 master gene-RORγt.

Long-term culture of primary naïve T lymphocytes with HCV-core expressing lenti-virus

We constructed the HCV-core expressing lenti-virus to analyze the long-term culture of primary naïve T lymphocytes with the expression of HCV core protein. The efficiency of lenti-virus infection was 27.7±3% (average±standard deviation). In addition to IL1β and IL23, the IL6 and TGF-β1 cytokine conditions could remarkably induce the RORγt mRNA (Fig.4C). Moreover, significantly higher amounts of RORγt mRNA were detected in the HCV-core expressing T lymphocytes in comparison with the control groups (Fig. 4C)($p<0.05$).

Discussion

Autoimmune thyroiditis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, autoimmune hepatitis and rheumatoid arthritis etc. could be classified not only as HCV-related diseases but also as Th17-related autoimmune diseases[16-19,34–36]. In this study, we clearly demonstrated the relevance of lymphotropic HCV to autoimmune-related diseases including an important role of Th17 cells in CH-C patients. This study revealed two important mechanisms by which Th17 development is enhanced. In the first, the existence of lymphotropic HCV can result in the IL6 and TGF-β1 double high condition that can enhance Th17 development. Previously, Machida et al. described that HCV replication in B cells induced IL6 production from B cells[24]. In the second, the existence of HCV in naïve T cells can enhance Th17 development in the IL6 and TGF-β1 double high condition by enhancing STAT-3/RORγt signaling. Previously, we showed that lymphotropic HCV could suppress IFN-γ/STAT-1/T-bet signaling, which could contribute to the persistence of hepatitis C virus infection[1,4,13]. STAT-3 signaling could be enhanced by the suppression of STAT-1 signaling. However, the finding of our research was surprising. Therefore, we examined this phenomenon studiously and carefully. First, we found a novel genotype, 1b lymphotropic HCV strain (Ly-HCV), that could infect Raji and human primary lymphocytes. Although the infectivity of this strain was lower than that of SB-HCV[29], we could detect negative and positive strand RNA in naïve T lymphocytes with stimulation.

Therefore, we used two lymphotropic HCV strains to analyze the effect on Th17 development. Moreover, two kinds of expression experiments showed that HCV-Core could enhance the STAT-3/RORγt signaling, since the method of gene expression and the period of incubation might affect the result of T cell development. However, the results of our two kinds of experiments (plasmids and lenti-virus) consistently showed that the expression of HCV-core protein in T lymphocytes could enhance the Th17 development. Other groups previously reported that HCV-core protein could affect anergy-related genes and T cell responses by inducing spontaneous and alternating T-cell receptor-triggered Ca2+ oscillations[37,38]. Therefore, the expression of HCV-core protein in T lymphocyte might be important for the functional changes in T lymphocytes. Although our study confirmed that the replication of HCV in lymphocytes is important, there was a bystander effect by exosome produced from HCV-infected lymphocytes. Previously, our group reported that exosome could transport miRNA and proteins in the microenviroment[39,40]. This phenomenon could explain the significant effects of a low level of HCV infection in lymphocytes. Moreover, the naïve T lymphocyte is located upstream of Th17 development. Therefore, we should not underestimate the effect of a low-level of HCV replication in lymphocytes.

In conclusion, we report the detailed mechanism of Th17 development and HCV infection, which might be involved in the pathogenesis of autoimmune-related disease in CH-C patients (Fig S2). Recently, a novel therapy targeting STAT-3 signaling was reported[23,41,42]. We should consider the clinical use of such treatments for autoimmune-related diseases in CH-C patients.

Supporting Information

Figure S1 Phylogenetic trees constructed based on the nearly entire nucleotide sequence of HCV by using the unweighted pair group method with the arithmetic mean (Michener 1957). The tree includes the three genotype 1a isolates, forty 1b, one 1c, three 2a, two 2b, one 2c and one 3a, 3b, 6b, 7b, 9b, 10a, whose nucleotide sequence data were retrievable from the GenBank/EMBL/DDBJ database (A). Mapping to the consensus HCV genome sequence. For Ly-HCV 0183-4, 197,414 reads were mapped (Fig S1B). The coverage was 100.0%, and the average depth was 2092.1x. For Ly-HCV 0186-1, 410 reads were aligned. The coverage was 95.9%, and the average depth was 4.3× (B).
(TIFF)

Figure S2 The schema of Th17 induction in perihepatic lymph node of CH-C patient with lymphotropic HCV are shown.
(TIFF)

Table S1 Various kinds of cytokines conditions for in vitro analysis are shown in Table S1.
(DOC)

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Author Contributions

Conceived and designed the experiments: YK MN OK TK ML TS. Performed the experiments: YK MN OK KM RF TN KK EK KN. Analyzed the data: YK MN OK TN KN ML TS. Contributed reagents/materials/analysis tools: KM. Wrote the paper: YK MN TN ML TS.

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Research Article

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A MAVS/TICAM-1-Independent Interferon-Inducing Pathway Contributes to Regulation of Hepatitis B Virus Replication in the Mouse Hydrodynamic Injection Model

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Key Words
Type I interferon · Hepatitis B virus regulation · Toll/IL-1R homology domain-containing adaptor molecule 1 · Mitochondrial antiviral signaling protein · Pathogen-associated molecular patterns

Abstract
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 interferon regulatory factor 3 (IRF-3) and IRF-7 transcription factors, as well as the interferon (IFN) receptor, had an adverse effect on the inhibition of HBV replication, demonstrating the dispensability of MAVS and TICAM-1 pathways in the early innate response against HBV. *Myd88*$^{-/-}$ mice did not have a significant increase in the initial viremia, but substantial viral antigen persisted in the mice sera, a response similar to *Rag2*$^{-/-}$ mice, suggesting that the 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.

Introduction

Hepatitis B virus (HBV) is a noncytopathic human DNA (hepadna) virus that infects hepatocytes causing acute and chronic hepatitis [1]. More than 360 million people are chronically infected with HBV worldwide. Although less than 5% of HBV-infected patients develop persistent infections that progress to liver cirrhosis and hepatocellular carcinoma, HBV causes about 20% of hepatocellular carcinoma deaths [2]. The adaptive immune response is widely acknowledged as pivotal in the defense against HBV. However, the role of innate immunity during HBV infection remains controversial since analysis in patients at the early stage of infection is unfeasible. In ad-

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dition, no reliable cell-based in vitro infection system or convenient animal model is available.

During HBV infection, the HBV genome is delivered into the nucleus. Infection is defined by the formation of covalently closed circular DNA. Following formation of covalently closed circular DNA, viral mRNA and pregenomic RNA are transcribed [3, 4]. The pregenomic RNA is subsequently converted to a partially double-stranded genome by the viral DNA polymerase. Unlike other DNA viruses, HBV uses an RNA proviral intermediate that must be copied back into DNA for replication. Although these replication steps are sequestered in the nucleus of infected cells, cytoplasmic DNA/RNA sensors are reported to affect the efficacy of HBV replication [5, 6]. The association between cytoplasmic pattern recognition receptors and the dynamics of the HBV life cycle in HBV-infected cells needs to be clarified.

Viral RNA is sensed by the innate immune system by either Toll-like receptor 3 (TLR3) or cytoplasmic sensors such as retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). RIG-I and MDA5 mainly participate in type I interferon (IFN) induction in conjunction with the adaptor molecule, mitochondrial antiviral signaling protein (MAVS; also called IPS-1, Cardif, or VISA) [7–9]. The Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1; also called TRIF) is the adaptor of TLR3, which senses viral RNA on the endosomal membrane [8–10]. Several DNA sensors, most of which signal through STING for type I IFN induction, have been reported in recent years [11]. A few reports have also mentioned that MAVS participates in DNA sensing in certain human cells whereby poly-dA/dT DNA is found to signal via RIG-I. Later, it was also shown that poly-dA/dT serves as a template for RNA polymerase III to make RIG-I ligands [12–14]. Nevertheless, this hypothesis is unresolved in mouse cells. Once stimulated by the viral DNA/RNA, these adaptor proteins activate IFN regulatory factor (IRF)-3 and IRF-7, which induce type I IFN production [7–9]. These pattern recognition receptor-mediated early innate immune responses are crucial in controlling viral replication and spread before the initiation of more specific and powerful adaptive immune responses [8, 9, 15].

Despite numerous studies on HBV pathogenesis, the putative molecular patterns of HBV that trigger cellular responses remain unknown. A few 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 [16–18]. However, no definitive evidence in vivo is available because analysis on the gene expression and effectors required for elimination of the replicative template has been especially difficult. Since viral clearance is a multifaceted process, 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/IRF-7, IFNAR, MyD88, or RAG2 to identify and characterize the immunological events for HBV clearance. With the availability of various gene-disrupted mice, our study allows the identification of pathways crucial for the clearance of HBV.

Materials and Methods

Animal Studies

All mice were backcrossed with C57BL/6 mice more than seven times before use. *Ticam-1*$^{-/-}$ [19] and *Mavs*$^{-/-}$ [20] mice were generated in our laboratory as described previously, while *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 CLEA Japan (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 were previously shown to produce a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers [21] was used in this study. A total of 50 μg of the plasmid was injected into the tail vein of 7- to 9-week-old mice in a volume of 2.0 ml of TransIT-QR hydrodynamic delivery solution (Mirus, USA). The total volume was delivered within 3–8 s. Plasmid DNA was prepared by using an EndoFree plasmid system (Qiagen, Germany) according to the manufacturer's instructions.

Quantification of HBV DNA by Real-Time PCR

To determine the HBV DNA in the serum, 30 μl of each serum sample 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 instructions and dissolved in 20 μl of TE-buffer. The purified viral genome was quantified by real-time PCR using the SYBR green master mix (Life Technologies, USA) and the HBV-DNA-F/R primer (see suppl. table 1 for primer sequences; for all online suppl. material, see www.karger.com/doi/10.1159/000365113). Amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of

denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay is 1,000 copies.

Immunohistochemical Staining for HBV Core Antigen

For immunohistochemical staining of the HBV core antigen (HBcAg), 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 (anticore polyclonal rabbit antibody, DAKO) overnight, followed by incubation with an immunoperoxidase technique involving avidin-biotin peroxidase complexes (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif., USA) according to a method reported previously [22].

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 HBV surface antigen (HBsAg) in the serum was quantified by sandwich ELISA in commercial ELISA kits following the manufacturer's protocol (XpressBio, USA). The reporting unit is the signal/cutoff ratio of the 1,000-fold diluted serum at an O.D. of 450 nm.

Southern Blotting to Detect Intracapsid HBV DNA

Viral DNA was isolated from intracellular viral capsids and detected with a specific DIG-labeled probe as described previously [21]. In brief, to isolate the viral DNA, mouse livers were homogenized and subjected to overnight sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. Twenty micrograms of the isolated DNA was separated in 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 [23] with slight modification. A 96-well plate was coated with antigen of HBs in carbonate buffer and followed by blocking of 2% BSA. Plasma samples were diluted 5× and then incubated in the antigen-coated wells for 3 h at room temperature. A horseradish peroxidase-conjugated goat anti-mouse IgGγ (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 A_{450} (sample)/A_{450} (2.1' normal mouse average) at which samples with a value >1 were considered to have 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 Biosystems) according to manufacturer's instructions. qPCR was performed using a Step One real-time PCR system (Applied Biosystems). The expression of cytokine 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 online supplementary table 1.

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 Biosystems) 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 a relative expression. The primer sequences are described in online supplementary table S1.

Reporter Gene Assay

To prepare the HBV RNA, immortalized mouse hepatocytes previously established in our laboratory [24] were transfected with either control plasmid or pTER1.4xHBV. Total RNA containing the HBV RNA was isolated after 12 h and confirmed with RT-PCR, while the RNA 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 a passive lysis buffer, and Firefly and Renilla luciferase activities were determined using a dual-luciferase reporter assay kit. Firefly luciferase activity was normalized by Renilla luciferase activity and was expressed as the fold stimulation relative to activity in nonstimulated cells.

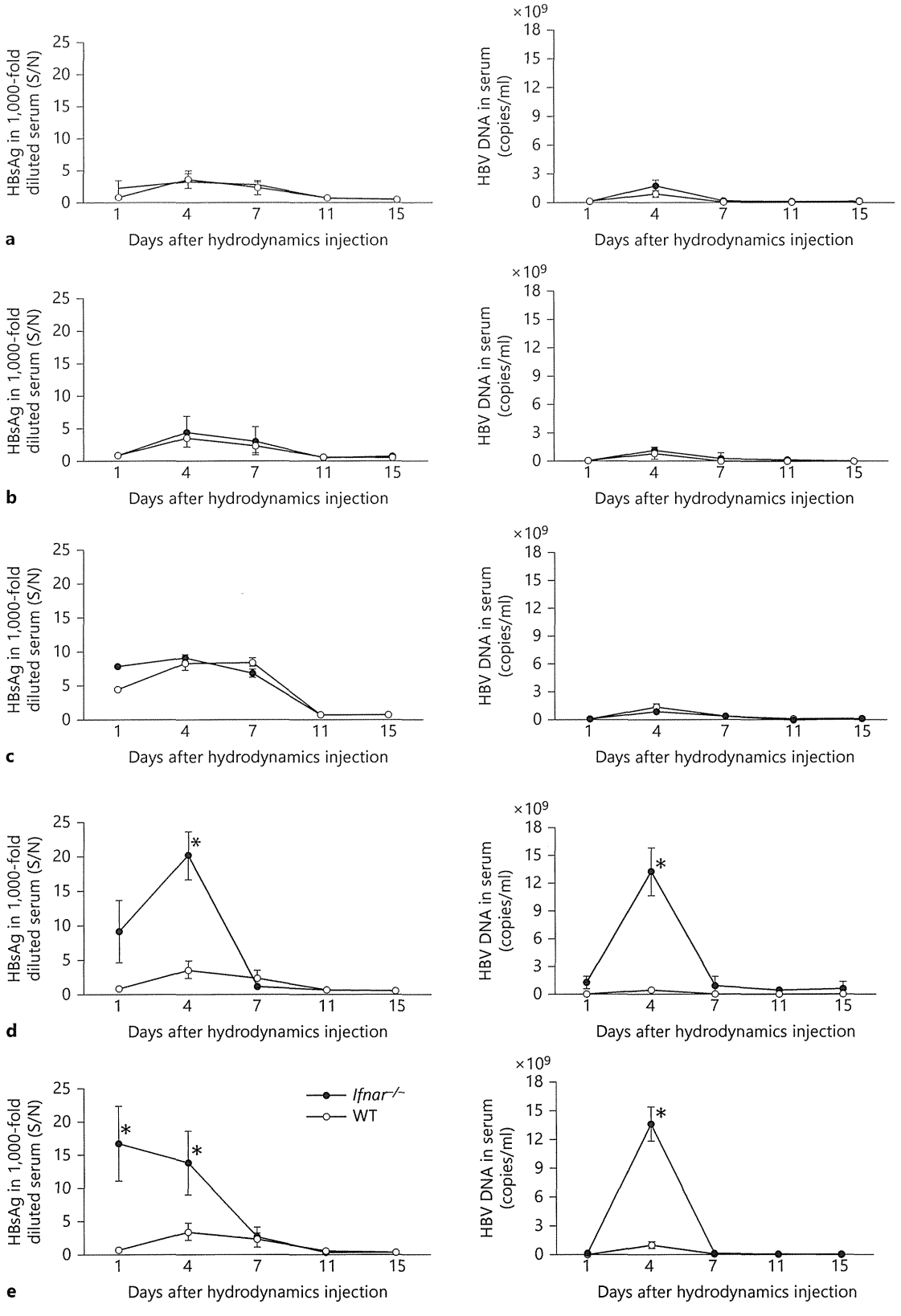
Statistical Analysis

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.

Results

MAVS and TICAM-1 Are Dispensable in Suppressing HBV Replication

We hydrodynamically transfected replication-competent HBV DNA into *Mavs*$^{-/-}$ or *Ticam-1*$^{-/-}$ and *Mavs*$^{-/-}$/*Ticam-1*$^{-/-}$ mice to access the role of these viral RNA-sensing pathways in response to HBV. Serum HBsAg 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 (fig. 1a–c). Subsequently, HBsAg in sera decreased and terminated by day 11. *Mavs*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice displayed HBsAg clearance



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kinetics that closely paralleled the WT mice response (fig. 1a, 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 1×10^4 copies/ml 1 day after injection and reached 2×10^9 copies/ml 4 days after injection (fig. 1a–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. 1a, b). The serum HBV-DNA and HBsAg 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. 1c). No notable differences were observed between WT and MAVS/TICAM-1 double-knockout mice in serum HBsAg and HBV-DNA levels, consistent with other data obtained. In addition, similar kinetics 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 (online suppl. fig. 1).

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 (online suppl. fig. 2). 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 not 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, and thymus (online suppl. fig. 3). Only weak HBV signals were detected in other organs in some types of knockout mice. These results demonstrated that HBV replication in vivo using the injection method was efficient and liver specific.

To further assess the possibility of HBV RNA acting as pathogen-associated molecular patterns 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 the 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 (online suppl. fig. 4). Unlike poly(I:C), neither the full genome of HBV nor RNA induced 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 hepatocyte cell lines in order to access the intrinsic RNA or DNA-sensing pathways (online suppl. fig. 5). We found that the hepatocyte cell lines, including those originating from mice and humans, expressed extremely low amounts of *Sting* compared to the intrinsic *Mavs*. However, other cell lines, including RAW 264.7 (murine macrophage cell line) and L929 (murine fibrosarcoma cell line), have higher endogenous expression of *Sting* in comparison to *Mavs*.

IRF-3/IRF-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-/IRF-7-deficient mice. Activation of transcription factors including IRF-3 or IRF-7 is essential for raising immune responses including IFN production [25]. Unlike *Mavs*$^{-/-}$, *Ticam-1*$^{-/-}$, or WT mice, mice lacking the transcription factors IRF-3/IRF-7 had

Fig. 1. IFNAR and IRF-3/IRF-7 are critically associated with regulation of HBV propagation in mice but not MAVS and/or TICAM-1. HBsAg or HBV DNA were measured with sera from *Mavs*$^{-/-}$ (n = 13) (**a**), *Ticam-1*$^{-/-}$ (n = 10) (**b**), *Ticam-1*$^{-/-}$/*Mavs*$^{-/-}$ (n = 6) (**c**), *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ (n = 12) (**d**), and *Ifnar*$^{-/-}$ (n = 13) (**e**) mice compared to WT mice (n = 15). These mice were hydrodynamically injected with 50 μg of the pTER-1.4xHBV plasmid containing full-genome HBV DNA. Mouse sera were isolated at the time points indicated. The HBsAg titers in the 1,000-fold diluted serum (left) and HBV DNA (right) in the knockout mice (●) were compared to the WT mice (○). Serum HBsAg titers were determined with an enzyme immunoassay at O.D. 450 nm [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. The statistical p values were analyzed and no significant differences were observed in **a–c**. * $p < 0.01$ in **d** and **e** are time points statistically different between WT and transgenic mice.

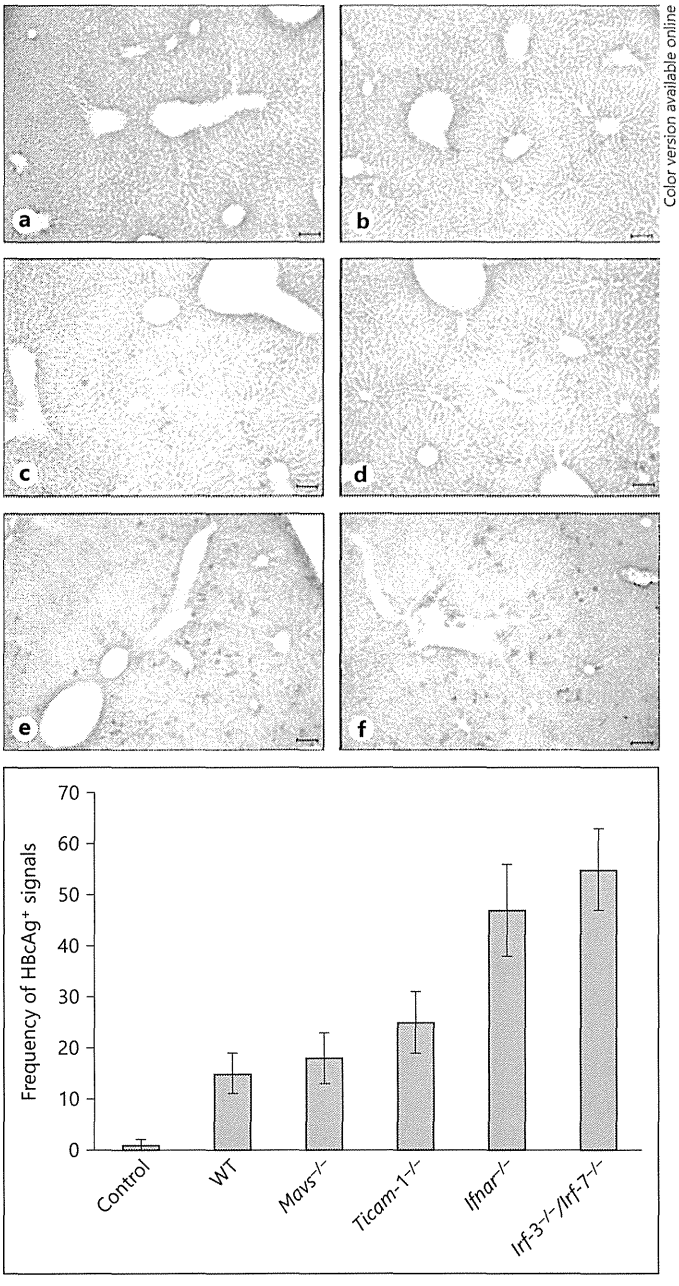

Fig. 2. Lacking IFNAR and IRF-3/IRF-7 causes an increase of HBcAg in mouse liver injected with the HBV replicative plasmid. The HBc protein in the livers on day 3 after injection was visualized with immunohistochemical staining of the mice liver sections embedded in OCT using an anti-HBc antibody for 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*$^{-/-}$/*Irf-7*$^{-/-}$ (**f**) mice are more prevalent compared to the WT mice. The scale bars represent 10 μm. The images are displayed at 200× magnification. Frequency of HBcAg-positive signals between the different mouse strains shown is based on 3 images of each.

markedly high amounts of HBsAg and HBV DNA in sera (fig. 1d). A sharp peak of HBsAg in sera occurred in *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice on day 4 after injection. However, in spite of the high virus titer at the early stage, HBsAg 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 in 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 in the early stage after 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. 1e) similar to *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice.

The presence of HBcAg-positive hepatocytes was also monitored by immunohistochemical staining of liver sections from mice of each strain at day 4 after the injections (fig. 2). Data from the observed HBcAg-positive hepatocytes were in good agreement with the results on sera HBsAg and HBV DNA: only deficiency of IRF-3/IRF-7 and IFNAR resulted in a sharp increase of viremia in mice in the early stage (earlier than day 4). Fewer HBcAg-positive hepatocytes were observed in *Mavs*$^{-/-}$ and *Ticam1*$^{-/-}$ as well as WT mice at day 4 after injection than in *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ or *Ifnar*$^{-/-}$ mice (fig. 2).

To gain insight into cytokine production in the liver in response to the HBV genome and its replication, we quantified the expression of type I IFN, IFN-γ, IL-7, IL-12p40, and chemokines including CXCL9, CXCL10, and CXCL11 mRNA in the livers of WT 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 (fig. 3a–h). A similar study was carried out in WT 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. 3i–k). This increase was not observed in *Ifnar*$^{-/-}$ mice lacking the INF 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 suppressing HBV

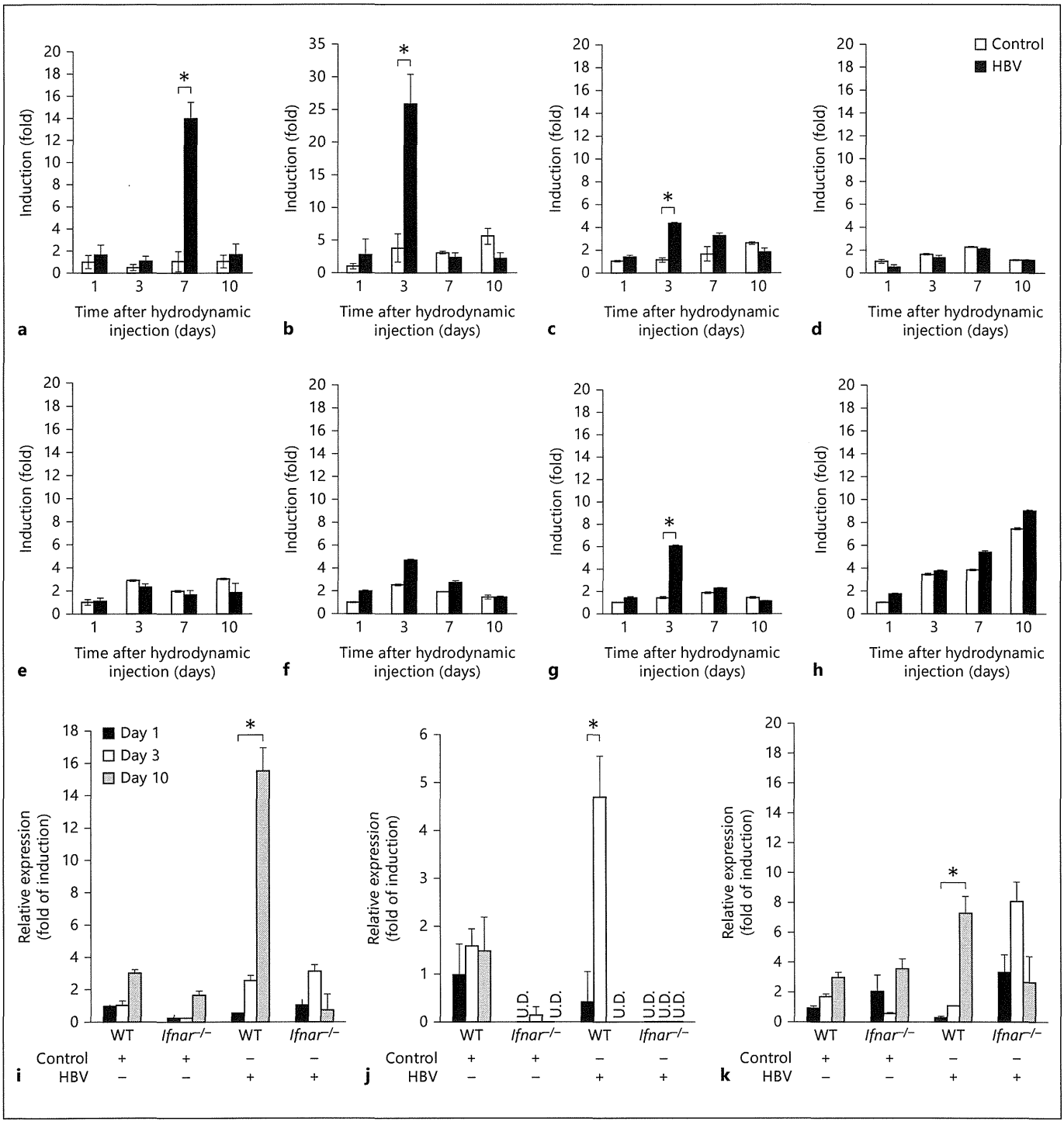

Fig. 3. Type I and II IFN expression is induced by HBV replication, and lacking the type I IFN receptor (IFNAR) causes failure of these inductions. WT mice were hydrodynamically injected with 50 μg of the pTER-1.4xHBV or control plasmid as described, and livers were isolated on days 1, 3, 7, and 10 after 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: IFN-α (**i**), IFN-β (**j**), and IFN-γ (**k**). *Ifnar*$^{-/-}$ mice show reduced expression of the IFNs compared to the WT. Data represent the mean of 3 mice on each strain and time point mentioned. * $p < 0.05$. U.D. = Undetected.

replication early. However, the reason for the lag in the induction of IFN-γ between the WT and *Ifnar*$^{-/-}$ mice remains unclear.

Taken together, these results suggest that type I IFN was indispensable for suppressing HBV replication in 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.

HBV Clearance in a Later Stage by Acquired Immunity

Previous studies by Yang et al. [23] 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 whether the elevated viral titer in *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice on day 4 after 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 resulted in failure to produce mature B or T lymphocytes. As shown in figure 4, the absence of mature T and B cells in the *Rag-2*$^{-/-}$ mice did not result 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 HBsAg and HBV DNA were detected up to day 15 (fig. 4a), by the time viral replication was terminated in all the other strains tested (fig. 4c, d). In other words, activation of the immune effectors such as the B and T cells is responsible for the intrahepatic HBV clearance, their activation being independent of IFN and IRF-3/IRF-7.

MyD88 Deficiency Leads to Slower HBV Clearance

The MyD88-dependent pathway has been known to lead to the production of inflammatory cytokines and is common to all TLRs, except TLR3 [22]. To examine whether a MyD88-dependent pathway is required in the intrahepatic clearance of the HBV, we monitored the serum HBsAg in MyD88-deficient mice. As shown in figure 4b, an increase in sera HBsAg 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. 4b, c). Instead, a delay in the elimination of the HBV was observed (fig. 4b, d). Typically, WT mice or other mouse strains lose serum HBsAg from day 11 after injection. However, serum antigen was detectable on day 15 in *Myd88*$^{-/-}$ mice. Delayed elimination of HBV plasmid and single-strand DNA in the liver was observed in Southern analysis of the liver from *Myd88*$^{-/-}$ mice compared with WT, *Mavs*$^{-/-}$, and *Ticam-1*$^{-/-}$ mice (online suppl. fig. 1).

Additionally, ELISA to determine anti-HBsAg antibody production in mouse sera after hydrodynamic injection revealed that anti-HBs antibody was produced in WT mice from day 7 and peaked at day 15 (fig. 4e). RAG2-deficient mice lacking mature T and B cells failed to produce any antibody, and *Myd88*$^{-/-}$ mice also 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.

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 IRF-3 or IRF-7 transcription factors, as well as the IFN receptor, had an adverse effect on the inhibition of HBV replication. The results herein demonstrated that the 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 of HBV in a few reports [16–18, 26]. HBx or HBs inhibits IFN-β induction followed by activation of TLR3 or RIG-I pathways with poly(I:C) or SeV, respectively. However, these findings must be interpreted with caution, as poly(I:C) and SeV are heterologous inducers for evaluating either the TLR3 or RIG-I pathway [16, 17]. 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 the cytosol of infected cells [27, 28] and by TLR3 or

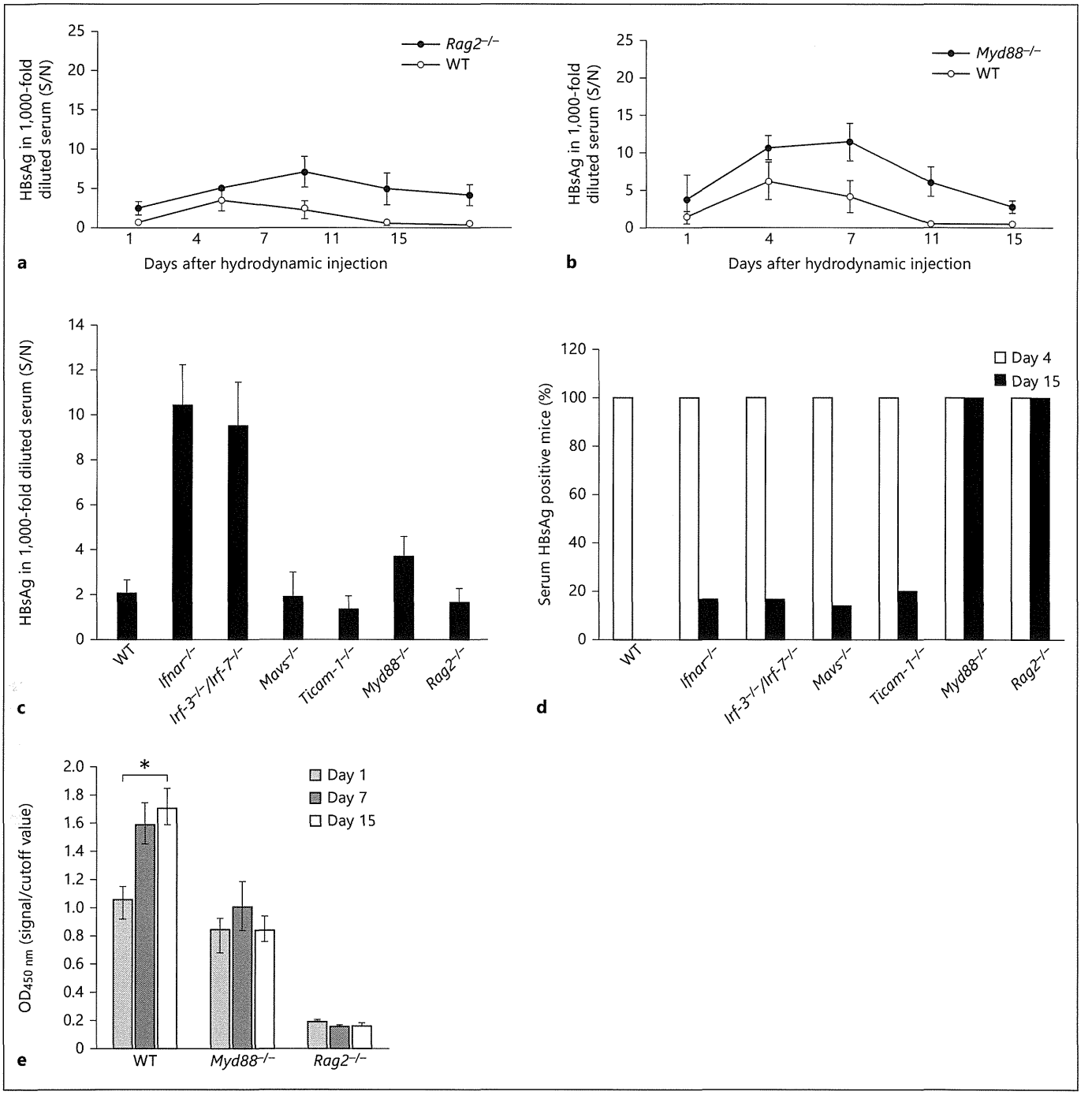

Fig. 4. Mice lacking RAG2 and MyD88 show insufficient clearance of HBV. **a**, **b** The *Rag2*$^{-/-}$, *Myd88*$^{-/-}$, and WT mice were hydrodynamically injected with 50 μg of pTER-1.4xHBV and HBsAg in the mouse sera at the time points indicated and analyzed with ELISA as described. **c** HBsAg in 1,000-fold diluted serum from all the mice strains including WT, *Ifnar*$^{-/-}$, *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$, *Mavs*$^{-/-}$, *Ticam-1*$^{-/-}$, *Myd88*$^{-/-}$, and *Rag2*$^{-/-}$ at day 4 after the hydrodynamic injections. Only *Ifnar*$^{-/-}$ and *Irf-3*$^{-/-}$/*Irf-7*$^{-/-}$ mice show a remarkable increase, while a moderate increase of sera HBsAg was seen 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 day 4 (□) and day 15 (■) after the hydrodynamic injections. Serum HBsAg was found to be persistent only in mice deficient in MyD88 and RAG2 on day 15 as 100% of the mice from these two strains were HBsAg positive (n = 8 for each mice strain). **e** Lacking MyD88 and RAG2 leads to the failure of the knockout mice to produce anti-HBs IgG compared to the WT mice on day 15 after injection as determined by ELISA using antigen of HBs (n = 3 for each mice strain). * $p < 0.05$. S/N = Signal-over-noise ratio.

TLR7/8 in the endosome of other noninfected cells [29, 30]. These RNA sensors require MAVS, TICAM-1, or MyD88 as adaptor proteins to induce type I IFN [28]. On the other hand, cytoplasmic DNA is recognized by DNA sensors including DAI, IFI16, RIG-I, DHX9 (helicase), and cGAS [31]. STING is the only adaptor for all IFN-inducing DNA sensors in mouse cells reported so far [30, 32, 33], although some of these sensors are reported to induce type I IFN via MAVS in human cells. These adaptors, TICAM-1, MAVS, and STING, are all linked to activation of IRF-3/IRF-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 dependent on the virus species and cell type. Cell type-specific contributions of other sensors, including DEAD box helicases, might occur in some cases of infection. However, in hepatocytes, the control plasmid per se exhibited no IFN-inducing response, suggesting that the HBV replication is a critical step for IFN induction. Actually, 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 WT counterparts and result in elevated HBV titers in mice sera and livers. These findings imply that type I IFN acting on IFNAR is indispensable for evoking anti-HBV protective responses although such a 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 [34]. There are a few possibilities of how type I IFN is produced in mice 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 leads 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/IRF-7, and genomic DNA is an important part of HBV replication. It would be interesting to clarify such hypotheses 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 immortalized hepatocytes. To our surprise, we were unable to detect significant IFN-β induction with either HBV replicative DNA or HBV RNA. As we looked into the possible reasons to account for the lack of innate immune responses against HBV in hepatocytes, we found that the endogenous expression of STING in hepatocyte cell lines including HepG2 and immortalized mouse hepatocytes is extremely low compared to other cell lines like macrophages or dendritic cells, thus 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. 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 pDCs, Isogawa et al. [5], demonstrated that freshly isolated CD11c^{+} cells of intrahepatic myeloid cells rather than the hepatocytes expressed TLRs including TLR2, 3, and 9. 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 an 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 mouse model. Notably, MyD88 is the adaptor molecule for TLR7 and 9 in pDCs [35, 36]. Deficiency of MyD88 in pDCs may result in failure to induce acquired immunity for HBV. Our findings show that HBV-specific antibodies are efficiently produced in WT, but not in *Myd88*$^{-/-}$ mice. In addition, the number of pDCs has been previously reported to be reduced in vivo during several systemic viral infections including HBV [37]. In one of the most recent reports, Lv et al. [38], showed that HBV-derived CpG induces potent IFN-α production by human pDCs, which may partially explain how pDCs interact with HBV in infection. 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% improvement in HBV patients and little is known about why most chronic HBV patients do not respond to IFN therapy [39]. 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 in the initial days after injection. Conversely, type I IFN did not have any influential effects on clearance of the HBV template in the later stages. Such observations coincide with the latest study conducted in patients with chronic HBV infection by Tan et al. [40], in which IFN-α treatment was shown to modulate innate immune parameters in the patients, but without any detectable effect on HBV-specific adaptive immunity. The missing link between the induction of type I IFN and anti-HBV cellular effectors needs to be further investigated in mouse models, including the mechanism of MyD88 participation in activation of the cellular immune response during infection. Elucidating molecular mechanisms between innate pattern sensing and evoking cellular effectors may provide a reasonable explanation for the failure of IFN-treatment in HBV infection.

Collectively, our study validates the use of the hydrodynamic transfection method in mimicking acute HBV infection in 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 the dispensability of RNA sensors for induction of IFN by HBV RNA and the complexity of innate and adaptive immunity during HBV clearance.

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Disclosure Statement

The authors declare no financial or commercial conflict of interest.

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Construction and characterization of an infectious cDNA clone of rat hepatitis E virus

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Abstract

Rat hepatitis E virus (ratHEV) is related to human HEV and has been detected in wild rats worldwide. Here, the complete genome of ratHEV strain R63/DEU/2009 was cloned downstream a T7 RNA polymerase promotor and capped genomic RNA generated by *in vitro* transcription was injected into nude rats. RNA of ratHEV could be subsequently detected in serum and faeces of rats injected intrahepatically, but not in those injected intravenously. The ratHEV RNA-positive faecal suspension was intravenously inoculated into nude rats and Wistar rats leading to ratHEV RNA detection in serum and faeces of nude rats and to seroconversion in Wistar rats. In addition, ratHEV was isolated in PLC/PRF/5 cells from the ratHEV RNA-positive faecal suspension of nude rats, and passaged subsequently. The cell culture supernatant was infectious for nude rats. Genome analysis identified 9 point mutations of the cell culture-passaged virus in comparison to the originally cloned ratHEV genome. The results indicate that infectious ratHEV could be generated from the cDNA clone. Since rats are widely used and well characterized laboratory animals, studies on genetically engineered ratHEV may give novel insights into organ tropism, replication and excretion kinetics as well as immunological changes induced by hepeviruses.

Introduction

Rat hepatitis E virus (ratHEV) was first identified in 2010 in faeces of wild rats from Germany (Johne et al., 2010a). Thereafter, the virus has been detected in animals from several countries of Europe, Asia, and in the USA (Johne et al., 2014a). The host species are mainly rat species (*Rattus norvegicus*, *Rattus rattus*, and other), but ratHEV sequences have also been detected in the Greater Bandicoot (*Bandicota indica*) and the Asian musk shrew (*Suncus murinus*) (Li et al., 2013a; Guan et al., 2013; Johne et al., 2014a).

Analysis of the complete genome sequence indicated that ratHEV is a member of the family *Hepeviridae*, with a distant relationship to the human pathogenic HEV genotypes 1-4 (Johne et al., 2010b). Phylogenetic analysis indicates that ratHEV is most closely related to the recently identified ferret HEV (Raj et al., 2012); both viruses constitute a group clustering between human HEV genotypes 1-4 and hepeviruses from bats and birds (Johne et al., 2014a; Smith et al., 2014). All members of the family *Hepeviridae* have a genome of single-stranded RNA with positive polarity and a length of approximately 7 kb, which is capped at the 5'-end and polyadenylated at the 3'-end. The ratHEV genome contains the open reading frames (ORFs) 1, 2 and 3 encoding a non-structural polyprotein, the capsid protein and a small phosphoprotein, respectively, which are present in all HEV-related viruses. In addition, the ratHEV genome has a small ORF4 overlapping with the 5'-region of ORF1, with unknown function (Johne et al., 2010b; Mulyanti et al., 2014).

67

68 Analysis of the amount of ratHEV-RNA in organs of wild rats indicated a liver tropism of
69 the virus (Johne et al., 2010b). The hepatotropism of ratHEV was also confirmed with
70 laboratory rats intravenously inoculated with ratHEV-containing organ homogenates (Li
71 et al., 2013b). Immunodeficient nude rats inoculated with ratHEV developed persistent
72 infections with long-time shedding of high virus amounts (Li et al., 2013b).
73 Experimentally infected Wistar rats showed transient virus shedding and developed anti
74 ratHEV-specific antibodies (Li et al., 2013b). Clinical signs were not recorded after
75 experimental infection of rats (Li et al., 2013b; Purcell et al., 2011). Preliminary infection
76 trials with rat-derived cell cultures did not result in ratHEV replication (Johne et al.,
77 2010a). However, ratHEV was successfully propagated in the human hepatoma cell
78 lines PLC/PRF/5, HuH-7 and HepG2 recently (Jirintai et al., 2014).
79

80 The zoonotic potential of ratHEV is not clear so far. Experimental infections of rhesus
81 monkeys and pigs with ratHEV did not result in signs of virus replication (Purcell et al.,
82 2011; Cossaboom et al., 2012). Serological analysis of human and porcine sera
83 identified only a few sera showing antibodies with higher reactivity to ratHEV as
84 compared to human HEV, indicating a very rare transmission of ratHEV-related viruses
85 (Dremsek et al., 2012; Krumbholz et al., 2013).
86

87 Reverse genetics systems, which enable the generation of infectious virus from cloned
88 cDNA, have been widely used for site-directed mutagenesis of viral RNA genomes

following various applications in basic and applied virology (Stobart & Moore, 2014; Ye et al., 2014; Hoenen et al., 2011). A reverse genetics system for human HEV has been first developed by Panda et al. (2000). In this system, the cDNA of the genome of a genotype 1 strain was cloned downstream a T7 RNA polymerase promotor. The linearized cDNA clone was transcribed *in vitro* and thereafter transfected into cell cultures. The cell culture supernatant was shown to be infectious for rhesus monkeys; however, direct inoculation of the RNA into the monkeys did not result in generation of virus. Emerson et al. (2001) showed, that a capping step during *in vitro* transcription enables the generation of infectious virus by intrahepatically inoculation into rhesus monkeys and chimpanzees. Similar systems have been subsequently developed for HEV genotypes 3 and 4, rabbit HEV and avian HEV, some of them using transfection of RNA into cell cultures for generation of infectious virus, others use direct inoculation into the liver of laboratory animals (Huang et al., 2005a, b; Yamada et al., 2009; Cordoba et al., 2012; Cossaboom et al., 2014; Kwon et al., 2011).

In order to develop a reverse genetics system for ratHEV, its complete genome was cloned here under control of the T7 RNA polymerase promotor. Capped *in vitro*-transcribed ratHEV RNA was thereafter used to inoculate laboratory rats and the generation of infectious ratHEV was monitored. Generated virus was characterized by infectivity testings in rats and cell cultures as well as by genome sequence analysis. The results should contribute to the development of a reliable reverse genetics system for ratHEV, enabling site-directed mutagenesis and subsequent phenotypic studies on the