Table 3. Quasispecies Complexity and Diversity Among Patients With an Optimal or Partial Response to Entecavir

Variable	Optimal Responders (n = 34)	Partial Responders (n = 63)	*P*
Complexity-normalized Shannon Entropy			
Nucleotide level	0.9673 (0.6868–1)	0.9316 (0.3324–1)	.036
Amino acid level	0.8668 (0.4930–1)	0.7869 (0.2192–1)	.087
Diversity			
d (nucleotide level; 10^{-3} substitutions)	8.7083 (3.2747–36.4403)	5.2904 (0.8906–56.1354)	.019
d (amino acid level; 10^{-3} substitutions)	14.0147 (3.3561–66.2545)	8.6151 (0.8923–94.822)	.032
dS (10^{-3} substitutions/site)	12.4076 (3.8811–42.0139)	7.4706 (0.6533–78.0112)	.015
dN (10^{-3} substitutions/site)	6.6121 (1.6396–30.8776)	3.9649 (0.4040–43.8353)	.039

Abbreviations: d, mean genetic distance; dN, no. of nonsynonymous substitutions per nonsynonymous site; dS, no. of synonymous substitutions per synonymous site.

DNA, while HBV DNA became undetectable in the majority of patients (41/55 [75%]). rt124N variant was found in 9 of 14 patients (64%) with a detectable year 3 HBV DNA level and in 18 of 41 patients (44%) with an undetectable year 3 HBV DNA level ($P = .314$).

We further investigated the role of rt124N in patients who tested positive for HBeAg and had a high HBV DNA level at baseline, the 2 risk factors for suboptimal responses at year 1 that were identified in the present study, and also for a slower 3-year entecavir response, which was identified by a previous study [25]. Among the 55 suboptimal responders with year 3 HBV DNA data available, 32 (58%) were HBeAg positive and had an HBV DNA level of > 8 logs at baseline. Of these 32 HBeAg-positive patients with a high baseline HBV DNA level, 9 (28%) and 23 (72%) had detectable and undetectable HBV DNA at year 3, respectively. The proportion of patients with rt124N was higher in those with detectable year 3 HBV DNA (7/9 [78%]) than in those with undetectable year 3 HBV DNA (8/23 [35%]; $P = .049$).

DISCUSSION

The present study showed that 21% of patients still had detectable HBV DNA after 1 year of therapy, which is comparable to the detectability rate of HBV DNA at year 1 in other studies [3, 5, 6, 25–27]. Although HBV DNA may become undetectable in some partial responders upon long-term treatment, it has been shown that patients with detectable HBV DNA after 1 year of entecavir treatment have a lower probability of having undetectable HBV DNA at year 3 [3]. This partial treatment response also has a long-term clinical implication, as it has been demonstrated that patients with intermediate levels of HBV DNA still carry an increased risk of development of hepatocellular carcinoma and cirrhosis-related complications [28]. A recent study has also demonstrated that a posttreatment cutoff HBV DNA level of < 2000 IU/mL is not sufficient to indicate a reduced probability of disease progression, pinpointing the importance of complete HBV DNA suppression (to a level of <12 IU/mL) as the so-called optimal response [29]. Thus, investigating the molecular reasons for the slow/incomplete virological response to entecavir is of clinical importance. To examine this, studies with 2 important properties are required: (1) a large number of subjects for testing, because only 20%–30% of patients are expected to have suboptimal responses, and (2) a sensitive and robust viral sequence revelation of the whole genomic constitution of the HBV rt region. Our present study is specifically designed to solve these issues.

In the present study, HBV rt sequence analysis of 64 partial responders and 241 optimal responders showed that 4 variations (rt53N, rt118N, rt124N, and rt332S) were found to be present in a higher proportion in the partial responders. Detailed analyses showed that these 4 rt variants were also associated with other baseline parameters, such as HBV genotype and cirrhosis. In silico analysis showed that they were mostly associated with HBV genotype B, which was found to be more prevalent in the partial responders in the present study. However, logistic regression analysis showed that HBV genotype B was not an independent factor for partial response to entecavir. This is in accordance with the findings observed in other studies that the difference in the entecavir response has not been observed with different HBV genotypes in different studies [5, 6]. The prevalence of the 4 rt variants was also lower in patients with cirrhosis, which was also found to be associated with a better entecavir response in this present study. This is in accordance with a recent study showing that cirrhosis predicts early HBV DNA clearance upon entecavir therapy [30]. However, it should be noted that the patients with cirrhosis were generally HBeAg negative, had a lower HBV DNA level, and were older ($P = .013$, .002, and < .0001, respectively; data not shown), all of which were associated with a better entecavir response. Thus, a multivariate analysis was performed to identify the independent factors associated with a partial entecavir response.

Multivariate analysis showed that high baseline HBV DNA level, HBeAg positivity, and the rt variant rt124N were

associated with a partial entecavir response. This agrees with a previous study from our center, which found that patients who tested positive for HBeAg and had an HBV DNA level of ≥8 logs copies/mL (7.3 logs IU/mL) had a lower rate of HBV DNA undetectability at years 1–3 [25]. One novel finding of the present study is that the rt124N variant was significantly associated with a partial entecavir response at year 1. Furthermore, rt124N may also be associated with a higher chance of persistently detectable HBV DNA at year 3. Although the percentage of subjects with the rt124N variant was comparable between patients with detectable and those with undetectable HBV DNA at year 3 (64% vs 44%, respectively), it may be due to the limited number of subjects being tested. In particular, among the HBeAg-positive patients with a high baseline HBV DNA level, rt124N was significantly more frequently found in the patients who still had detectable HBV DNA at year 3. These data suggested that rt124N, although not the sole factor, is associated with a slower long-term response to entecavir, particularly when other coexisting adverse factors are present.

At the molecular level, the effect of rt124N on entecavir response was further studied by adopting our molecular docking simulation model. HBV rt124 is located in the rt fingers domain, but it is not inside the catalytic pocket within the palm domain of rt [17, 31]. Thus, it is less likely that rt124 variants will cause a remarkable conformational change to the entecavir binding site. Nevertheless, our molecular docking simulation model indicated that rt124N caused a slight interference with entecavir binding, suggesting that the rt124N may be slightly less susceptible to entecavir, without completely abolishing the binding. This may partly explain the slower response to entecavir in some patients who still achieve undetectable HBV DNA upon continuation of treatment beyond the first year. However, this modeling is based on the assumption that HBV rt forms a dimerized structure like that of HIV-1 rt. Should the crystal structure of HBV rt be available, the steric effect of HBV rt124N to entecavir binding will have to be confirmed with an HBV-based model. It should also be noted that the direct effect of rt124N should be best studied by an in vitro phenotypic assay. Nevertheless, this present study served as an initial identification of the HBV variants. An in vitro phenotypic study is required as a subsequent confirmation.

Another interesting finding from the clonal sequencing data is that the optimal responders had a higher quasispecies complexity and diversity than the partial responders. Studies of quasispecies complexity and diversity in NA-treated patients with chronic hepatitis B are rare. Our present finding differs from that in a recent study by Liu et al, which involved 31 entecavir recipients and showed that the baseline quasispecies complexity and diversity are comparable between the optimal and partial responders [21]. However, our study had a greater power because of the greater number of patients (n = 97) being studied.

The reason for the higher baseline quasispecies complexity and diversity in optimal responders in this present study is unclear. From the classical view of viral genetics, high quasispecies diversity would imply that the population has an increased possibility to harbor drug-resistant variants. However, according to the quasispecies theory, all variants within a viral population form an interacting network and react as a whole unit in response to stimulants such as antiviral therapy [19]. Under this quasispecies theory, a high quasispecies diversity may imply that the viral population reaches a critical status, termed self-organized criticality, in which the quasispecies network is maintained at an optimal capability [32]. At such a self-organized criticality status, the viral quasispecies population would be extremely sensitive to external perturbation [19, 33]. Therefore, a viral population with a high quasispecies diversity will be more prone to the external pressure exerted by entecavir. In accordance with the same line of thought, it has been demonstrated that higher baseline HCV quasispecies diversity and complexity are associated with an early virological response to interferon and ribavirin therapy [33]. It is also possible that, in the optimal responders with higher baseline complexity and diversity, the viral quasispecies were "less-fit" subpopulations coexisting in the total viral population with no single "good-fit" and replicatively competent dominant strain. Consequently, they are more susceptible to entecavir.

In conclusion, apart from the known factors of high baseline HBV DNA level and HBeAg positivity, a novel single nucleotide polymorphism, rt124N, was found to be a significant factor associated with partial entecavir response. In addition, lower baseline quasispecies complexity and diversity were also found to be associated with partial response to entecavir. These 4 factors may exert additive or synergistic influences on the suboptimal responsiveness to entecavir treatment.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Review Article

Immunobiology of hepatitis B virus infection

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The adaptive immune response, particularly the virus-specific CD8⁺ T-cell response, is largely responsible for viral clearance and disease pathogenesis during hepatitis B virus (HBV) infection. The HBV-specific CD8⁺ T-cell response is vigorous, polyclonal and multispecific in acutely infected patients who successfully clear the virus and relatively weak and narrowly focused in chronically infected patients. The immunological basis for this dichotomy is unclear. A recent study using HBV transgenic mice and HBV-specific T-cell receptor transgenic mice suggests that intrahepatic antigen presentation by HBV positive hepatocytes suppresses HBV-specific CD8⁺ T-cell responses through a co-inhibitory molecule, programmed cell death 1 (PD-1). In contrast, antigen presentation by activated professional antigen-presenting cells induces functional differentiation of HBV-specific CD8⁺ T cells. These findings suggest that the outcome of T-cell priming is largely dependent on the nature of antigen-presenting cells. Another study suggests that the timing of HBV-specific CD4⁺ T-cell priming regulates the magnitude of the HBV-specific CD8⁺ T-cell response. Other factors that could regulate HBV-specific cellular immune responses are high viral loads, mutational epitope inactivation, T-cell receptor antagonism and infection of immunologically privileged tissues. However, these pathways become apparent only in the setting of an ineffective cellular immune response, which is therefore the fundamental underlying cause. Understanding the cellular and molecular mechanisms by which HBV evades host immune responses will eventually help develop new immunotherapeutic strategies designed to terminate chronic HBV infection.

Key words: hepatitis B virus, immune response, immunological priming, immunotherapy, T cells

INTRODUCTION

HEPATITIS B VIRUS (HBV) is a partially double-stranded DNA virus that causes necroinflammatory liver disease of variable severity.[1] More than 240 million people worldwide are persistently infected with HBV. Persistent infection by HBV is often associated with chronic liver disease that can lead to the development of cirrhosis and hepatocellular carcinoma (HCC). HBV is thought to be largely non-cytopathic for the infected hepatocyte under normal circumstances,[2,3] but could be cytopathic in immunodeficient chimeric mice carrying human hepatocytes,[4] suggesting that unmitigated HBV replication and gene expression trigger cellular stresses and cell death. The immune response mediates the clearance of HBV and disease pathogenesis.[2] Interestingly, HBV appears to be a poor inducer of innate immune responses, acting as a stealth virus in this regard by evading recognition by innate immune sensor molecules.[5] Despite the weak innate immune responses, acute HBV infection of adults is usually self-limited and cleared by functional HBV-specific CD8⁺ T-cell responses. The induction of functional HBV-specific CD8⁺ T-cell responses is dependent on early CD4⁺ T-cell priming prior to HBV spread,[6] which might explain why patients infected with HIV become persistently infected following horizontal transmission of HBV.[7,8] Once chronic HBV infection is established, HBV-specific CD8⁺ T cells are subjected to powerful immunoregulatory mechanisms in the liver. Ample evidence suggests that HBV actively suppresses adoptive immune responses, particularly HBV-specific CD8⁺ T-cell responses. Reduction of viral antigen expression by antiviral treatment

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can overcome CD8+ T-cell hyporesponsiveness in patients with chronic HBV infection, suggesting that T cells are present in these subjects but suppressed by high viral loads.[9] In addition, recent evidence suggests that antigen recognition in the liver suppresses HBV-specific CD8+ T-cell effector functions by signaling through a co-inhibitory molecule, programmed cell death 1 (PD-1).[10,11] Furthermore, several viral proteins have been shown to regulate the adaptive immune response to HBV,[12–14] which may explain the varying chronicity rate between genotypes that express different levels of viral antigens.[15,16] We herein review our current knowledge of the host immune responses to HBV as well as the cellular and molecular mechanisms by which HBV evades these immune responses. We also discuss how the accumulating knowledge of HBV immunobiology will help us to develop more inventive therapeutic strategies that are designed to reinvigorate HBV-specific immune responses, thereby terminating chronic infection with this deadly virus.

INNATE IMMUNE RESPONSE

Absence of an innate response by infected cells during acute HBV infection

VIRUS INFECTION USUALLY triggers a robust innate immune response that is heralded by rapid induction of interferon (IFN)-α/β by the infected cell.[17] Production of IFN-α/β induces the transcriptional expression of a large number of IFN-inducible genes (ISG), which in turn exert a variety of intracellular antiviral mechanisms.[17,18] Surprisingly, intrahepatic gene expression profiling in acutely HBV-infected chimpanzees revealed that HBV acts like a stealth virus early after infection because it does not induce any cellular gene expression, including ISG, as it spreads through the liver.[5] This contrasts strikingly with the induction of many ISG during the spread of hepatitis C virus that is highly visible to the innate immune system.[19] The relative invisibility of HBV to the innate sensing machinery of the cells probably reflects its replication strategy. HBV retains its transcriptional template, namely cccDNA, in the nucleus[20] and the HBV mRNA is capped and polyadenylated, resembling normal cellular transcripts.[21,22] In addition, HBV replicates within viral capsid particles in the cytoplasm[20] and is therefore shielded from innate sensing machinery. Thus, the typical widespread expansion of HBV in the liver may reflect the absence of IFN-α/β production to which the virus is exquisitely sensitive, as shown in HBV transgenic mice.[23,24]

Natural killer and natural killer T-cell responses during HBV infection

The role of natural killer (NK) cells in acute HBV infection remains largely unknown. Studies that analyzed NK-cell functions during acute HBV infection have provided conflicting evidence. A recent study demonstrated that the activation and cytokine-producing ability of NK cells were impaired in acute HBV patients,[25] while others showed enhanced IFN-γ production by, and cytotoxicity of, NK cells.[26,27] The cause of this discrepancy is unclear. On the other hand, NK cells in chronic HBV patients seem generally cytolytic but their cytokine-producing ability is selectively impaired.[28,29] A recent report demonstrated a strong correlation between liver disease, IFN-α production and NK-cell expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) during chronic HBV infection,[30] suggesting a pathogenic role of NK cells (Fig. 1, grey area). Interestingly, NK cells seem highly immunoregulatory as well, because they can apparently delete HBV-specific, but not cytomegalovirus-specific, CD8+ T cells through TRAIL–TRAIL receptor 2 interaction.[31] Taken together, NK cells appear to exert a detrimental effect on the host during chronic HBV infection. The precise role of NK cells during chronic HBV infection must be fully elucidated to develop new NK-cell-based therapeutic approaches against chronic HBV infections. The role of natural killer T (NKT) cells during natural HBV infection is even less clear. The therapeutic potential of NKT-cell activation against HBV infection was initially indicated in a HBV transgenic mice model,[32] and subsequent studies with HBV and woodchuck hepatitis virus suggested that NKT cells were activated soon after infection.[33,34] However, the degree of NKT-cell activation was relatively modest in both cases, and the physiological importance of such activation has not been addressed in these studies. Studies with several mouse models provided more convincing evidence that CD1d-restricted NKT cells are important for the control of HBV replication and gene expression.[35,36] It is well known, however, that hepatic NKT-cell repertoires are phenotypically and functionally very different between humans and mice.[37] Further studies are required to confirm the role of NKT cells during HBV infections.

ADOPTIVE IMMUNE RESPONSE

Antibody response

ANTIBODIES TO THE hepatitis B surface antigen (HBsAg) neutralize HBV infection presumably by preventing virus attachment to hepatocytes. They also

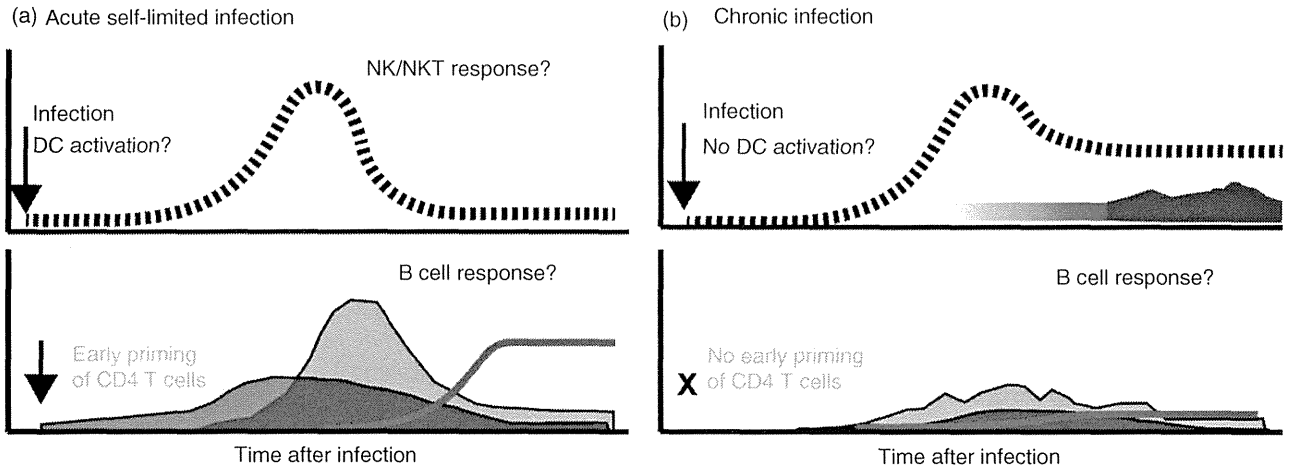

Figure 1 *Schematic representation of immune responses during acute and chronic hepatitis B virus (HBV) infections.* (a,b) Schematic representation of the time course of serum HBV titer and immune responses during acute and chronic HBV infections. The upper panel in each figure represents the timing of HBV infection as an arrow, the serum HBV DNA as a dashed black line, and the natural killer (NK) cell response as gray area. The lower panel in each figure represents the timing of CD4 T-cell priming to HBV as an arrow, the CD4 T-cell response as red area, the CD8 T-cell response as green area, the blue line as the antibody response. ▮▮▮▮, HBV titer; ▬, NK response; ▬, CD4 T-cell response; ▬, CD8 T-cell response; ▬, anti-HBs. DC, dendritic cells; NKT, natural killer T cell.

facilitate viral clearance by complexing with free viral particles and removing them from the circulation. The induction of anti-hepatitis B surface (HBs) by immunization is a T-cell-dependent process and requires $CD4^+$ T-cell help.[38] While anti-HBs neutralizing antibodies are essential for providing protection after vaccination, their importance in resolving natural HBV infection is somewhat underappreciated, mostly because the appearance of anti-HBs occurs relatively late after HBV exposure (Fig. 1, blue lines).[39] It is generally believed that anti-HBs neutralizing antibodies prevent viral spread from the rare cells that remain infected, even after resolution of HBV infection. Supporting this notion, HBV is frequently reactivated long after resolution of infection in patients who receive conventional chemotherapy or a newly developed monoclonal antibody (rituximab) therapy that targets the CD20 antigen expressed on the surface of normal and malignant B lymphocytes.[40] More detailed analysis of HBV-specific B-cell responses may reveal a heretofore unappreciated role of humoral responses in the control of HBV infection.

$CD4^+$ T-cell response

Ample evidence suggests a clear relationship between the $CD4^+$ T-cell response to HBV and the outcome of HBV infection. For example, the peripheral blood $CD4^+$ T-cell response to HBV is vigorous and multispecific in patients with acute hepatitis who ultimately clear the virus, while it is relatively weak in chronic HBV patients (Fig. 1, red areas).[39,41,42] Despite the association between a strong $CD4^+$ T-cell response and viral clearance, $CD4^+$ T-cell depletion at the peak of HBV infection had no effect on viral replication and liver disease in infected chimpanzees.[43] In contrast, $CD4^+$ T-cell depletion before HBV infection resulted in quantitatively and qualitatively reduced HBV-specific $CD8^+$ T-cell responses, leading to persistent HBV infection.[6] These results suggest that $CD4^+$ T cells probably contribute indirectly to the control of HBV infection by facilitating the induction and maintenance of the virus-specific $CD8^+$ T-cell responses. It is likely that $CD4^+$ T cells help induce functional HBV-specific $CD8^+$ T-cell responses by producing interleukin (IL)-2 that is essential for T-cell proliferation and by activating professional antigen-presenting cells (pAPC) that are capable of providing co-stimulation to T cells.[44] In contrast, in the absence of early $CD4^+$ T-cell responses, $CD8^+$ T-cell priming could occur in the liver, resulting in T-cell inactivation, tolerance or apoptosis,[11,45,46] as summarized later in this review. This requirement of $CD4^+$ T cells for the induction of HBV-specific $CD8^+$ T cells is one of the reasons patients infected with HIV (low CD4 count) become persistently infected after horizontal transmission of HBV[7,8] and why genetic variants in the human leukocyte antigen DP locus are associated with the risk of developing chronic HBV infection.[47] Interestingly, early $CD4^+$

T-cell priming does not occur when the size of inoculum is small (i.e. 1–10 copies) and the appearance of HBV DNA is delayed after infection,[6] suggesting that slow spread of HBV may facilitate chronicity, as often observed with genotype A infections.[15,16] Interestingly, recent studies indicated that the frequency of IL-17-producing CD4 T cells (T-helper [Th]17 cells) is increased in chronic HBV patients,[48] and IL-17 and another Th17-related cytokine IL-22 are shown to exacerbate liver disease.[49] These results suggest that CD4 T cells not only help induce antiviral cellular immune responses during acute HBV infections, but also mediate inflammatory responses during chronic HBV infection.

CD8⁺ T-cell response

The HBV-specific CD8⁺ T-cell response plays a fundamental role in viral clearance and liver disease. A vigorous polyclonal CD8⁺ T-cell response is readily detectable in the peripheral blood of patients with acute hepatitis B who ultimately clear the virus. In contrast, the peripheral blood T-cell response in chronically infected patients is weak and narrowly focused (Fig. 1, green areas).[2,50] The livers of these patients contain virus-specific T cells that likely contribute to disease pathogenesis but, for functional and/or quantitative reasons, are unable to clear the infection. Interestingly, a recent study that examined the relationship between the number of intrahepatic HBV-specific CD8⁺ T cells, extent of liver disease and levels of HBV replication in chronically infected patients indicated that inhibition of virus replication could be independent of liver damage and that the functionality of HBV-specific CD8⁺ T cells was more important than the number of T cells in controlling HBV replication.[51] Experiments in chimpanzees have shown that the viral clearance and the onset of liver disease coincide with the accumulation of virus-specific CD8⁺ T cells and the induction of IFN-γ and ISG in the liver.[52] Importantly, depletion of CD8⁺ T cells at the peak of viremia delays viral clearance and the onset of viral hepatitis until the T cells return, providing the most definitive evidence that viral clearance and liver disease are mediated by virus-specific CD8⁺ T cells.[43]

NON-CYTOLYTIC AND CYTOLYTIC T-CELL EFFECTOR FUNCTIONS EMERGE ALTERNATELY DURING HBV CLEARANCE

IT IS WIDELY believed that virus-specific CD8⁺ T cells clear the viral infection by killing infected cells. However, CD8⁺ T-cell killing is an inefficient process, requiring direct physical contact between the CD8⁺ T cells and the infected cells. Therefore, it may not be possible for HBV-specific CD8⁺ T cells to kill all the HBV infected cells if the CD8⁺ T cells are greatly outnumbered, as occurs during HBV infection in which as many as 10^{11} hepatocytes can be infected.[53] Hence, although the liver disease in HBV infection is clearly due to the cytopathic activity of the CD8⁺ T cells, viral clearance may require more efficient CD8⁺ T-cell functions than killing. Important insights into the pathogenic and non-cytopathic antiviral functions of the CD8⁺ T-cell response have come from studies of HBV transgenic mice that develop an acute necroinflammatory liver disease after adoptive transfer of HBV-specific effector or effector memory CD8⁺ T cells that were generated by immunizing non-transgenic mice.[10,54] In that model, the HBV-specific CD8⁺ T cells rapidly enter the liver and recognize viral antigen and secrete IFN-γ, which non-cytopathically inhibits HBV replication in the remaining hepatocytes[54] by preventing the assembly of HBV RNA-containing capsids in the cytoplasm[23,55] in a proteasome-[56] and kinase-dependent[57] process. During this remarkable process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes, yet the hepatocytes remain perfectly healthy.[54] Antibody blocking and knockout experiments in the HBV transgenic mouse model further demonstrated that the cytopathic and antiviral functions of CD8⁺ T cells are completely independent of each other.[58,59] Following the secretion of IFN-γ and the suppression of HBV replication, HBV-specific CD8⁺ T cells expand vigorously *in situ*. Surprisingly, however, the ability of HBV-specific CD8⁺ T cells to produce IFN-γ is quickly downregulated as they expand. Despite the suppression of IFN-γ secretion, the cytotoxic capabilities of the HBV-specific CD8⁺ T cells increase over time, causing necroinflammatory liver disease and complete inhibition of HBV mRNA expression.[10] Taken together, these results suggest that, in the context of a vigorous immune response during acute HBV infection, the sequential and altering patterns of non-cytolytic and cytolytic effector function, coupled with T-cell expansion and contraction, cooperate to minimize the extent of tissue injury required for viral clearance. Conversely, we suggest that diminution of the CD8⁺ T-cell response or poor coordination of these responses could lead to viral persistence and varying degrees of chronic liver disease.

WHERE, HOW AND WHAT KIND OF HBV-SPECIFIC CD8⁺ T CELLS ARE GENERATED?

THE RESULTS DESCRIBED above were generated by adoptively transferring splenocytes from

HBV-immunized mice to HBV transgenic mice. While these results suggest that intrahepatic antigen recognition has a profound impact on the distribution, expansion and effector functions of primed effector-memory $CD8^+$ T cells, they may not reflect the events that occur when a naïve individual becomes infected with HBV, because immunologically naïve T cells are known to behave very differently than memory T cells.[60–62] Accordingly, we generated transgenic mice whose $CD8^+$ T cells express T-cell receptors (TCR) specific for HBV, to study where and how immunologically naïve T cells are primed in response to intrahepatic HBV.

When HBV-specific naïve $CD8^+$ T cells were adoptively transferred into HBV transgenic mice, they were predominantly activated rapidly in the liver, independent of T-cell homing to lymphoid organs, suggesting that HBV-specific native T cells are primed in the liver.[11] While this intrahepatic priming of HBV-specific $CD8^+$ T cells challenges the current T-cell priming model postulating that naïve T cells are primed by activated pAPC in lymphoid organs,[63,64] several studies previously indicated that the liver is an exception to this rule.[45,46,65] This rather unusual ability of the liver to prime naïve T cells presumably reflects the unique architecture of the hepatic sinusoid, which is characterized by a discontinuous endothelium, the absence of a basement membrane and a very slow flow rate,[66] allowing circulating T cells to make prolonged direct contact with resident liver cells, including hepatocytes. Furthermore, the liver is replete with diverse and unique antigen-presenting cell populations, including liver sinusoidal endothelial cells (LSEC), hepatic stellate cells, Kupffer cells, and conventional and plasmacytoid dendritic cells (DC), all of which are capable of priming and/or tolerizing naïve T cells.[67] In our model, HBV-specific $CD8^+$ T cells are presumably primed by HBV-expressing hepatocytes, because HBV-specific $CD8^+$ T cells were activated when co-cultured with hepatocytes isolated from HBV transgenic mice but not with LSEC, liver residential DC or Kupffer cells. Importantly, the intrahepatically primed HBV-specific $CD8^+$ T cells expand vigorously in the liver but are functionally impaired because they do not express IFN-γ or the cytolytic granule granzyme B, suggesting that intrahepatic priming by HBV-expressing hepatocytes expands functionally defective $CD8^+$ T cells. Consequently, such intrahepatically primed T cells are incapable of suppressing HBV gene expression or inducing liver disease.[11] Interestingly, functionally defective HBV-specific $CD8^+$ T cells expressed a co-inhibitory molecule, PD-1, after antigen recognition in the liver.[11] Furthermore, a significant fraction of PD-1-deficient HBV-specific naïve T cells expanded more vigorously than PD-1 positive controls, and differentiated into effector T cells capable of producing IFN-γ, as well as granzyme B, which in turn suppressed HBV gene expression and caused liver disease.[11] Collectively, these results suggest that PD-1 signaling impairs the effector functions of HBV-specific $CD8^+$ T cells. Importantly, PD-1 expression was also observed on HBV-specific $CD8^+$ T cells in chronic HBV patients,[68] indicating that PD-1-mediated functional suppression is active during natural HBV infection. Taken together, these results suggest that intrahepatic priming induces T-cell tolerance and such tolerance is at least partially mediated by PD-1 signaling. This PD-1-meditated intrahepatic suppression may be the underlying cause of the weak T-cell responsiveness during chronic HBV infection.

OTHER MECHANISMS OF HBV PERSISTENCE

THE POTENTIAL MECHANISMS by which HBV evades the immune responses are listed in Table 1. Neonatal tolerance to HBV is probably responsible for viral persistence following mother–infant transmission.[69,70] The underlying cause of adult-onset chronic HBV infection is probably multifaceted. Potential contributing factors include mutational escape leading to inactivation of B- and T-cell epitopes and specific inhibition of the adaptive immune response by viral proteins.[71] For example, hepatitis B e-antigen (HBeAg) has

Table 1 Causes of HBV persistence

Potential mechanisms of HBV persistence
• Neonatal tolerance
• Peripheral deletion/exhaustion (due to high viral load and negative signaling)
• Anergy
• Ignorance
• Weak CD4 T-cell response (associated with low viral titer and HIV infection)
• Reduced dendritic cell numbers and functions
• Negative signaling (PD-1/CTLA-4/Tim-3)
• Immunosuppressive microenvironment (IL-10, TGF-β)
• Regulatory T cells/myeloid-derived suppressor cells
• Escape mutations in T- and B-cell epitopes
• Inhibit antigen processing or presentation
• Inhibit cytokine signaling

CTLA-4, cytotoxic T-lymphocyte antigen-4; HBV, hepatitis B virus; IL, interleukin; PD-1, programmed cell death 1; Tim-3, T-cell immunoglobulin and mucin 3; TGF, transforming growth factor.

been shown to suppress the antibody and T-cell response to hepatitis B core antigen in T-cell receptor transgenic mice.[12,13] The immunosuppressive potential of HBeAg may explain the clinical observation that viral mutations precluding the production of HBeAg are often associated with exacerbations of liver disease and, sometimes, even with viral clearance from chronically infected patients.[72,73] HBsAg may also prevent immune elimination of infected cells by functioning as a high-dose tolerogen, because extremely high serum HBsAg titers in the mg/mL range are often seen in chronically infected patients[74,75] and chronically infected patients have an absence or subnormal levels of HBsAg-specific $CD8^+$ T cells.[75] In addition, HBV X protein, a transcriptional transactivator that is required for the initiation of infection,[76,77] can inhibit cellular proteasome activity when it is overexpressed[78] and may interfere with antigen processing and presentation. Finally, genotypic variation clearly influences the outcome of HBV infection. Up to 10% of adult-onset infections with genotype A become persistent, while less than 5% of infections with other genotypes do so.[15] The precise mechanism for the difference is unclear, but the high levels of HBsAg expression and low replication activity of genotype A infections may facilitate immune evasion.[16]

In both neonate and adult onset of chronic HBV infections, weak CD4 and CD8 T-cell responses are clearly responsible for the viral persistence. The mechanism that suppresses HBV-specific immune responses during chronic HBV infection is likely multifactorial. In addition to PD-1, other negative signaling molecules, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and T-cell immunoglobulin and mucin 3 (Tim-3), are expressed on functionally compromised HBV-specific CD8 T cells in chronic HBV patients.[79] For example, a fraction of CD8 T cells in patients with chronic hepatitis B express the co-inhibitory molecule CTLA-4, and its expression level is correlated with serum HBV DNA titer and pro-apoptotic protein Bim expression. Blockade of CTLA-4 increases the expansion of IFN-γ producing HBV-specific CD8 T cells *in vitro*. Similarly, Tim-3 is also highly expressed on functionally impaired HBV-specific CD8 T cells in chronic HBV patients, and the blockade of Tim-3 *in vitro* restored effector functions of the CD8 T cells,[79] suggesting that multiple layers of negative co-regulation contribute to T-cell exhaustion in chronic HBV infection. Interestingly, Tim-3-expressing CD8 T cells appear to have a immunosuppressive function similar to regulatory T cells (Treg), limiting proliferation of antigen-specific effector T cells *in vitro* and *in vivo*.[80] Extrinsic factors could also contribute to the reduced T-cell responses during chronic HBV infection. The livers of chronic HBV patients have been shown to express elevated levels of IL-10,[81] transforming growth factor-β[82] and arginase,[83] all of which are known to suppress cellular immune responses. These immunosuppressive soluble factors may be produced by regulatory cells, as the frequency of Treg is increased in chronic HBV patients.[84] Interestingly, recently identified myeloid-derived suppressor cells have been shown to induce T-cell tolerances in mouse models of HBV infection.[85,86] In addition, several studies suggest that the number and functionality of DC are reduced in chronic HBV patients compared with healthy donors, and the reduction of HBV DNA titer by a nucleotide analog adefovir dipivoxil significantly restored the number and functionality of DC. Caution should be exercised, however, to interpret the physiological relevance of the antigen non-specific immunosuppression during chronic HBV infections, because chronic HBV patients are no more susceptible than healthy people to other infectious agents such as influenza virus.

NEW IMMUNOTHERAPEUTIC STRATEGIES TO CURE CHRONIC HBV INFECTIONS

THE MANAGEMENT OF chronic HBV has improved significantly in the last decade, mainly because of the development of very effective and safe nucleoside analogs (NA) that primarily inhibit reverse transcription and DNA replication.[87] Although NA strongly inhibit HBV replication and have much fewer side-effects than IFN-based therapies, they usually do not achieve sustained viral suppression. Failure to sustain viral suppression by NA presumably reflects the persistence of cccDNA, which is untouched by reverse transcriptase inhibitors. Because the elimination of cccDNA usually requires the turnover of infected hepatocytes, the induction of functional HBV-specific $CD8^+$ T cells that can specifically kill virus-infected hepatocytes has been deemed the most promising approach to cure chronic HBV infections. Immunization of chronically infected patients is the most straightforward approach to boost HBV-specific $CD8^+$ T-cell responses. Several strategies have been tested in clinical trials with disappointing results,[88] indicating that more inventive approaches are required to induce functional HBV-specific $CD8^+$ T-cell responses. There are three approaches that could effectively reinvigorate HBV-specific $CD8^+$ T-cell responses (Table 2).

The first approach is to block signaling pathways that negatively regulate HBV-specific $CD8^+$ T-cell responses.

Table 2 Immunotherapeutic strategies to terminate chronic HBV infection

Strategies to reinvigorate T-cell responses
1. Block negative signaling pathways (e.g. PD-1, CTLA-4, Tim-3)
2. Stimulate pAPC (e.g. anti-CD40, TLR) and cytokine signaling
3. Redirect peripheral T cells with high avidity TCR

CTLA-4, cytotoxic T-lymphocyte antigen-4; HBV, hepatitis B virus; pAPC, professional antigen-presenting cells; PD-1, programmed cell death 1; TCR, T-cell receptor; Tim-3, T-cell immunoglobulin and mucin 3; TLR, Toll-like receptor.

As described in "Where, How and What Kind of HBV-Specific CD8+ T Cells are Generated", we showed that blockade of PD-1 signaling induced functional differentiation of intrahepatically primed, HBV-specific CD8 T cells in a HBV transgenic mouse model, indicating that PD-1 could be a new therapeutic target to treat chronic HBV infection. HBV-specific CD8 T-cell responses may be restored by blocking other negative signaling molecules, such as CTLA-4, Tim-3 and arginase, as well as by depleting Treg. More evidence is needed to support this approach.

The second approach is to provide extra stimulus to override negative signaling that suppresses effector functions of HBV-specific CD8 T cells. One of the promising approaches is to activate pAPC, particularly DC. Activated DC are capable of providing the second and third signals necessary for the functional differentiation of HBV-specific $CD8^+$ T cells. Indeed, we showed that activation of myeloid DC (mDC) with an agonistic anti-CD40 antibody (αCD40) restored HBV-specific $CD8^+$ T-cell responses that are otherwise suppressed by PD-1 signaling.[11] Interestingly, PD-1 expression on the functional HBV-specific $CD8^+$ T cells is strongly downregulated in αCD40-treated HBV transgenic mice. These results suggest that activation of mDC directly or indirectly suppresses PD-1 expression, thereby rescuing HBV-specific $CD8^+$ T cells from PD-1-mediated functional suppression. The data also illustrate the therapeutic potential of mDC activation by αCD40 for the treatment of chronic HBV infection. Other signaling pathways that stimulate pAPC, such as Toll-like receptor signaling pathways, should be tested for their ability to restore HBV-specific $CD8^+$ T-cell responses. In addition, cytokines required for T-cell expansion and differentiation, particularly common γ-chain cytokines such as IL-2, IL-4, IL-7, IL-15 and IL-21, could serve as effective adjuvants for a therapeutic vaccine against chronic HBV infections.

The third approach is to genetically engineer peripheral blood T cells from chronic HBV patients to express TCR specific for HBV-derived epitopes. The first and second strategies described above require the presence of HBV-specific $CD8^+$ T cells that can efficiently recognize HBV-derived T-cell epitopes presented on infected hepatocytes. However, such T cells are often deleted in chronic HBV patients after years of exposure to HBV antigens. To circumvent this problem, genes encoding TCR that efficiently recognize HBV-derived epitopes can be introduced into the patient's peripheral blood T cells using retroviral or lentiviral vectors. These TCR redirected autologous T cells could be subsequently transferred into the chronic HBV patient. Such approaches are employed in treating cancer patients[89,90] and the results so far have been very encouraging. In addition, T cells with redirected specificity toward the HBV envelope protein have been shown to recognize and lyse HCC cell lines with natural HBV DNA integration in *in vitro* and animal models.[91] Safety concerns, cost-effectiveness, and ethical issues should be fully addressed before this promising approach is adapted to chronic HBV patients.

SUMMARY AND CONCLUSIONS

IN SUMMARY, HBV acts like a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response several weeks later. The relative invisibility of HBV to the innate sensing machinery of the cells probably reflects its replication strategy, with the replicating viral genome being sheltered within viral capsid particles in the cytoplasm. On the other hand, HBV can be controlled when properly activated HBV-specific $CD8^+$ T cells enter the liver, recognize antigens, kill infected cells and secrete IFN-γ, which triggers a broad-based cascade that amplifies the inflammatory process and has non-cytopathic antiviral activity against HBV. However, HBV-specific $CD8^+$ T-cell responses are subjected to a powerful immunoregulatory mechanism in the liver and the induction of an effective HBV-specific $CD8^+$ T-cell response is dependent on activation of pAPC. Failure to activate pAPC induces functionally impaired $CD8^+$ T-cell responses due to intrahepatic priming by HBV-expressing hepatocytes, leading to persistent infection (Fig. 2). The precise mechanism by which pAPC are activated during HBV infection remains to be elucidated but HBV-specific $CD4^+$ T cells probably play a critical role. The functional impairment is mediated, at least partially, by PD-1 signaling but other co-inhibitory molecules are likely to be

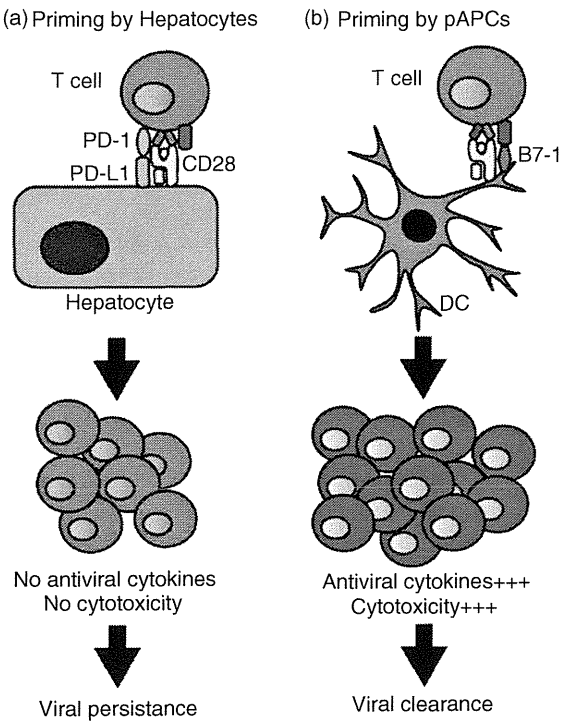

Figure 2 Outcome of T-cell priming is determined by the antigen-presenting cell population. (a) Hepatitis B virus (HBV)-specific naive CD8 T cells that are primed by HBV positive hepatocytes receive co-inhibitory signals rather than co-stimulatory signals, resulting in the expansion of functionally impaired CD8 T cells. (b) T-cell priming by professional antigen-presenting cells (pAPC), such as dendritic cells (DC), that can deliver co-stimulatory signals is presumably required to induce functional HBV-specific CD8 T-cell responses.

involved as well. Thus, activating pAPC and suppressing PD-1 and other negative signaling pathways may have therapeutic potential to treat chronic HBV patients. Adoptive transfer of genetically engineered T cells with redirected specificity towards HBV may represent a powerful immune strategy to cure chronic HBV infection, but safety, cost and ethical issues should be fully addressed before clinical trials.

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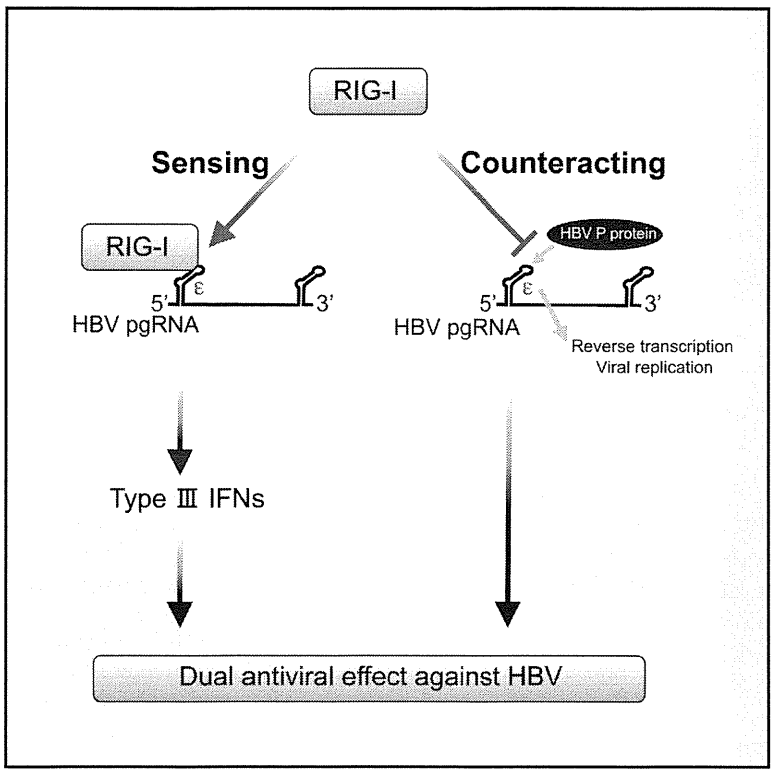
Immunity

The RNA Sensor RIG-I Dually Functions as an Innate Sensor and Direct Antiviral Factor for Hepatitis B Virus

Graphical Abstract



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In Brief

The sensing mechanism of hepatitis B virus (HBV) and the subsequent signaling events remain to be fully clarified. Sato and colleagues demonstrate that the RNA sensor RIG-I not only senses HBV pregenomic RNA to preferentially induce type III interferon production but also counteracts the interaction of viral polymerase with the pregenomic RNA for antiviral defense against HBV.

Highlights

- Type III IFNs are predominantly induced in human hepatocytes during HBV infection
- RIG-I senses the HBV genotype A, B, and C for the induction of type III IFNs
- The 5′-ε region of HBV pgRNA is a key element for the RIG-I-mediated recognition
- RIG-I counteracts the interaction of HBV P with pgRNA to suppress viral replication

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Article

The RNA Sensor RIG-I Dually Functions as an Innate Sensor and Direct Antiviral Factor for Hepatitis B Virus

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SUMMARY

Host innate recognition triggers key immune responses for viral elimination. The sensing mechanism of hepatitis B virus (HBV), a DNA virus, and the subsequent downstream signaling events remain to be fully clarified. Here we found that type III but not type I interferons are predominantly induced in human primary hepatocytes in response to HBV infection, through retinoic acid-inducible gene-I (RIG-I)-mediated sensing of the 5′-ε region of HBV pregenomic RNA. In addition, RIG-I could also counteract the interaction of HBV polymerase (P protein) with the 5′-ε region in an RNA-binding dependent manner, which consistently suppressed viral replication. Liposome-mediated delivery and vector-based expression of this ε region-derived RNA in liver abolished the HBV replication in human hepatocyte-chimeric mice. These findings identify an innate-recognition mechanism by which RIG-I dually functions as an HBV sensor activating innate signaling and to counteract viral polymerase in human hepatocytes.

INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic virus of the *Hepadnaviridae* family and contains a circular, partially double-stranded DNA genome of about 3.2 k base pairs that is replicated via reverse transcription of a pregenomic RNA (pgRNA). HBV causes hepatic inflammation associated with substantial morbidity worldwide (Rehermann and Nascimbeni, 2005; Protzer et al., 2012; Revill and Yuan, 2013). Around four hundred million people worldwide are persistently infected with HBV, which is a major causative factor associated with not only inflammation but also cirrhosis and even cancer of the liver. Currently, interferon (IFN) and nucleoside/nucleotide analogs are available for HBV treatment (Rehermann and Nascimbeni, 2005; Halegoua-De Marzio and Hann, 2014). However, the long-term response rates are still not satisfactory. Elucidation of host immune response against HBV infection is crucial for better understanding of the pathological processes and viral elimination to control HBV infection.

The type I IFNs, IFN-α and IFN-β, are representative cytokines that elicit host innate immune responses against viral infections. In addition, another IFN family, type III IFNs (IFN-λ, also known as IL-28 and IL-29) exhibits potent antiviral activity similar to IFN-α and IFN-β (Sheppard et al., 2003; Kotenko, 2011; Kotenko et al., 2003). Production of type I and type III IFNs is massively induced in many types of cells upon infection with various viruses, which is known to be mediated by the activation of pattern-recognition receptors (PRRs). During virus infection, virus-derived nucleic acids (both RNA and DNA) are mainly sensed by certain PRRs, such as retinoic acid-inducible gene-I (RIG-I) (Yoneyama et al., 2004; Choi et al., 2009; Chiu et al., 2009; Ablasser et al., 2009), melanoma differentiation-associated gene 5 (MDA5) (Yoneyama et al., 2005), cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013), and IFN-γ-inducible protein 16 (IFI16) (Unterholzner et al., 2010). Particularly, RIG-I is a key PRR that can detect virus-derived RNAs in the cytoplasm during infection with a variety of viruses, such as influenza virus, hepatitis C virus (HCV), and measles virus, which are closely related to human disease pathogenesis (Rehwinkel and Reis e Sousa, 2010). Binding of RIG-I to its ligand RNAs, such as 5′-triphosphorylated RNA or short double-stranded RNAs (Takeuchi and Akira, 2009; Hornung et al., 2006), activates the downstream signaling pathways in a manner dependent on the adaptor protein mitochondrial antiviral

signaling protein (MAVS; also known as IPS-1, VISA, or Cardif) (Takeuchi and Akira, 2009), leading to the induction of the IFN-regulatory factor-3 (IRF-3) and NF-κB-dependent gene expression and the subsequent production of type I and type III IFNs and inflammatory cytokines (Takeuchi and Akira, 2009). Thus, RIG-I sensing of viral RNA is a crucial process to activate the antiviral innate responses to limit viral replication and the activation of adaptive immunity (Takeuchi and Akira, 2009).

As for the viruses that are known to be the leading cause of hepatic inflammation, RIG-I is the major PRR that initiates innate immune responses against HCV. RIG-I sensing of HCV is mediated through its recognition of the poly-U/UC motif of the HCV RNA genome 3′ nontranslated region, which leads to the activation of type I IFN response (Saito et al., 2008). On the other hand, earlier studies have shown that the innate immune activation is impaired and the induction of type I IFNs such as IFN-α or IFN-β is hardly detected in animal models of HBV infection, as compared with HCV infection (Wieland et al., 2004; Nakagawa et al., 2013). However, it is still not fully clarified how HBV is recognized by human hepatocytes and the role of type III IFNs as well.

Here we report that HBV infection predominately induces type III, but not type I, IFN gene induction, which is mediated by RIG-I through its recognition of the 5′-ε region of HBV-derived pgRNA. We also show that RIG-I can counteract the interaction of HBV polymerase (P protein) with the 5′-ε region of pgRNA in an RNA-binding dependent manner, resulting in the suppression of HBV replication. Furthermore, liposome-mediated delivery and expression of the 5′-ε region-derived RNA in liver suppressed the HBV replication in vivo in chimeric mice with humanized livers. Thus, our findings demonstrate the innate defense mechanisms based on the viral RNA-RIG-I interaction, whereby RIG-I functions not only as a HBV sensor for the activation of IFN response but also as a direct antiviral factor.

RESULTS

Type III IFNs Are Predominantly Induced in Hepatocytes during HBV Infection

To investigate the innate immune activation during HBV infection, we examined type I and type III IFN responses in human hepatocytes. Consistent with the previous reports (Wieland et al., 2004; Nakagawa et al., 2013), we hardly observed the induction of type I IFNs, IFN-α4, and IFN-β in response to transfection with plasmids carrying 1.24-fold the HBV genome of three major different genotypes, Ae (HBV-Ae), Bj (HBV-Bj), and C (HBV-C) (Figure 1A and Figure S1A available online) at least up to seven days after transfection, although the expression of HBV RNAs was detectable (Figure S1B). On the other hand, type III IFN, IFN-λ1, was induced in all of the three types of human hepatocyte cell line tested (Figures 1A and S1A). In HepG2 cells, HBV-C shows the highest IFN-λ1 response, which was also confirmed by ELISA, albeit weakly (Figures 1A and 1B). Moreover, IFN-λ1 in culture supernatant could inhibit vesicular stomatitis virus (VSV) replication in plaque reduction assay, as well as HBV replication (Figure S1C), indicating the physiological relevance of the induced IFN-λ1 to antiviral activities. Consistent with these results, we observed the significant induction of not only IFN-λ1 but also IFN-λ2 and -λ3 in primary human hepatocytes (PHH) in vitro 24 hr after infection with HBV-C (Figure 1C); however, neither of type I nor type II IFN tested was induced (Figures S1D and S1E). Although it is difficult to simply compare the amount of IFN induced by different types of virus, the induction of IFN-λ1, λ2, and λ3 mRNAs in response to HBV infection was much weaker than that of Newcastle disease virus (NDV) infection (Figure 1C). In this regard, in order to rule out the possibility that the IFN-λ response is due to contaminants in the inocula, we used Lamivudine (LAM), an HBV inhibitor, in this assay. Treatment with LAM inhibited IFN-λ mRNA induction in response to HBV infection in PHH (Figure 1C), suggesting that the IFN response is actually induced by HBV replication. Furthermore, we analyzed HepG2-sodium taurocholate cotransporting polypeptide (NTCP)-C4 cell line (Iwamoto et al., 2014) stably expressing human NTCP, a functional receptor for HBV (Yan et al., 2012), and confirmed that IFN-λ1 and IFN-inducible genes such as *OAS2* and *RSAD2*, but not IFN-β, were induced in these cells after infection with HBV-C, and that these inductions were abolished by treatment with LAM (Figure 1D). To next assess the innate immune responses in vivo during HBV infection, we exploited severe combined immunodeficiency mice that carry the urokinase-type plasminogen activator transgene controlled by an albumin promoter ($uPA^{+/+}$/SCID mice), in which more than 70% of murine hepatocytes were replaced by human hepatocytes (Tateno et al., 2004) (hereinafter referred to as chimeric mice). After the chimeric mice were intravenously infected with HBV-C, which was derived from patients with chronic hepatitis, the expression of type III IFN mRNAs increased in the liver tissue, whereas IFN-α4 and IFN-β mRNAs were not upregulated (Figure 1E). In parallel with this type III IFN response, we also observed the expression of IFN-inducible genes, such as *CXCL10*, *OAS2*, and *RSAD2*, in the human liver of these infected mice (Figure 1E). These findings indicate that a moderate type III but not type I or type II IFN response is activated in human hepatocytes in response to HBV infection.

HBV-Induced Type III IFN Expression Depends on RIG-I

We next determined which sensor-mediated signaling pathway is responsible for the HBV-induced type III IFN response. As HBV is a DNA virus (Rehermann and Nascimbeni, 2005; Protzer et al., 2012; Revill and Yuan, 2013), we assessed the contribution of previously reported cytosolic DNA sensors including RIG-I (Chiu et al., 2009; Ablasser et al., 2009; Choi et al., 2009), IFI16 (Unterholzner et al., 2010), and cGAS (Sun et al., 2013) in human hepatocytes. Knockdown analyses revealed that IFN-λ1 induction in HepG2 or Huh-7 cells by plasmid transfection for HBV-C or HBV-Ae, respectively, was suppressed by the knockdown of RIG-I, but not that of the other sensors (Figures 2A, S2A and S2B). To further confirm the involvement of RIG-I in HBV-triggered type III IFN response, we measured IFN-λ1 mRNA expression induced by plasmid expression in Huh-7.5 cells that carry a dominant-negative mutant RIG-I allele that prevents RIG-I signaling (Saito et al., 2007), as compared with Huh-7 cells that have an intact RIG-I pathway. Huh-7.5 cells failed to induce IFN-λ1 mRNA expression in response to HBV-Ae genome plasmid transfection, as in the case of stimulation with 5′-triphosphate RNA (3pRNA), a RIG-I ligand (Takeuchi and Akira, 2009; Hornung et al., 2006) (Figure 2B). In concordance with this result, knockdown of tripartite motif containing protein

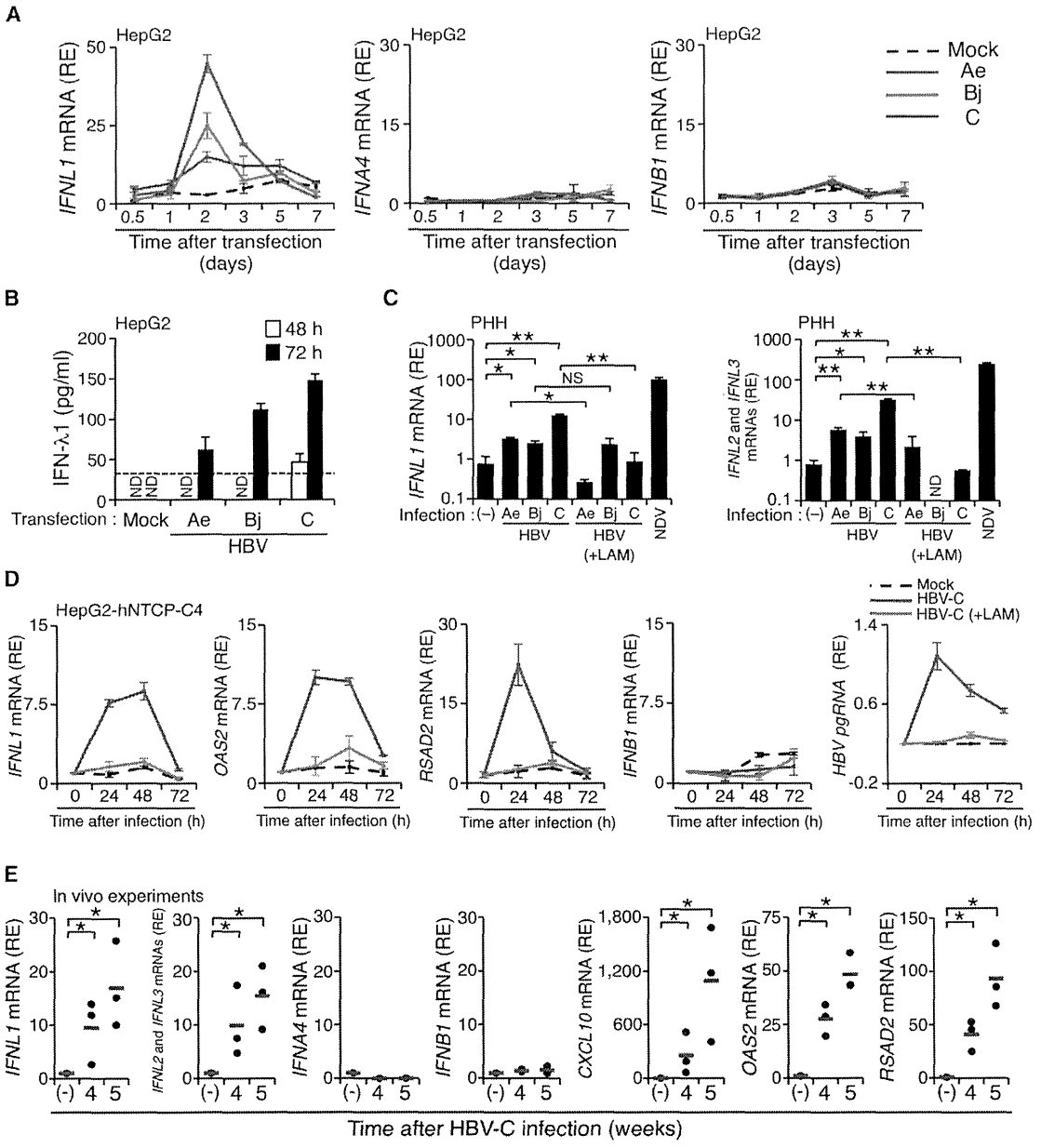

Figure 1. IFN-λ Induction in Human Hepatocytes in Response to HBV Infection

(A) Quantitative RT-PCR (qRT-PCR) analysis of *IFNL1* (left), *IFNA4* (middle), and *IFNB1* (right) mRNA at the indicated times after transfection with 1.24-fold the HBV genome (genotype Ae, Bj, or C) or empty vector (Mock) in HepG2 cells.

(B) ELISA of IFN-λ1 at 48 or 72 hr after transfection with the HBV genome in HepG2 cells. The dot line indicates the minimum detectable amount (31.2 pg/ml) of IFN-λ1 by the ELISA kit. ND, not detected, indicates below the minimum detectable amount.

(C) qRT-PCR analysis of *IFNL1*, *IFNL2*, and *IFNL3* mRNA at 24 hr after infection with HBV, NDV (multiplicity of infection = 10) or mock (–), or media-treated Lamivudine (LAM) as control in primary human hepatocytes (PHH). The mRNA copy number (±SD) of each subtype of type III IFN per 1 μg total RNA upon HBV-C infection is as follows: *IFNL1* (83,197.6 ± 6,241.4) and *IFNL2/3* (409,280.6 ± 119,676.2).

(D) Time course analyses by qRT-PCR of *IFNL1*, *OAS2*, *RSAD2*, *IFNB1* mRNA, and *pgRNA* at the indicated times after HBV infection in HepG2-hNTCP-C4 cells. The effect of Lamivudine treatment was also analyzed.

(E) qRT-PCR analysis of *IFNL1*, *IFNL2*, *IFNL3*, *IFNA4*, *IFNB1*, *CXCL10*, *OAS2*, and *RSAD2* mRNA of liver tissues at 4 or 5 weeks after infection with HBV-C in chimeric mice. (–), uninfected mice. Red lines represent the mean of each dataset. *$p < 0.05$ and **$p < 0.01$ versus control. RE, relative expression. (A–D) Data are presented as mean and SD (n = 3) and are representative of at least three independent experiments. See also Figure S1.

25 (TRIM25), MAVS, TANK-binding kinase 1 (TBK1), and IRF-3, all of which are signaling molecules essentially involved in the RIG-I-mediated IFN pathway (Takeuchi and Akira, 2009), resulted in the suppression of IFN-λ1 mRNA induction in HepG2 cells in response to transfection with the HBV-C genome. On the other hand, such an effect was not observed in cells treated

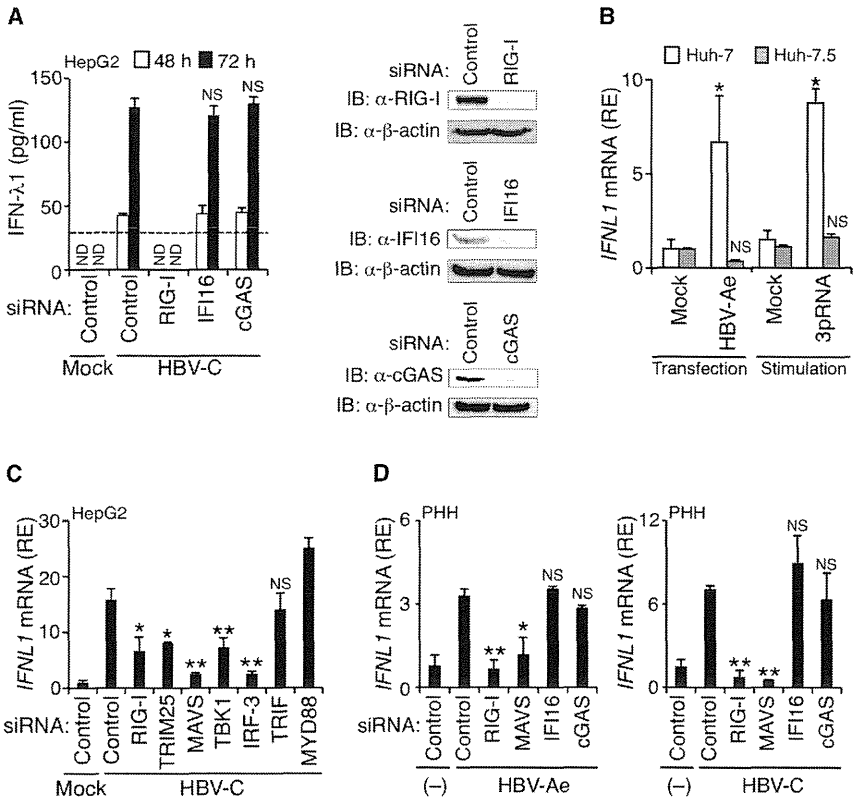

Figure 2. RIG-I-Dependent IFN-λ Induction in Response to HBV Infection

(A) HepG2 cells treated with control siRNA (Control) or siRNA targeting RIG-I, IFI16, or cGAS were transfected with the HBV-C genome for 48 or 72 hr. The amount of IFN-λ1 were measured by ELISA. The dot line indicates the minimum cytokine expression detected (31.2 pg/ml) of IFN-λ1 by the ELISA kit. ND, not detected, indicates below detectable concentrations (left), and knockdown efficiency was analyzed by immunoblotting (IB) (right).

(B) qRT-PCR analysis of *IFNL1* mRNA in Huh-7 or Huh-7.5 cells transfected with the HBV-Ae genome (at 24 hr after transfection) or stimulated with 3pRNA (1 μg/ml) for 6 hr.

(C) HepG2 cells treated with control siRNA (Control) or the indicated siRNAs were transfected with the HBV-C genome. At 48 hr after transfection, total RNAs were subjected to qRT-PCR analysis for *IFNL1*.

(D) qRT-PCR analysis of *IFNL1* mRNA in siRNA-treated PHH at 24 hr postinfection with indicated HBV genotype. Mock, empty vector-transfected. (–), uninfected. Data were normalized to the expression of *GAPDH*. Data are presented as mean and SD (n = 3) and are representative of at least three independent experiments. *p < 0.05 and **p < 0.01 versus control in (B) or HBV-infected control group in (A, C, and D). NS, not significant. See also Figure S2.

with either TRIF (also known as TICAM-1) or MYD88 siRNA (Figures 2C and S2C). In addition, we confirmed that the knockdown of RIG-I and MAVS abolished IFN-λ1 induction in PHH infected with each genotype (Figure 2D). Furthermore, we also confirmed by knockdown assay that the induction of IFN-λ1 and OAS2 mRNA in HepG2-hNTCP-C4 cells in response to infection with HBV-C was dependent on RIG-I (Figure S2E). These data indicate that IFN-λ1 gene induction during HBV infection depends largely on RIG-I signaling pathway.

The 5′-ε Region of HBV pgRNA Is a Key Element for RIG-I-Dependent IFN-λ1 Induction

RIG-I can recognize not only virus-derived RNA but also DNA in the cytoplasm (Yoneyama et al., 2004; Choi et al., 2009; Chiu et al., 2009; Ablasser et al., 2009). To further clarify how RIG-I recognizes HBV, we first examined either or both of which nucleic acid (DNA and RNA) derived from HBV-infected cells can activate IFN-λ1 gene expression. Transfection with nucleic acid fractions extracted from HBV infected Huh-7 cells after pretreatment with RNase A, but not DNase I resulted in marked inhibition of the *IFNL1* promoter activation, suggesting that virus-derived RNAs might be candidates of the RIG-I ligand during HBV infection (Figure 3A).

The HBV genome comprises a partially double-stranded 3.2 kb DNA. During a life cycle of HBV in hepatocytes, its covalently closed circular DNA (cccDNA) is transcribed to generate four major RNA species: the 3.5, 2.4, 2.1, and 0.7 kb viral RNA transcripts (Rehermann and Nascimbeni, 2005; Protzer et al., 2012; Revill and Yuan, 2013). We created an siRNA to suppress the expression of all of these RNA transcripts and tested its effect on HBV-induced IFN-λ1 expression. As shown in Figure 3B, knockdown with this siRNA (Figure S3A) suppressed IFN-λ1 induction in Huh-7 cells transfected with HBV-Ae. Next, to determine which of these HBV RNA transcripts is/are involved in the RIG-I-mediated IFN-λ1 induction, we prepared expression vectors to express each of these four viral transcripts in HEK293T cells that are often used to analyze RIG-I signaling pathway in human cells. As a result, it is only the longest 3.5 kb transcript, that is, pgRNA, that has the potential to elicit a significant induction of IFN-λ1 mRNA (Figures 3C and S3B). It was also confirmed by knockdown analysis with pgRNA-targeted siRNA, which showed significant suppression of IFN-λ1 induction in HepG2 cells transfected with HBV-Ae (Figure S3C). These results suggest that 5′-1.1 kb region of HBV pgRNA is critical for the activation of RIG-I pathway to induce IFN-λ1 expression. On the other hand, the remaining three transcripts, which also contain the same sequence of part of this 1.1 kb region of HBV pgRNA at the 3′ end of their transcripts, failed to induce IFN-λ1 mRNA (Figure 3C). An artificially deleted form of pgRNA, which lacks this overlapping sequence at the 3′-region (Δ3), showed IFN-λ1 induction, whereas such response was not observed for another mutant pgRNA lacking it at the 5′-region (Δ5) (Figure 3D). These data also support a possible important role of the 5′-overlapping sequence of HBV pgRNA for RIG-I-mediated IFN-λ1 induction.

The 5′-end of HBV pgRNA is known to contain the encapsidation sequence, called "epsilon (ε)," which takes a stem-loop secondary structure (Junker-Niepmann et al., 1990; Pollack and

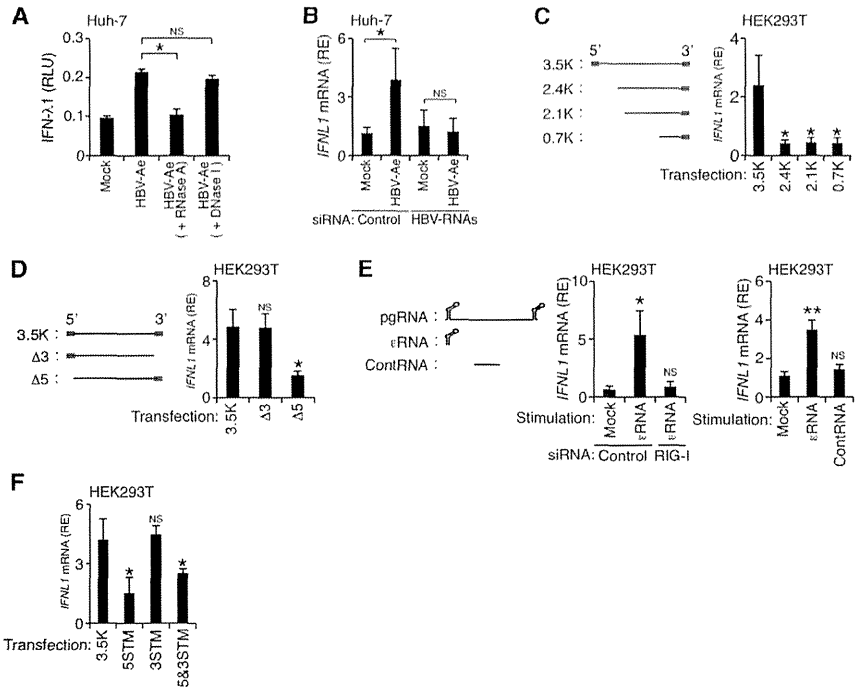

Figure 3. RIG-I Activation Is Mediated by Its Recognition of the 5′-ε Region of HBV pgRNA

(A) Luciferase activity of an IFN-λ1 reporter plasmid after 24 hr of stimulation with nucleic acids (2 μg/ml) extracted from Huh-7 cells transfected with control plasmid (Mock) or the HBV-Ae genome with or without RNase A or DNase I treatment. RLU, relative luciferase units.

(B) Huh-7 cells treated with control or HBV RNA-targeted siRNA were transfected with the HBV-Ae genome or mock. After 24 hr of transfection, total RNAs were subjected to qRT-PCR for *IFNL1*.

(C and D) A schematic representation of four types of HBV RNAs, pgRNA (3.5 kb), 2.4 kb, 2.1 kb, and 0.7 kb RNAs in (C), and two deleted forms of pgRNA, Δ5 and Δ3, in (D). The overlapping region is shown in blue. qRT-PCR analysis of *IFNL1* mRNA of HEK293T cells after 24 hr of transfection with the indicated expression vectors. Data were normalized to the amount of each HBV RNA expression (C and D).

(E) A schematic representation of pgRNA, εRNA, or control RNA (ContRNA) (left). HEK293T cells treated with control or RIG-I siRNA were unstimulated (Mock) or stimulated with εRNA for 12 hr. Total RNAs were subjected to qRT-PCR for *IFNL1* (middle). qRT-PCR analysis of *IFNL1* mRNA in HEK293T cells after 12 hr of stimulation with εRNA or ContRNA (right). Each of the RNAs was prepared by in vitro transcription.

(F) HEK293T cells were transfected with each plasmid for stem-loop mutants of pgRNA (5STM, 3STM, or 5 and 3STM), and then subjected to qRT-PCR analysis as described in (C). *$p < 0.05$ and **$p < 0.01$ versus control in (A, B, and E) or versus 3.5K in (C, D, and F). NS, not significant. See also Figure S3.

Ganem, 1993; Knaus and Nassal, 1993; Jeong et al., 2000). Therefore, we hypothesized that this 5′-ε structure might confer a possible pathogen-associated molecular pattern (PAMP) motif for RIG-I recognition. To test this hypothesis, we stimulated HEK293T and HepG2 cells with the ε region-derived RNA (hereafter called εRNA). Consequently, IFN-λ1 mRNA was significantly induced, which was dependent on RIG-I, while such a response was not detected upon stimulation with the equivalent length of RNA that is derived from HBV pgRNA but does not contain any ε element (ContRNA) (Figure 3E and S3D). We also confirmed RIG-I-dependent IRF-3 activation in response to stimulation with εRNA (Figures S3D and S3E). Due to the overlapping sequence of 5′- and 3′-ends of HBV pgRNA as mentioned above, this ε element is found at both ends of pgRNA. We next generated several mutant forms of HBV pgRNA, each of which carries mutations within 5′- or 3′-ε region or both to disrupt the stem-loop structure (5STM, 3STM, or 5 and 3STM, respectively). In concordance with the results shown in Figures 3C and 3D and S3B, IFN-λ1 mRNA induction was detected upon expression of the 3STM transcript that has an intact 5′-ε region, as similar to that of intact 3.5-kb pgRNA (Figure 3F). In contrast, either 5STM or 5 and 3STM did not show significant response. These findings indicate that the 5′-ε region of HBV pgRNA is critical for IFN-λ1 induction possibly through the recognition by RIG-I.

RIG-I Interacts with the ε-Region of pgRNA

Next, we assessed the interaction of RIG-I with the ε region of HBV pgRNA, that is, εRNA. Pull-down assays showed that Flag-tagged RIG-I was coprecipitated with εRNA, but not with ContRNA, in HEK293T cells (Figure 4A, top). Similarly, endogenous RIG-I interacted with εRNA albeit weakly (Figure 4A, bottom). We also demonstrated the intracellular colocalization of RIG-I with εRNA in Huh-7.5 cells (Figure 4B). In addition, RNA-binding protein immunoprecipitation (RIP) assay revealed that the full length of HBV pgRNA was detected in the RIG-I-immunoprecipitated complex, and Δ5 pgRNA and Δ3 pgRNA were also detected (Figure S4A), which is seemingly inconsistent with the results by the functional assay (Figures 3C, 3D, 3F and S3C). These results suggest that the ε region is required for its interaction with RIG-I, but only the 5′-ε region is necessary to activate RIG-I pathway. We further tried to determine which region of RIG-I mediates its interaction with HBV pgRNA. Both RIP assay and RNA pull-down assay with several deletion mutants of RIG-I showed that the C-terminal portion of RIG-I (C-RIG) including its helicase domain and repressor domain (RD) except for CARDs can bind to HBV pgRNA (Figure 4C; Figures S4B and S4C). In addition, gel shift assay showed that the interaction of HBV εRNA or pgRNA was impaired with the RD or C-RIG mutant, respectively, each of which carries a point mutation (K888E) that abolishes its RNA-binding activity (Cui et al., 2008) (Figure 4D). A similar result was also obtained by RIP assay, wherein the wild-type (WT) C-RIG, but not the K888E mutant, was coimmunoprecipitated with HBV pgRNA (Figure S4D), like HCV RNA that was previously reported to interact with RIG-I (Figure S4E). We also confirmed the interaction of HBV pgRNA with endogenous RIG-I in HepG2 cells, whereas its interaction with other nucleic acid sensors, such as IFI16 and MDA5 (Yoneyama et al., 2005), was not detected (Figure 4E). These data indicate that