in serum-free hepatocyte maintenance medium (Gibco, NY, USA) for one week before starting each experiment.

Treatment with Neutralizing Antibodies (nAb) and Recombinant IFN

Two days prior to stimulation or infection, HuS-E/2 cells were seeded on the collagen coated 12 well plate (8×10^4 cells/well) to yield a confluent cell layer within 24 h. In the case of infection experiment, the cells were treated with nAb mentioned below for 12 hours (hrs). Then the cultured medium contained nAb were replaced with new culture medium containing Sendai virus (SeV) or cell culture derived recombinant HCV (HCVcc) after wash with phosphate buffer saline (PBS). SeV was prepared as described previously [10]. HCVcc was prepared from the HuH-7 or Huh-7.5 cells transfected with in vitro synthesized Jikei Fulminant Hepatitis (JFH) 1^{E2FL} RNA as described previously [11]. Recombinant human IFN-α was obtained from Merck (Darmstadt, Germany). Blocking Antibodies targeting IFN-α (MMHA-2), IFN-β (Rabbit polyclonal antibody), and IFNAR2 (MMHAR-2) were purchased from PBL Biomedical Laboratories (Piscataway, NJ).

RNA Extraction, Reverse-transcriptase Polymerase Chain Reaction (RT-PCR), and Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cell cultures with Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Using 200 ng of total RNA as a template, RT-PCR was done with one-step RNA PCR kit (Takara, Kyoto, Japan) according to the manufacturer's instruction. qRT-PCR was performed with One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara, Kyoto, Japan) by using 7500 Real-time PCR system (Applied Biosystems, Carlsbad CA) according to the manufacturer's instruction. The primer sets used in those PCRs are detailed in Table S1. Real-time PCR data are given as the mean of triplicate samples with standard deviation. The value obtained for the untreated control sample was generally set to 1.

Primer Design and Selection

The primers were designed based on the conserved specific sequence of IFN genes, using primer design software Primer-BLAST (National Center for Biotechnology Information. USA).

To evaluate the sensitivity and specificity of designed primer sets, RT-PCR using those primer sets and in vitro synthesized RNAs for subtypes of IFN as templates was performed. To make the in vitro expression plasmids for the IFNs, the cDNA fragment of each subtype of IFN was synthesized from total RNA from IFN-α stimulated HuS-E/2 cells by RT-PCR and subcloned in to the multiclonning sites of pcDNA3. The RNA fragment of each IFN subtype was synthesized with the plasmid in vitro using the MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's protocol. Synthesized RNA was treated with DNase I followed by acid phenol extraction to remove any remaining template DNA and used for RT-PCR by using one-step RNA PCR kit (Takara, Kyoto, Japan). The amplification conditions were 2 min preheating at 94°C, followed by from 25 to 35 cycles of 10 sec denaturation at 94°C, 30 sec annealing at 55°C, and 1 min elongation at 72°C.

Quantification of Type I IFN

Quantification of active type I IFN was performed by HEK-Blue IFN-α/β cells (Invivogen, San Diego, CA) according to the manufacturer's instruction. Type I IFN concentration (U/ml) was extrapolated from the linear range of a standard curve generated using known amounts of recombinant IFN-α (Merck Darmstadt, Germany).

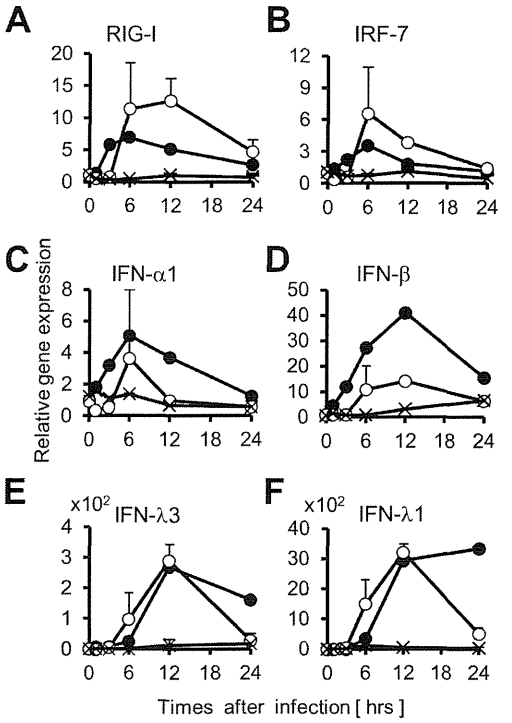

Figure 2. Expression profiles of genes associated with IFN signals in the cells after infection of SeV. Total RNAs were purified from PHH (closed circles), HuS-E/2 cells (open circles), and HuH-7 cells (cross marks) at indicated time points after infection of SeV. Relative quantities of RIG-I (*A*), IRF-7 (*B*), IFN-α1 (*C*), IFN-β (*D*), IFN-λ3 (*E*), and IFN-λ1 (*F*) mRNAs in each total RNA sample were examined by qRT-PCR and plotted using the RNA level firstly detected as a benchmark. The quantity of each RNA sample was normalized by the amount of GAPDH mRNA as relative gene expression. Error bars represent standard deviation (SD) of the mean of determinations from three experiments.
doi:10.1371/journal.pone.0089869.g002

Immunoblotting

Immunoblotting analysis was performed essentially as described previously [12], with slight modifications. Samples of cell lysates were prepared in lysis buffer (50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, 1 mM Orth vanadate (sigma-aldrich, St.Louis, USA), 10 mM NaF, Protease Inhibitor Cocktail (sigma-aldrich St.Louis, USA)). Antibodies used in this study were polyclonal rabbit antiserum against RIG-I at a 1:1000 dilution, IRF-7 at a 1:1000 dilution, STAT1 at a 1:1000 dilution, or p-STAT1 at a 1:1000 dilution. These antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Immune-complexes were detected using Western Lightning reagent (PerkinElmer, Waltham, MA) by LAS-4000 system (Fujifilm, Tokyo, Japan).

ELISA for Human IFN-α Protein

Conditioned medium from culture system of HuS-E/2 cells infected with SeV, was collected at 3 and 12 hrs post-infection. The concentration of IFN-α in the conditioned medium was measured with human IFN-α enzyme-linked immunosorbent assay (ELISA) kit (PBL Biomedical Laboratories).

Indirect Immunofluorescence Analysis

Indirect immunofluorescence (IF) analysis was performed essentially as described previously [11]. The primary antibody was anti-SeV polyclonal antibody (1:200) (MBL International

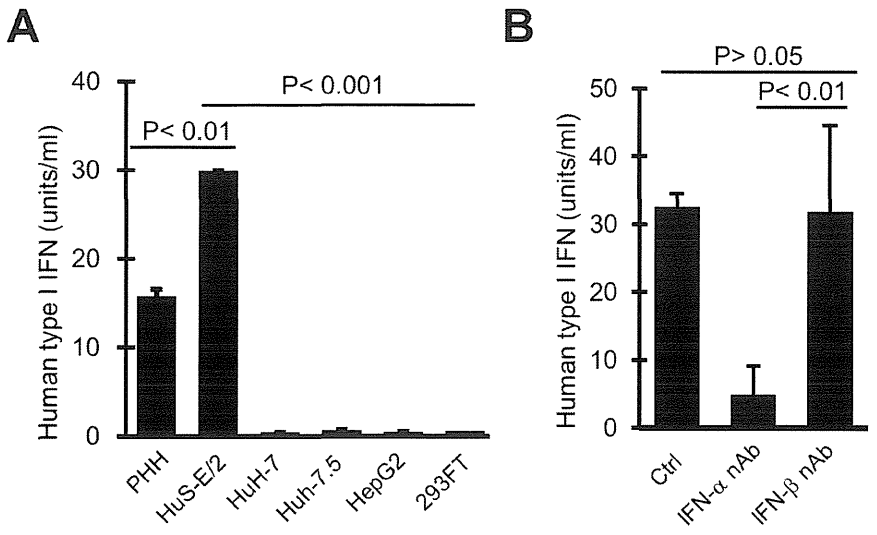

Figure 3. Activity of IFN-α constitutively produced into the culture medium. *A*, Activity of type I IFN produced from the cells without virus infection. Activities of type I IFN produced from PHH, HuS-E/2, HuH-7, Huh-7.5, HepG2, and 293FT cells in the culture media were examined by HEK-Blue™ IFN-α/β cells as described in experimental procedures section and plotted in the graph. *B*, Production of IFN-α from the cells without virus infection. Activity of type I IFN produced from HuS-E/2cells in the culture medium over night was examined as *A*, after treatment with 5 μg/ml neutralizing antibody (nAb) against IFN-α (IFN-α nAb) or IFN-β (IFN-β nAb) for 2 hrs. Error bars represent SD calculated from results of three independent experiments. Probability value (*P* value) was calculated with Student's t test.
doi:10.1371/journal.pone.0089869.g003

Corporation, MA, USA). The fluorescent secondary antibody was Alexa 568-conjugated anti-rabbit (Invitrogen, Carlsbad, CA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescent signals were visualized by fluorescence microscopy (Bio Zero Keyence Co.).

Results

Induction of IFN Genes and ISGs in PHH and HuS-E/2 Cells by the Infection of Sendai Virus

We examined the antiviral responses of IFN system in some human hepatocyte derived cells against RNA viral infection using sendai virus (SeV) which is a negative strand RNA virus and has been widely used in studies on induction of IFN system [13]. The constitutive expression of IRF-3 and RIG-I genes was commonly found in all those cells as already repoted (data not shown, [10]). Our previous observation that IRF-7 gene was expressed in PHH and HuS-E/2 cells but not in HuH-7 cells under this condition was also confirmed (data not shown, [10]). In addition, the expression of IFN-α1 gene was newly observed in PHH and HuS-E/2 cells albeit at low level (Fig. 1*A*). That, however, was not in the case in HuH-7 and HepG2 cells, although the RT-PCR product was sometimes seen in HuH-7 and HepG2 cells but only vaguely (Fig.1*A*). These results were also confirmed quantitatively by qRT-

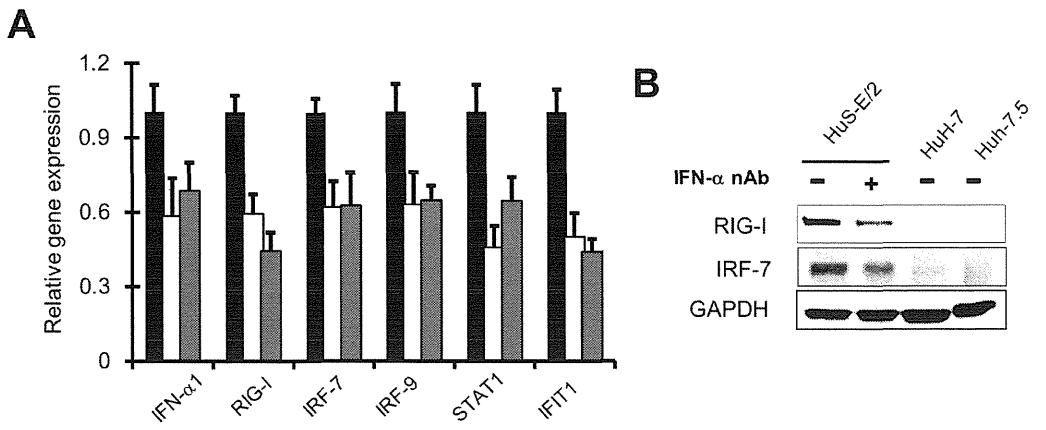

Figure 4. The function of constitutive IFN-α1 on the expression of the genes associated with IFN signals. *A*, The mRNA levels of the genes associated with IFN signals, RIG-I, IRF-7, IRF-9, STAT1, IFIT1, and IFN-α1 itself were examined in HuS-E/2 cells treated with and without nAb against IFN-α (IFN-α nAb, gray bars) (5 μg/ml) or IFNAR2 (IFNAR2 nAb, white bars) (5 μg/ml) for 12 hrs by qRT-PCR. Relative expression level of those genes are plotted using the RNA levels detected in the cells treated without nAb (Mock, black bars) as a benchmark. Results were derived from three independent experiments and error bars represent the calculated SD. *B*, Total cell lysates of HuS-E/2 cells treated with (+) or without (−) 5 μg/ml IFN-α nAb for 12 hrs were analyzed with immunoblotting using antibodies against RIG-I, and IRF-7. Those of HuH-7 and Huh-7.5 cells without treatment were also investigated. Protein levels was normalized among the samples by levels of GAPDH detected with anti-GAPDH antibody.
doi:10.1371/journal.pone.0089869.g004

PCR (Fig. 1*B*). In order to examine the gene expression of the other IFN subtypes in those cells, the primer sets for RT-PCR to detect mRNAs for those factors sensitively and specifically were designed and obtained to use for specific amplification of about one hundred copies of IFN-α4, IFN-α6, IFN-α8, IFN-β, IFN-λ1, or IFN-λ3 mRNAs (Fig. 1*C* and Table S1). Those mRNAs, however, were not detected at all in all cells used in this study by this RT-PCR condition (Fig. 1*A*). These results suggested that IFN-α, at least IFN-α1, but not IFN-β and IFN–λ, is slightly produced in human hepatocytes without virus infection.

Next, the effect of SeV infection on mRNA levels of those genes was investigated in those cells. The mRNA levels of IRF-7 and IFN-α1, a target gene product of IRF-7, were transiently increased and reached peaks 6 hrs post-infection (p.i.) in both PHH and HuS-E/2 cells (Fig. 2*B and 2C*, closed and open circles). Those of IFN-β, and IFN-λ3 reached peaks 12 hrs p.i. in those cells in similar ways (Fig. 2*D and 2E*, open and closed circles). IFN-λ1 mRNA was also increased by 12 hrs p.i. in both HuS-E/2 cells and PHH (Fig. 2*F*, open and closed circles). It decreased from 12 to 24 hrs p.i. in HuS-E/2 cells (Fig. 2*F*, open circles) whereas it slightly increased in PHH (Fig. 2*F*, closed circles). The gene expression of RIG-I, a key factor in the innate immune response to RNA virus infection in many cells [14,15], was also investigated. The increase of RIG-I mRNA was observed from 1 hr to 6 hrs p.i. in PHH (Fig. 2*A*, closed circles). Although that was observed from 3 hrs to 12 hrs p.i., the similar pattern of the increase was observed in HuS-E/2 cells (Fig. 2*A*, open circles). The expressions of RIG-I, IRF-7, and IFNs genes were also assessed in HuH-7 cells following infection. Obvious increase of those mRNAs, however, was not detected in telling contrast to PHH and HuS-E/2 cells (Fig. 2, crosses). These observations indicated that this early innate immune response to SeV infection did not occur in HuH-7 cells. The absence of this early response may explain why HuH-7 cells support efficient proliferation of recombinant HCV [16]. On the other hand, the innate immune response of HuS-E/2 cells against SeV infection seemed to be relatively similar to that of PHH, although the responsiveness curves of those gene expression were slightly different. Thus, we supporsed that HuS-E/2 cells provide a valuable model for studying innate immune response of human hepatocytes against viral infection.

Active IFN-α was Constitutively Produced in HuS-E/2 Cells at a Low Level

In this study, we addressed the constitutive expression of IFN-α1 gene observed in PHH and HuS-E/2 cells, since the detection of IFN-α1 mRNA in the liver tissues from normal human individuals was already detected by RNA blot hybridization, but its function was not cleared yet [8].

The presence of IFN-α in the conditioned medium from HuS-E/2 cell culture system was examined firstly by the ELISA system for IFN-α. However, that was not detected probably because of low concentration of secreted IFN-α and low sensitivity of the system. Then the activity of type I IFN in the culture medium assessed by using HEK-Blue type I IFN assay system in which the cells are designed to produce embryonic alkaline phosphatase protein in type I IFN receptor signaling-dependent manner. As shown in Fig. 3*A*, the culture medium from HuS-E/2 cell culture system showed significantly higher activity of type I IFN than those from the culture systems for Huh-7.5, HuH-7, and HepG2 cells as well as 293FT cells, a cell line derived from human embryonic kidney. To confirm above results further, the effect of neutralizing antibody (nAb) targeting IFN-α on the above conditioned medium was examined similarly. As shown in Fig. 3*B*, the pretreatment of the conditioned medium from HuS-E/2 cell culture with this antibody effectively suppressed the activity of IFN receptor signaling, while nAb targeting IFN-β did not affected (Fig.3*B*). These observations indicated that functional type I IFN was included in the conditioned medium from HuS-E/2 cell culture system and, at least, the majority of the type I IFN in the medium was IFN-α and not IFN-β. From above results, we concluded that HuS-E/2 cells produce functional IFN-α into the culture medium without virus infection.

The Basal Expression of IFN-α1 and ISGs was Elevated by Type I IFN Receptor Signaling

To see whether the IFN-α1 produced in the cells without virus infection affects the cells through the autocrine or paracrine signaling regardless of low level production, the basal expression of several ISGs in HuS-E/2 cells cultured in the medium containing nAb targeting IFNα/β receptor (IFNAR) 2, one of the receptors for type I IFN, were investigated. As shown in Fig. 4*A*, it was clearly observed that the mRNA levels of RIG-I, IRF-7, IRF-9, signal transducer and activator of transcription (STAT) 1, and IFN-induced protein with Tetratricopeptide 1 (IFIT1) were diminished in the cells with the nAb treatment, compared to mock treated cells. Quite similar results were observed in the cells treated with nAb targeting IFN-α (data not shown). The basal expression level of IFN-α1 gene was also reduced by these treatments (Fig. 4*A*). Decreased protein levels of RIG-I and IRF-7 were also observed in HuS-E/2 cells treated with nAb targeting IFN-α, while these proteins were below the detectable level in HuH-7 and Huh-7.5 cells as predicted (Fig. 4*B*). As the treatments of two different nAbs showed the similar results to each other, it was suggested that the type I IFN receptor signaling upregulates the expression of those ISGs, as well as IFN-α1 gene, in basal level in HuS-E/2 cells to some extent.

Basal Production of IFN-α in HuS-E/2 Cells Contributes to Rapid Antiviral Response during Early Phase of Infection

To study the functional role of basal level production of IFN-α in HuS-E/2 cells, the innate immune responses of the cells treated with the above nAbs against the RNA virus infection was investigated. In this experiment, HuS-E/2 cells pretreated with nAbs for 12 hrs to interrupt the basal IFN signaling were exposed to the fresh culture medium contained SeV for 30 min after rinsing the nAbs off from the cells. After the infection, the cells were cultured for 3 hrs and then used as follows (Fig. 5*A*). As the induced expression of RIG-I gene, used as a representative of ISGs, by the treatment with recombinant IFN-α was observed similarly in both cells pretreated with and without nAbs (Fig. 5*B*), it was clearly shown that the cells pretreated with the nAbs retained responsiveness to IFN-α rinsing procedure. At first, the levels of mRNAs for RIG-I, IRF-7, IFN-α1, IFN-β, IFN-λ1, and IFN-λ3 were examined in the cells with or without SeV infection. As shown in Fig. 5*C*, almost the similar patterns of increases of those mRNAs were observed in the cells with SeV infection, although only minor levels of the induction were found in the cases of IRF-7 and IFN-α mRNAs even in the mock pretreated cells. In all the cases, nAb pretreatments effectively suppressed the increases of those mRNAs 3 hrs after SeV infection (Fig. 5*C*). As shown in Fig. 5*D*, the suppression of IFN-α protein production in the culture medium 12 hrs after the infection was also observed by using ELISA.

Next, to examine the effect of the pretreatments with nAbs on virus-induced STAT1 activation, phosphorylated form of STAT1, activated form of STAT1, was detected in SeV infected cells pretreated with nAbs by western blot analysis using anti-

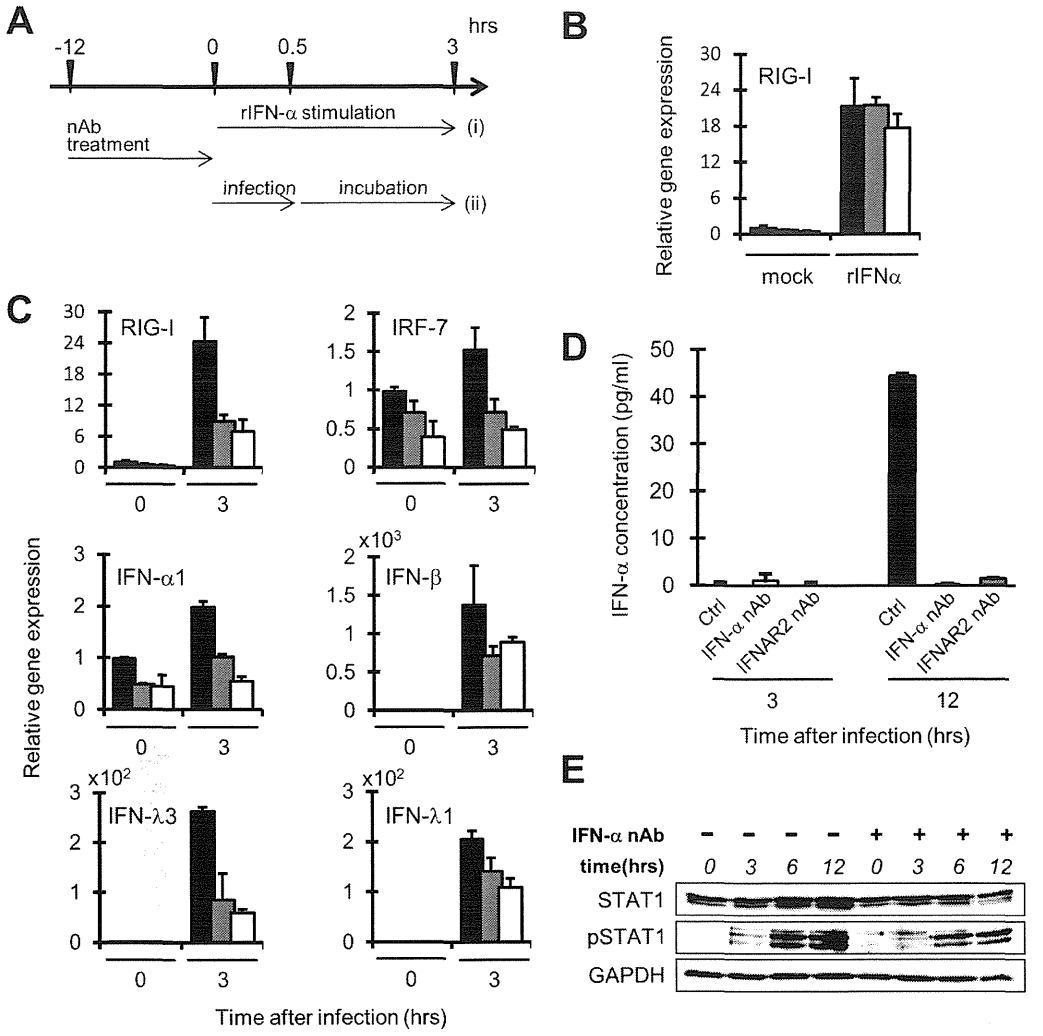

Figure 5. Roles of Constitutive IFN-α on the virus-induced antiviral responses in HuS-E/2 cells. *A*, Schematic of the time schedules for the experiments of recombinant human IFN-α (rIFN-α treatment (i) or SeV infection (ii) after nAb treatment. *B*, Responses of the cells pretreated with IFN-α or IFNAR2 nAbs against exogenous stimulation of rIFN-α. RIG-I mRNA in the cells treated with rIFN-α (2.5 unit/ml) (i) for 0 and 3 hrs after the treatment with IFNAR2 nAb (gray bars), IFN-α nAb (white bars) or mock (black bars) were analyzed by qRT-PCR. Relative expression level of those genes are plotted using each RNA level detected in the cells treated with mock for 0 hr as a benchmark (*B*, *C*). Error bars represent the calculated SD from the results obtained in three independent experiments (*B*, *C*, *D*). *C*, Responses of the cells pretreated with IFN-α or IFNAR2 nAbs against SeV infection. IFN-α1, IFN-β, IFN-λ1, IFN-λ3, and IRF-7 mRNAs in the cells processed as described for panel *B* except infection of SeV (ii) instead of rIFN-α treatment, were analyzed by qRT-PCR. *D*, SeV infection induced IFN-α production from the cells pretreated with IFN-α or IFNAR2 nAbs. Quantity of IFN-α protein in the culture medium was determined by ELISA at 3 and 12 hrs post-infection of SeV. The cells were processed as described for panels *C*. *E*. STAT1 phosphorylation in the cells pretreated with IFN-α nAbs after SeV infection. The phosphorylation status of STAT1 in the cells processed as described for panel *D* except pretreatment with (+) or without (−) IFN-α nAb only was analyzed by western blot analysis using anti-STAT1 antibody (STAT1), anti-phosphorylated STAT1 antibody (pSTAT1) at 3, 6, and 12 hrs post-infection of SeV. Protein levels were normalized among the samples by levels of GAPDH detected with anti-GAPDH antibody.
doi:10.1371/journal.pone.0089869.g005

phosphorylated STAT1 (pSTAT1). As shown in Fig. 5*E*, the level of pSTAT1 found in the SeV infected cells without IFN-α nAb pretreatment was apparently reduced in the cells with pretreatment at 3, 6 and 12 hrs post-infection, although the protein levels of STAT1 were not affected by the pretreatment, suggesting that pretreatment by IFN-α nAb suppressed the IFN signaling induced by virus infection.

These results suggested that IFN-α produced in the HuS-E/2 cells without virus infection plays a role in the enhancement of initial response of IFN system.

IFN-α Released from HuS-E/2 cells without Viral Infection Contributes to Inhibit Initial Infection and Proliferation of RNA Viruses, Including HCV

To examine whether IFN-α produced in the cells without virus infection actually plays a role in prevention of viral infection, the permissiveness of HuS-E/2 cells, which were pretreated with and without IFNAR2 nAb, against SeV infection was investigated. Compared to the cells without pretreatment of nAb (shown as control panels in Fig. 6*A*), the number of SeV infected cells (shown in red) was increased in the cells with treatment both 6 and 9 hrs after infection in time-dependent manner (Fig. 6*B*). We also assessed

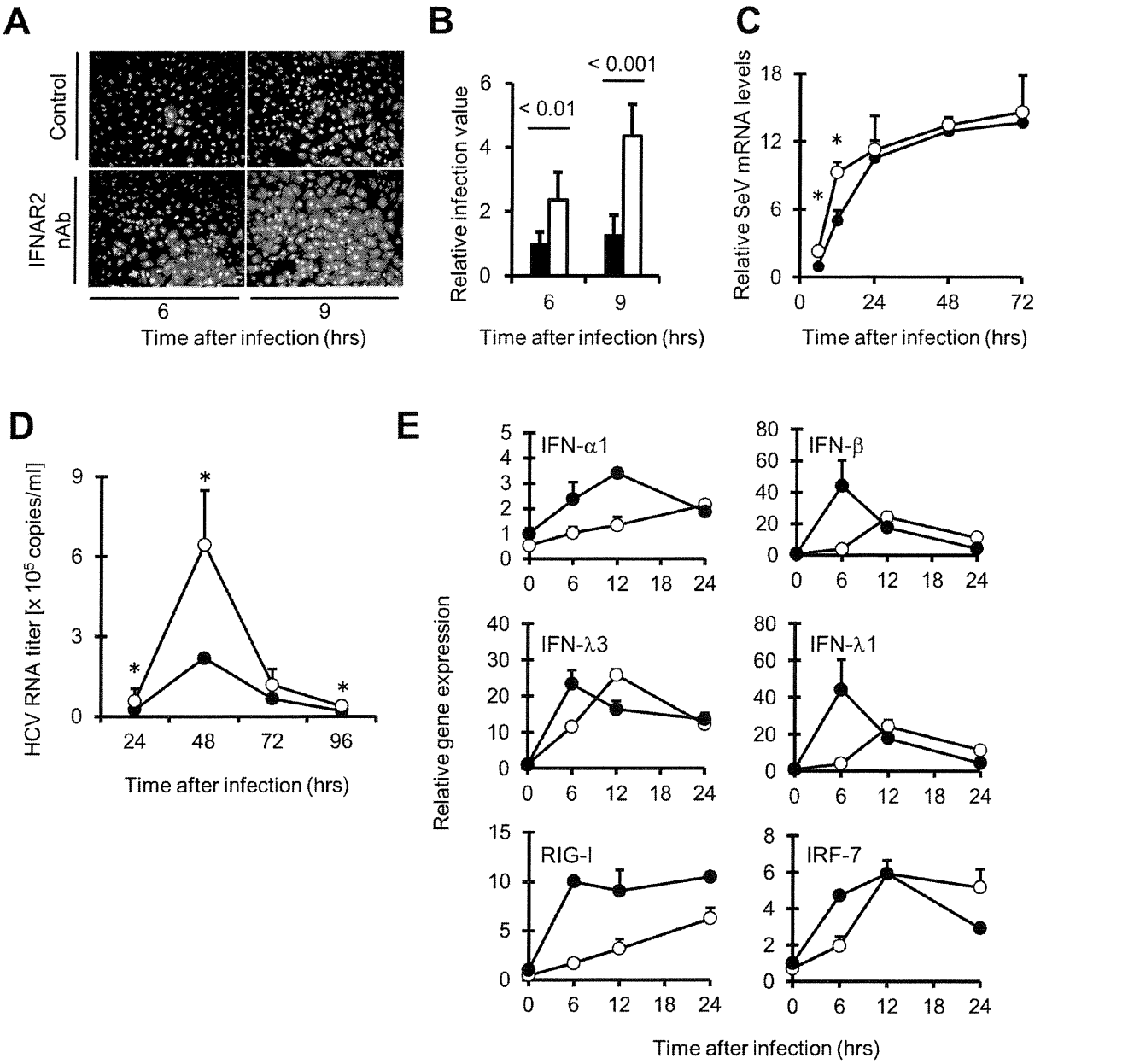

Figure 6. Suppressive role of constitutive IFN-α on viral infection or initial viral proliferation. *A*, Enhanced infection of SeV by neutralization of constitutive IFN-α visualized with IF. Cells were processed basically as described in the figure legend for Fig. 5, panel *C* (*A, B, C, D, E*). At 6 and 9 hrs after SeV infection, the cells were fixed and studied by IF using anti SeV antibodies (red). Nuclei were stained with DAPI (blue). *B*, Numerical conversion of the results in panel *A*. Infection of SeV was quantified by counting of the fluorescent positive cells in ten fields of views per well of two wells. Relative infection values of the cells treated with (white bars) and without (black bars) IFNAR2 nAb were calculated by using the averaged number of infected cells without nAb treatment at 6 hrs postinfection as a benchmark. *P* value was calculated with Student's t test. *C*, Quantification of SeV RNA were measured by qRT-PCR. RNA samples from HuS-E/2 cells pre-treated with (open circles) and without (closed circles) nAb against IFN-α (5 μg/ml) were prepared at the indicated time points (24, 48, and 72 hrs post-infection with SeV). *D*, HCV RNA copies in HuS-E/2 cells pre-treated with (open circles) and without (closed circles) IFN-α nAb at the indicated time points (24, 48, 72, and 96 hrs post-infection with HCVcc) were measured by qRT-PCR. *E*, Time course expression of IFN-α1, IFN-β, IFN-λ1, IFN-λ3, RIG-I and IRF-7 mRNAs in the cells pre-treated with (open circles) or without (closed circles) IFN-α nAb during early stage of HCV infection (6, 12, 18, and 24 hrs post-infection with HCVcc). For each analysis, the results are normalized to the value obtained from the mock treatment. Error bars represent the calculated SD from the results obtained in three independent experiments (*B, C, D, E*).
doi:10.1371/journal.pone.0089869.g006

the proliferation of SeV in HuS-E/2 cells with or without pretreatment of IFN-α nAb by quantitative estimation of SeV genomic RNA. As shown in Fig. 6*C*, the increase of SeV genomic RNA levels was clearly observed in the cells with the pre-treatment at 12 hrs post-infection, suggesting that the preexisting IFN-α play a suppressive role on SeV proliferation during early phase of infection. In addition, to investigate the effect of constitutive IFN-α on the hepatotropic virus, we examined the infection and proliferation of HCVcc in HuS-E/2 cells with or without pretreatment of IFN-α nAb. As shown in Fig. 6*D*, the transient

infection and proliferation of HCVcc was observed in the HuS-E/2 cells as previously reported [10]. It was clearly observed that HCV RNA levels in the cells with the pretreatment were significantly higher than the cells without pretreatment from 24 to 48 hrs post-infection, although the significant difference was not observed in each cells from 72 to 96 hrs post-infection (Fig. 6*D*). Next, the expression of several IFN related genes in HuS-E/2 cells with or without the pretreatment was examined after HCVcc infection. As shown in Fig. 6*E*, compared to the cells without pretreatment, the delayed increases of RNAs for IFN-α1, IFN-β, IFN-λ1, IFN-λ3, RIG-I and IRF-7 after HCV infection were observed in HuS-E/2 cells with the pretreatment, although induction patterns were varied among those genes. These results suggested that IFN-α produced from HuS-E/2 cells without virus infection contributes to rapid antiviral innate immune response of the cells to limit the proliferation of HCV during the initial stage of infection.

Discussion

In this study, we showed that IFN-α1 gene is expressed in HuS-E/2 cells without virus infection as in the case of PHH, albeit at a low level and that the IFN-α, including IFN-α1, functions to elevate the expression of the genes related with anti-viral innate immune system in the cells. Previously, the expression pattern of the genes related with innate immunity of the HuS-E/2 cells, was shown to be similar to that of PHH [10]. The constitutive expression of IFN-α1 gene was also previously observed in human liver tissue [8]. Our results, therefore, suggested that the previous detection of IFN-α1 mRNA in normal human liver is due in part at least to the gene expression in hepatocytes in that tissue. The constitutive production of type I IFN has been reported previously in several tissues mainly concerning IFN-β [17,18,19,20]. The mechanisms that support the constitutive production of IFN-β have been relatively studied well and revealed to be involved with multiple transcription factors, such as c-Jun and RelA [6]. However, molecular mechanism that controls the steady-state production of IFN-α has been largely unclear. The transcriptional promoter region of IFN-α1 gene contains two regulatory elements, one is homologous to the positive regulatory domain I (PRDI) of the IFN-β gene promoter [21,22,23] and another is virus-responsive enhancer module, as proposed to a TG-like domain [24,25]. Our previous report showed that IRF-7 gene is constitutively expressed in HuS-E/2 cells [10]. IRF-7, together with IRF-3, is known to play a pivotal role in the induction of IFN-α and IFN-β genes through binding with PRDI in cells infected with virus [3,4], although it was suggested that IRF-7, rather than IRF-3, is important to suppress the infection and replication of HCV in the cells [10]. The basal expression of IFN-α and IFN-β genes, however, was reported not to depend on these regulatory factors [26]. We also observed that silencing IRF-7 in HuS-E/2 cells with shRNA method did not diminish the steady-state level expression of IFN-α1 (data not shown). We found some sequences homologous to the binding sites for some hepatocyte-specific transcription factors, including hepatocyte nuclear factor 1α (HNF1α), HNF1β, and HNF4α within the region 5000 base pairs upstream of the transcription start site of IFN-α1 gene using computational promoter analysis (data not shown). It, therefore, may be possible that the tissue-specific transcription factor contributes to the expression of IFN-α1 gene in a tissue-specific manner. As a recent study showed that tissue–specific differences in IFN genes or ISG expression can be attributed in part to the epigenetic regulation [27], it is probable that the innate immune phenotypes of IFN-α gene in human hepatocytes is also associated with tissue-specific patterns of histone modification. Further study is needed to clarify the molecular mechanisms of constitutive expression of IFN-α1 gene in human hepatocytes.

The results obtained from this study showed the functional role of the constitutive IFN-α in human hepatocytes on the immediate innate immune response against RNA virus infection, including HCV, through augmentation of the steady-state level expression of several genes related to detection of the infection and induction of IFN systems, such as RIG-I, IRF-7, and IFNs genes. Rapid activation of IFN system should be important to suppress the expansion of viral infection. The constitutive IFN-β has been reported previously in several tissues and was demonstrated to strengthen IFN response toward viral infection [17,18,19,20]. This constitutive IFN-β involves a positive feedback loop as proposed in a "revving-up model" in such tissues [28]. The weak cellular signals constantly introduced by constitutive IFN-β allows cells to elicit a more robust response against viral infection than the cells without such signals [17]. This signaling likely occasion induction of the IRF-7 gene without viral infection as a priming effect [29]. Cardiac myocytes was reported to produce higher basal IRF-7 without viral infection through the Jak-STAT pathway activated with preexisting IFN-β for instant antiviral response [20]. Plasmacytoid dendritic cells (pDCs) are known to produce IFN-α and IRF-7 constitutively just like human hepatocytes reported in this study. pDCs respond rapidly and effectively to a range of viral pathogens with high production of IFN-α in constitutive IRF-7 production dependent manner [30,31,32]. These suggested that IRF7, of which gene expression is induced by constitutive type-I interferon, both IFN-α and IFN-β, in those cells, plays a crucial role in the priming effect on the consecutive and rapid anti-viral innate immune response.

The liver is the largest solid organ in the body with dual inputs for its blood supply. It receives 80% of its blood supply from the gut through the portal vein, which is rich in bacterial products, environment toxins, and food borne pathogens. The remaining 20% of the blood is supplied from vascularization by the hepatic artery [33]. This high exposure to pathogens may require that the liver has an efficient and rapid defensive mechanism against possible frequent infection. Although most pathogens that get at the liver are killed by local innate and adaptive immune responses, hepatitis viruses (such as HBV and HCV) which gain the ingenious function to escape immune control persist in hepatocytes [34,35,36,37,38]. Therefore, further study to reveal the role of steady-state production of IFN-α1 in human hepatocytes may provide new insights into the virus-cell interaction and chronic infection of hepatotropic viruses.

In addition to the importance for the antiviral effect, it has been also proposed that the constitutive IFN-β primes for an efficient subsequent response to other cytokines and are also important for immune homeostasis [39,40,41], maintenance of bone density [42] and antitumor immunity [43]. Further analysis of the possible role of constitutive IFN-α on the other physiological events in human hepatocytes may be required.

Supporting Information

Table S1 List of names and sequences of the primers and expected sizes of RT-PCR products using those primers.
(TIF)

Author Contributions

Conceived and designed the experiments: YT MH. Performed the experiments: YT HK. Analyzed the data: YT MH. Contributed reagents/materials/analysis tools: TF KS MH. Wrote the paper: YT MH.

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Interleukin-1 and Tumor Necrosis Factor-α Trigger Restriction of Hepatitis B Virus Infection via a Cytidine Deaminase Activation-induced Cytidine Deaminase (AID)*

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Background: Cytokines and host factors triggering innate immunity against hepatitis B virus (HBV) are not well understood.
Results: IL-1 and TNFα induced cytidine deaminase AID, an anti-HBV host factor, and reduced HBV infection into hepatocytes.
Conclusion: IL-1/TNFα reduced host susceptibility to HBV infection through AID up-regulation.
Significance: Proinflammatory cytokines modulate HBV infection through a novel innate immune pathway involving AID.

Virus infection is restricted by intracellular immune responses in host cells, and this is typically modulated by stimulation of cytokines. The cytokines and host factors that determine the host cell restriction against hepatitis B virus (HBV) infection are not well understood. We screened 36 cytokines and chemokines to determine which were able to reduce the susceptibility of HepaRG cells to HBV infection. Here, we found that pretreatment with IL-1β and TNFα remarkably reduced the host cell susceptibility to HBV infection. This effect was mediated by activation of the NF-κB signaling pathway. A cytidine deaminase, activation-induced cytidine deaminase (AID), was up-regulated by both IL-1β and TNFα in a variety of hepatocyte cell lines and primary human hepatocytes. Another deaminase APOBEC3G was not induced by these proinflammatory cytokines. Knockdown of AID expression impaired the anti-HBV effect of IL-1β, and overexpression of AID antagonized HBV infection, suggesting that AID was one of the responsible factors for the anti-HBV activity of IL-1/TNFα. Although AID induced hypermutation of HBV DNA, this activity was dispensable for the anti-HBV activity. The antiviral effect of IL-1/TNFα was also observed on different HBV genotypes but not on hepatitis C virus. These results demonstrate that proinflammatory cytokines IL-1/TNFα trigger a novel antiviral mechanism involving AID to regulate host cell permissiveness to HBV infection.

The intracellular immune response can eliminate pathogens from a host, and host cells possess different mechanisms to counteract viral infection depending on the virus type. Human immunodeficiency virus (HIV) infection is restricted by cellular proteins designated as restriction factors, including APOBEC3G (A3G),[3] TRIM5α, tetherin/BST-2, and SAMHD1 (1, 2). All of these factors can be induced by stimulation with interferon (IFN). Hepatitis C virus (HCV) is eliminated by type I and III IFNs derived from dendritic cells or infected hepatocytes (3–6). In hepatocytes, this process involves a series of antiviral factors that are downstream genes of IFN, IFN-stimulated genes (ISGs). Influenza virus spread and virulence is inhibited by cytokines such as IFNs and TNFα. Responsive genes for these mechanisms include IFN-induced cellular Mx proteins that are dynamin-like GTPases (7, 8). However, these cytokine-induced antiviral immune responses are poorly understood in hepatitis B virus (HBV) infection.

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[3] The abbreviations used are: A3G, APOBEC3G; AID, activation-induced cytidine deaminase; HBV, hepatitis B virus; HCV, hepatitis C virus; ISG, IFN-stimulated gene; QNZ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; GEq, genome equivalent; PHH, primary human hepatocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ISRE, interferon sensitivity-responsive element; cccDNA, covalently closed circular DNA.

HBV infection is a worldwide health problem affecting more than 350 million people and is a major cause of the development of liver cirrhosis and hepatocellular carcinoma (9–11). During the course of infection, a number of cytokines and chemokines are up-regulated in HBV-infected patients, including IFNα/γ/λ, TNFα, IL-1, IL-6, IL-10, IL-12, IL-15, and IL-8 (12–15). Some of these cytokines are reported to suppress HBV replication (3, 16–21). In particular, type I, II, and III IFNs suppress the replication of HBV *in vitro* and *in vivo* (19, 20, 22–26). Although one of the downstream genes of IFN, *A3G*, has the potential to reduce HBV replication (27–34), it is still under discussion whether this protein is responsible for the anti-HBV activity of type I IFN, because it has been previously reported by Trono and co-workers (28, 35) that the induction of A3G does not explain the IFN-induced inhibition of HBV replication. Moreover, these studies were carried out using an HBV transgene that only reproduces a portion of the whole HBV life cycle, mainly focusing on intracellular HBV replication.

Here, we screened for cytokines and chemokines that affected HBV infection in HepaRG cells, a human hepatocyte cell line susceptible to HBV infection and reproducing the whole HBV life cycle (36, 37). IL-1 and TNFα decreased the host cell permissiveness to HBV infection, and this effect was at least partly mediated by the induction of activation-induced cytidine deaminase (AID). The anti-HBV activity of IL-1/TNFα was mechanistically different from that of IFNα. This study presents the activity of IL-1/TNFα to suppress HBV infection into hepatocytes independent of the effect on immune cells and the physiological role of AID in this machinery. Moreover, as far as we know, this is the first report to show the AID function to inhibit the infection of human pathogenic virus.

EXPERIMENTAL PROCEDURES

Reagents—All cytokines were purchased from PeproTech or R & D Systems. Heparin was obtained from Mochida Pharmaceutical. Lamivudine, PD98059, SP600125, SB203580, and Bay11-7082 were obtained from Sigma. Entecavir was obtained from Santa Cruz Biotechnology. BMS-345541 and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from Merck.

Cell Culture—HepaRG cells (Biopredic) were cultured with Williams' medium E (Invitrogen) supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 5 μg/ml insulin (Wako), 20 ng/ml EGF (PeproTech), 50 μM hydrocortisone (Sigma), and 2% DMSO (Sigma). HepG2, HepAD38 (kindly provided by Dr. Seeger at Fox Chase Cancer Center) (38), and HepG2.2.15 cells (a kind gift from Dr. Urban at Heidelberg University) (39) were cultured with DMEM/F-12 + GlutaMAX (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 50 μM hydrocortisone, and 5 μg/ml insulin in the presence (HepAD38 and HepG2.2.15) or absence (HepG2) of 400 μg/ml G418 (Nacalai Tesque). HepAD38 cells were cultured with 0.3 μg/ml tetracycline when terminating HBV induction. Huh-7.5.1 cells (kindly provided from Dr. Chisari at Scripps Research Institute) were cultured as described previously (40). Primary human hepatocytes (PHH) isolated from urokinase-type plasminogen activator transgenic/SCID mice inoculated with PHH (PhoenixBio) or purchased from Lonza were cultured with DMEM supplemented with 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 44 mM $NaHCO_3$ or with 1 mM pyruvate, nonessential amino acids, 20 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 0.25 μg/ml insulin (Wako), 5 ng/ml EGF, and 50 nM dexamethasone.

HBV Preparation and Infection—HBV used in this study was mainly derived from HepAD38 cells, which is classified as genotype D (38). Media from HepAD38 cells at days 7–31 post-induction of HBV by depletion of tetracycline were recovered every 3 days. Media were cleared through a 0.45-μm filter and precipitated with 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with medium at ~200-fold concentration. The HBV DNA was quantified by real time PCR. HBV genotype A and C in Fig. 7*B* was recovered from the media of HepG2 cells transfected with the plasmid pHBV/Aeus and pHBV/C-AT (41).

HepaRG cells were infected with HBV at 2000 (Fig. 7*B*) or 6000 (other figures) genome equivalent (GEq)/cell in the presence of 4% PEG8000 for 16 h as described previously (36). Urban and co-workers (42) reported that more than 10^3 GEq/cell amount of HBV derived from HepAD38 or HepG2.2.15 cells (*i.e.* $1.25–40 \times 10^4$ GEq/cell) as inoculum was required for efficient infection into HepaRG cells. The anti-HBV effect of IL-1/TNFα shown in this study was also observed when inoculated with HBV at 300 GEq/cell (data not shown).

Extraction of DNA and RNA—HBV DNA was extracted from the cells or from the medium using a DNA kit (Qiagen) according to the manufacturer's protocol. Total RNA was recovered with RNeasy mini kit (Qiagen) according to the manufacturer's protocol.

Real Time PCR and RT-PCR—HBV DNA was quantified by real time PCR analysis using the primer set 5′-ACTCACCAACCTCCTGTCCT-3′ and 5′-GACAAACGGGCAACATACCT-3′ and probe 5′-carboxyfluorescein (FAM)-TATCGCTGGATGTGTCTGCGGCGT-carboxytetramethylrhodamine (TAMRA)-3′ (43). The PCR was performed at 50 °C for 2 min, 94 °C for 10 min, and 50 cycles of 94 °C for 15 s and 60 °C for 1 min. Detection of cccDNA was achieved using 5′-CGTCTGTGCCTTCTCATCTGC-3′ and 5′-GCACAGCTTGGAGGCTTGAA-3′ as primers and 5′-CTGTAGGCATAAATTGGT (MGB)-3′ as a probe (44). This primer-probe set theoretically detected neither relaxed circular DNA nor HBV DNA integrated into host genome but can capture cccDNA as described previously (44). For quantification of cellular mRNA, cDNA was synthesized from extracted RNA using SuperScriptIII (Invitrogen), followed by PCR with TaqMan Gene Expression Master Mix (Applied Biosystems) and primer-probe set (TaqMan Gene Expression Assay, Applied Biosystems) or with Power SYBR Green PCR Master Mix (Applied Biosystems) and 5′-AAATGTCCGCTGGGCTAAGG-3′ and 5′-GGAGGAAGAGCAATTCCACGT-3′ as primers for *AID*.

RT-PCR was performed as described previously (45) using a one-step RNA PCR kit (Takara). Primers for amplifying each gene were as follows: 5′-CTCTGAGGTTTAGCATTTCA-3′ and 5′-CTCCAGGTCCAAAATGAATA-3′ for *cIAP*; 5′-GCA-

GATTTATCAACGGCTTT-3′ and 5′-CAGTTTTCCACCACAACAAA-3′ for *XIAP*; 5′-TAGCCAACATGTCCTCACAGAC-3′ and 5′-TCTTCTACCACTGGTTTCATGC-3′ for *ISG56*; 5′-GCCTTTTCATCCAAATGGAATTC-3′ and 5′-GAAATCTGTTCTGGGCTCATG-3′ for *PKR*; and 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for *GAPDH*, respectively.

ELISA—HBs protein was quantified by ELISA using plates incubated at 4 °C overnight with a sheep anti-HBs antibody at 1:5000 dilution (Maxisorp nunc-immuno plate, Nunc catalog no. 439454) followed by coating with 0.2% BSA, 0.02% NaN_3, 1× PBS at 4 °C until use. Samples were incubated with the plates for 2 h and after washing with TBST four times, horseradish peroxidase-labeled rabbit anti-HBs antibody was added for 2 h. The substrate solution (HCV core ELISA kit: Ortho) was reacted for 30 min before the A_{450} values were measured.

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed essentially as described previously (45). After fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100, an anti-HBc antibody (DAKO, catalog no. B0586) was used as the primary antibody.

MTT Assay—The MTT assay was performed as described previously (46).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (47). The polyclonal antibody against AID was generated using a peptide derived from AID protein as an immunogen as described previously for preparation of the anti-AID antibody 1 (48). The specificity of the antibody was described previously (48, 49).

Lentiviral Vector-mediated Gene Transduction—Lentivirus carrying shRNAs was prepared with 293T cells transfected with expression plasmids for HIV-1 Gag-Pol, VSV G, and shRNAs (sh-control, sh-cyclophilin A, sh-AID(1), sh-AID(2); Mission shRNA) (Sigma) with Lipofectamine 2000 (Invitrogen). Recovered lentiviral vector was transduced into HepaRG cells followed by selection with 1.5 μg/ml puromycin. Lentivirus overexpressing AID, AID mutant, A3G, or the control lentivirus was recovered using expression plasmids for HIV-1 Gag-Pol, Rev, VSV G, and the corresponding expression vector as described previously (50).

Southern Blot Analysis—Southern blot was performed as described previously (41). After digestion of free nucleic acids with DNase I and RNase A, cell lysates were digested with proteinase K, and HBV DNA in the core particles was extracted with phenol/chloroform, followed by isopropyl alcohol precipitation. Probe was prepared by cutting pHBV/D-IND60 (41) with SacII and BspHI to generate a full-length HBV DNA probe and labeled with AlkPhos direct labeling reagents (GE Healthcare). Labeled bands were visualized with CDP-star detection reagent (GE Healthcare).

Quantification of Nucleocapsid-associated HBV RNA—After digestion of free nucleic acids with DNase I and RNase A, nucleocapsid was precipitated with PEG8000 (41). Total RNA was then extracted from the resuspended precipitates. HBV RNA was quantified by real time RT-PCR with 5′-TCCCTCGCCTCGCAGACG-3′ and 5′-GTTTCCCACCTTATGAGTC-3′ as primers with Power SYBR Green PCR Master Mix (Applied Biosystems).

Co-immunoprecipitation Assay—Co-immunoprecipitation assay was essentially performed as described (45).

Differential DNA Denaturation PCR—Differential DNA denaturation PCR was performed as described previously (51).

Reporter Assay—DNA transfection was performed with pNF-κB-luc or pISRE-TA-luc (Stratagene) and pRL-TK (Promega), which express firefly luciferase driven by NF-κB or ISRE and *Renilla* luciferase by herpes simplex virus thymidine kinase promoter, respectively, and Polyethylenimine Max (Polysciences Inc., catalog no. 24765). After compound or cytokine treatment, cells were lysed, and luciferase activities were measured as described previously (52). A reporter carrying HBV core promoter was constructed by inserting the DNA fragment (1413–1788 nucleotide number) of HBV DNA (D-IND60) into pGL4.28 vector (Promega) (41). In the reporter assay using this construct (Fig. 1*H*), HX531, a retinoid X receptor antagonist was used as a positive control as retinoid X receptor was involved in the transcription from the core promoter (53).

RESULTS

IL-1 Reduced Host Cell Susceptibility to HBV Infection—To evaluate the effect of cytokines and chemokines on susceptibility to HBV infection, we treated HepaRG cells (36) with cytokines for 3 h prior to and 16 h during HBV infection, followed by culture without stimuli for an additional 12 days (Fig. 1*A*, *lower scheme*). Heparin, a competitive inhibitor of HBV attachment (54), was used as a positive control and decreased secretion of the viral envelope surface protein (HBs) from HBV-infected cells (Fig. 1*A*, *upper graph, lane 38*), which suggests a successful HBV infection in this experiment. Examination of 36 cytokines and chemokines revealed that IL-1β drastically decreased protein secretion from HBs (Fig. 1*A*, *upper graph, lane 8*). Although IFNs had a strong anti-HBV effect by a continuous treatment after HBV infection (Fig. 3*C*, *panel b*, and data not shown), they had only a limited effect in this screening where cytokines were only pretreated and cotreated with HBV (Fig. 1*A*, *lanes 2–7*). HBc protein expression (Fig. 1*B*) and HBV DNA (Fig. 1*C*) in the cells and medium (Fig. 1*D*) were significantly decreased by treatment with IL-1β without cytotoxicity (Fig. 1*G*). HBV cccDNA and HBV RNA was also decreased in infected cells treated with IL-1β (Fig. 1, *E* and *F*). IL-1β did not decrease HBV core promoter activity at least in HepG2 cells (Fig. 1*H*). These results suggest that IL-1β suppressed HBV infection to HepaRG cells. IL-1β did not decrease the expression of sodium taurocholate cotransporting polypeptide (*NTCP*), a recently reported HBV entry receptor (data not shown) (55). Similar results were obtained using primary human hepatocytes (Fig. 1*I*).

NF-κB Signaling Was Critical for Anti-HBV Activity—As shown in Fig. 2*A*, IL-1β suppressed HBV infection in a dose-dependent manner. This anti-HBV effect was reversed by cotreatment with a neutralizing antibody for the IL-1 receptor, IL-1RI (Fig. 2*B*), suggesting that receptor engagement was required for anti-HBV activity. IL-1Ra is a natural antagonist that associates with IL-1RI but does not trigger downstream signal transduc-

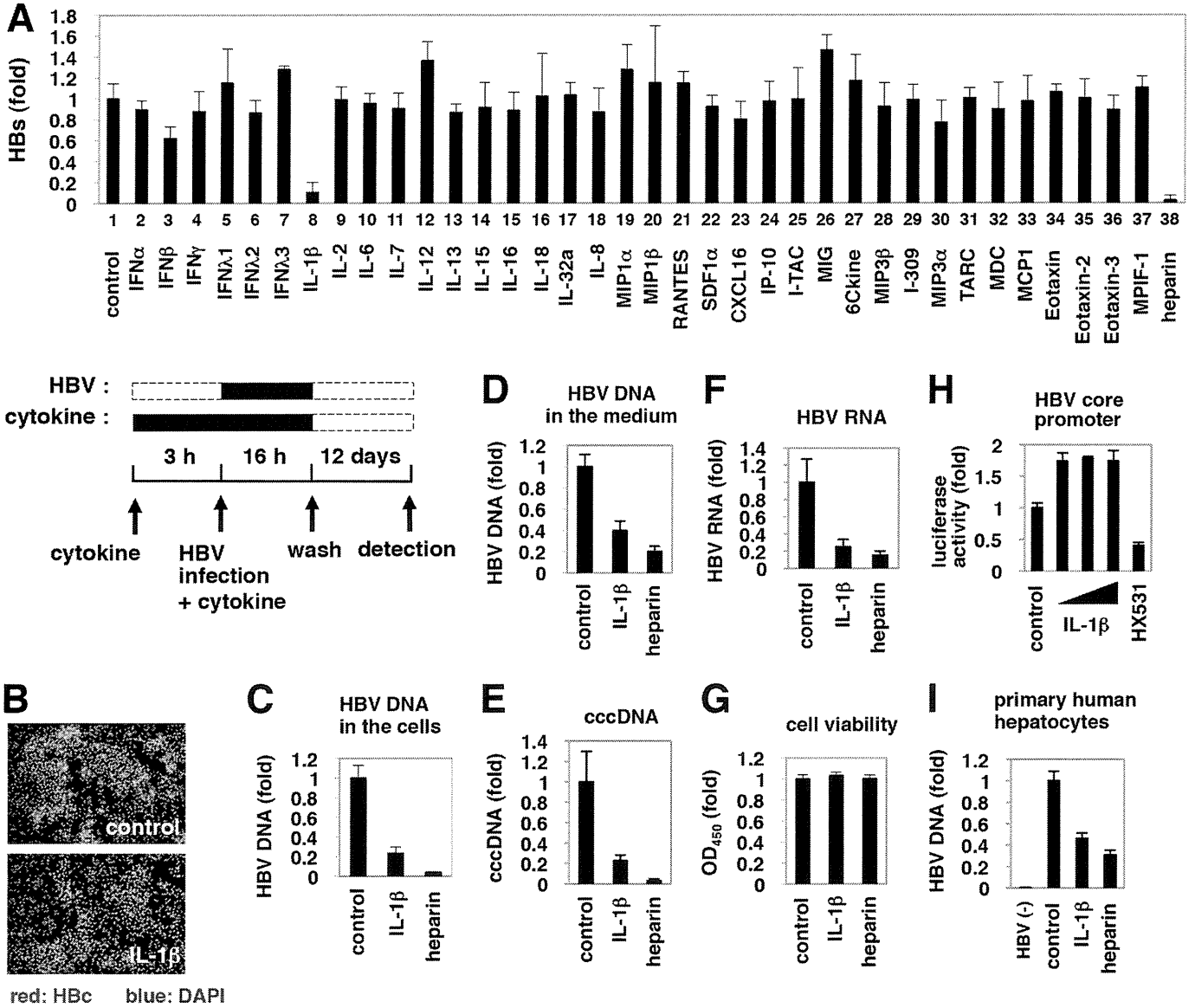

FIGURE 1. **Suppression of HBV infection by IL-1β.** *A, upper graph*, HepaRG cells were pretreated with cytokines at 100 ng/ml (except for IFNα and IFNβ at 100 IU/ml) or heparin at 25 units/ml as a positive control or were left untreated (*control*) for 3 h and then infected with HBV in the presence of each stimuli for 16 h. After washing, cells were cultured in normal growth medium for 12 days. HBs protein secreted into the medium was quantified by ELISA. *Lower scheme* indicates the treatment procedure for HepaRG cells. *Black* and *dashed line boxes* indicate the periods with and without treatment, respectively. *B–G* and *I*, HepaRG cells (*B–G*) or PHH (*I*) were treated as shown in *A* with or without 100 ng/ml IL-1β or 25 units/ml heparin as a positive control. HBc protein in the cells (*red*) was detected by indirect immunofluorescence analysis, and the nucleus was stained with DAPI (*blue*) at 12 days post-infection (*B*). HBV DNA (*C* and *I*), cccDNA (*E*), and HBV RNA (*F*) in the cells as well as HBV DNA in the medium (*D*) were detected. Cell viability was quantified by MTT assay (*G*). *HBV(−)* in *I* indicates uninfected cells. All of the data, except in *I*, are based on the average of three independent experiments. *I* shows the average results from one representative experiment, but the reproducibility of the data were confirmed in three independent experiments. *H*, reporter plasmid carrying the HBV core promoter was transfected with HepG2 cells and then treated with or without IL-1β (1, 10, and 100 ng/ml) and an retinoid X receptor antagonist HX531 as a positive control for 6 h. Luciferase activity was measured.

tion (56). Treatment with IL-1Ra did not decrease HBV infectivity (Fig. 2*C*), suggesting that signal transduction triggered by IL-1 was required for anti-HBV activity.

To identify the signal transduction pathway essential for anti-HBV activity, we treated HepaRG cells with PD98059, SP600125, SB203580, and Bay11-7082, which are inhibitors for MEK, JNK, p38, and NF-κB, respectively (57). As shown in Fig. 2*D*, only cotreatment with Bay11-7082 significantly removed the anti-HBV effect of IL-1β. Luciferase assay and RT-PCR analysis indicated that Bay11-7082, but not other inhibitors, blocked the transactivation of NF-κB (Fig. 2*E*, *upper panels*) and NF-κB downstream genes, *cIAP* and *XIAP* (Fig. 2*E*, *lower panels*). Additional NF-kB inhibitors, BMS-345541 and QNZ (Fig. 2*G*), also reversed the anti-HBV effect of IL-1β (Fig. 2*F*). These data suggest a critical role for NF-κB activation in the anti-HBV activity. Additionally, IL-1β did not augment the activity of interferon sensitivity-responsive element (ISRE) and mRNAs for ISGs, *ISG56*, and double-stranded RNA-dependent protein kinase (*PKR*) in HepaRG cells (Fig. 2*H*), suggesting that the anti-HBV activity is independent of ISG up-regulation. TNFα, another cytokine that activates NF-κB signaling (Fig. 2*E*, *lower panels*), also inhibited HBV infection (Fig. 2*I*). Thus, NF-κB activation in host hepatocytes was critical for the anti-HBV activity of proinflammatory cytokines.

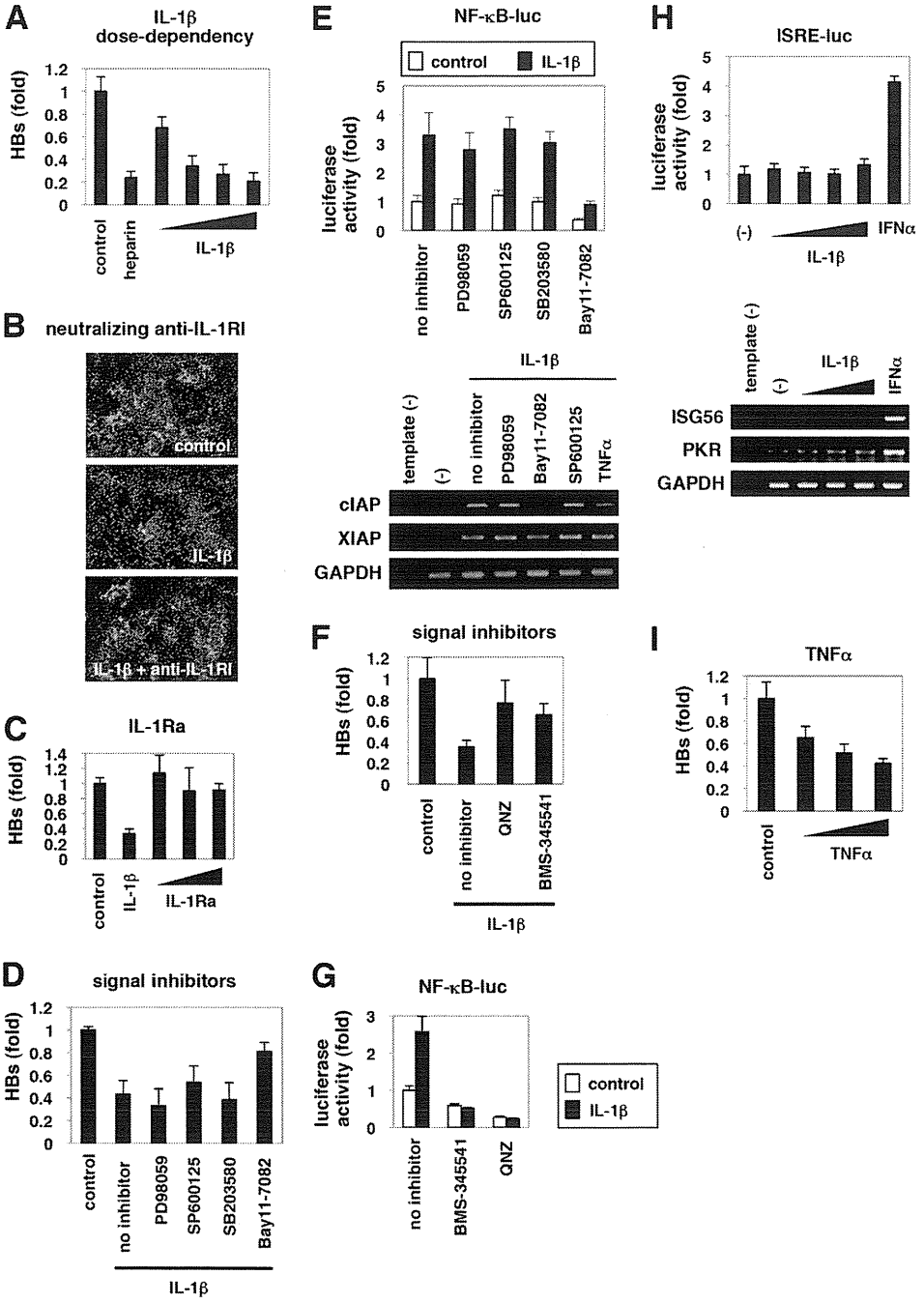

FIGURE 2. **NF-κB activation triggered by IL-1 and TNFα was critical for anti-HBV activity.** *A–D, F,* and *I,* HepaRG cells were left untreated (*control*) or treated with varying concentrations of IL-1β (1, 10, 30, and 100 ng/ml) or 25 units/ml heparin (*A*), with 30 ng/ml IL-1β together with or without a neutralizing anti-IL-1RI antibody at 20 μg/ml (*B*), with 10 ng/ml IL-1β or varying concentrations of IL-1Ra (10, 30, and 100 ng/ml) (*C*), with 3 ng/ml IL-1β together with or without PD98059, SP600125, SB203580, or Bay11-7082 (*D*), or QNZ or BMS-345541 (*F*), or with TNFα (10, 100, and 300 ng/ml) (*I*) according to the treatment schedule shown in Fig. 1*A*. HBV infection was monitored by HBs protein secretion into the medium in *A, C, D, F,* and *I* and with HBc protein in the cells in *B*. *E, G,* and *H,* NF-κB (*E* and *G*) and ISRE activity (*H*) were measured by reporter assay in the cells transfected with the reporter plasmid expressing luciferase driven from five tandem repeats of NF-κB elements (*E, upper graph,* and *G*) or ISRE (*H, upper graph*) or by RT-PCR in the cells (*E* and *H, lower panels*) upon signaling inhibitors used in *D* and *F* together with or without IL-1β (*E* and *G*), or upon IL-1β (10, 30, and 100 ng/ml) or IFNα 100 IU/ml as a positive control (*H*) for 6 h. The *white* and *black bars* in the *upper graph* of *E* and *G* show the data in the absence or presence of IL-1β, respectively. Bands for mRNA for *cIAP, XIAP,* and *GAPDH* (*E*) or *ISG56, PKR,* or *GAPDH* (*H*) are presented in the *lower panels*. All of the data are based on averages of three independent experiments.

Early Phase of HBV Infection as Well as HBV Replication Were Impaired by IL-1 Treatment—Although heparin, an attachment inhibitor, could block HBV infection only if added together with the HBV inoculum, pretreatment with IL-1β before HBV infection was sufficient to show anti-HBV activity (Fig. 3*A, panel b*). This activity was amplified by a prolonged treatment time of up to 12 h (Fig. 3*B*). Intriguingly, HBV cellular DNA was also reduced by IL-1β treatment following HBV infection (Fig. 3*C, panel b*). In contrast, IFNα was not effective by pretreatment (Figs. 3*C, panel a,* and 1*A*), although it did decrease HBV DNA by treatment after HBV infection (Fig. 3*C, panel b*), consistent with previous reports that IFNα can sup-

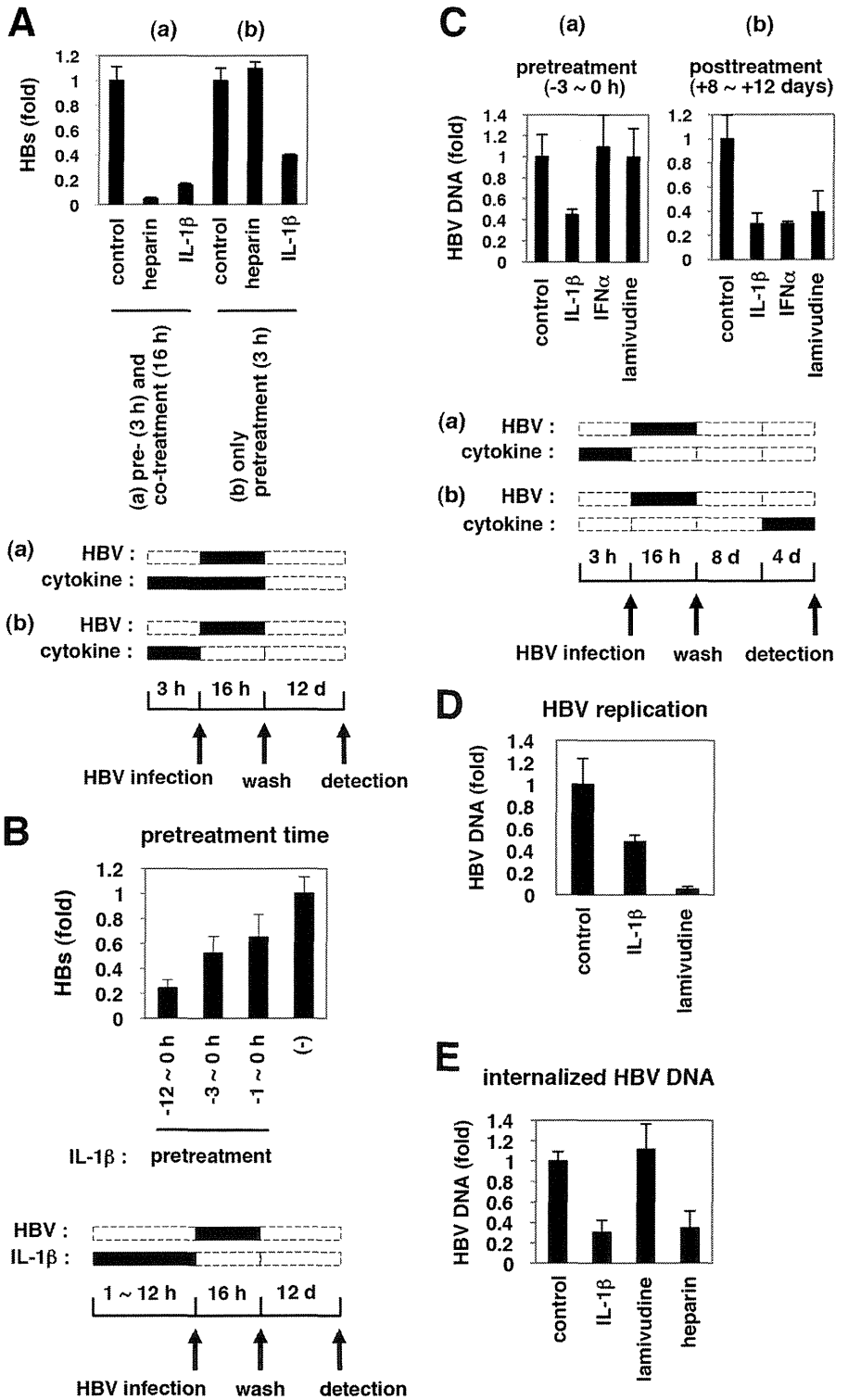

FIGURE 3. **Defining the steps of the HBV life cycle targeted by IL-1β.** *A*, HepaRG cells were pretreated with IL-1β or heparin for 3 h and then infected with HBV in the presence (*A, panel a*) or absence (*A, panel b*) of IL-1β or heparin for 16 h. HBV infection was monitored with HBs protein secretion from the infected cells. Only pretreatment with IL-1β and not heparin could inhibit HBV infectivity. *d*, day. *B*, HepaRG cells were pretreated with IL-1β or left untreated (−) for the indicated time (*h*) and infected with HBV without IL-1β. Anti-HBV activity was amplified by a prolonged treatment time. *C, panel a*, HepaRG cells were pretreated with 10 ng/ml IL-1β, 100 IU/ml IFNα, or 1 μM lamivudine for 3 h, followed by infection with HBV for 16 h in the absence of cytokines (*pretreatment*). *C, panel b*, HepaRG cells were infected with HBV for 16 h without pretreatment. After washing out the input virus, cells were cultured in normal medium for the first 8 days and then cultured with IL-1β, IFNα, or lamivudine for the following 4 days (*post-treatment*). HBV DNA in the cells was measured by real time PCR. IL-1β showed an anti-HBV activity in both pretreatment and post-treatment, although an anti-HBV effect of IFNα was seen only with post-treatment. *D*, HepAD38 cells were treated with 100 ng/ml IL-1β or 1 μM lamivudine, or left untreated for 6 days in the absence of tetracycline. HBV replication was evaluated by measurement of HBV DNA in the medium. *E*, HepaRG cells were pretreated with IL-1β, lamivudine, or heparin for 3 h or left untreated and infected with HBV for 16 h in the presence or absence of each compound. After trypsinization and extensive washing of the cells, cellular DNA was immediately recovered to detect HBV DNA. HBV DNA at 16 h post-infection was decreased by treatment with IL-1β but not lamivudine.

press HBV replication (19, 20, 26). Thus, the anti-HBV activity of IL-1β is likely to be mechanistically different from that of IFNα.

The HBV life cycle can be divided into at least two phases as follows: 1) the early phase of infection that includes attachment, entry, nuclear import, and cccDNA formation; and 2) the late phase representing HBV replication, including transcription, assembly, reverse transcription, DNA synthesis, and viral release (58). The early phase of HBV infection is not supported, but HBV DNAs persistently replicate in HepAD38 cells in the presence of tetracycline (38). IL-1β decreased the HBV DNA levels in HepAD38 cells (Fig. 3*D*), suggesting suppression of HBV replication. In addition, to examine the early phase preceding HBV replication, we infected HepaRG cells with HBV in the presence of IL-1β for 16 h and then immediately recovered cellular DNA in the trypsinized cells for quantification of HBV DNA (Fig. 3*E*). This procedure likely detected HBV DNA that had been internalized and evaded the host restriction before initiation of HBV replication because lamivudine showed no effect on the amount of DNA detected (Fig. 3*E*). In this experiment, IL-1β significantly decreased HBV DNA (Fig. 3*E*). cccDNA was also decreased by IL-1β, suggesting that the early phase of HBV infection before cccDNA formation was also interrupted by IL-1β.

IL-1 and TNFα Induced the Expression of AID—The innate immune pathway against HBV infection remains largely unknown. Recently, accumulating evidence suggested that several APOBEC family proteins, especially A3G, suppressed HBV replication when overexpressed (27–33). In contrast, there was no report available suggesting the anti-HBV function of other restriction factors against HIV, TRIM5α, tetherin/BST-2, and SAMHD1. We then investigated APOBEC family proteins as a candidate for an anti-HBV effector. The APOBEC family includes APOBEC1 (A1), A2, A3s, A4, and AID (59). Because some of these proteins are reported to be up-regulated in cytokine-stimulated hepatocytes (27, 28, 60, 61), we examined the expression of these genes in cells treated with IL-1β, TNFα, and IFNα as a control for 12 h. The mRNA levels of *A1*, *A2*, and *A3A* were below the detection threshold. *A3G* and *A3F* mRNA were significantly expressed in HepaRG cells, and their expression levels were remarkably increased by IFNα treatment (Fig. 4*A*), as observed in other reports (27, 28, 61). IL-1β and TNFα did not significantly up-regulate A3s, and only AID was up-regulated 6–10-fold by both cytokines (Fig. 4*A*). Induction of A3s by both IL-1β and TNFα was not observed at any time point examined until 12 h (data not shown). In contrast, induction of *AID* mRNA by IL-1β and TNFα was conserved in human hepatocyte cell lines, such as HepG2 and FLC4 cells, and in primary human hepatocytes (Fig. 4*B*). AID protein production was also increased in primary human hepatocytes by treatment with IL-1β and TNFα (Fig. 4*C*). This AID induction by IL-1β was suggested to be NF-κB-dependent, because the up-regulation of *AID* mRNA was canceled by addition of NF-κB inhibitors, Bay11-7082 or QNZ (Fig. 4*D*).

AID Played a Significant Role in the IL-1-mediated restriction of HBV—To examine the function of AID during HBV infection, we transduced AID ectopically into HepaRG cells using a lentiviral vector (Fig. 5*A*, *left panel*). The susceptibility of these AID-overexpressing cells to HBV was decreased by approximately one-third compared with the parental or empty vector-transduced HepaRG cells (Fig. 5*A*, *right panel*), suggesting that AID can restrict HBV infection. An AID mutant AID(M139V), with reported diminished activity to support class switching (48), also decreased the susceptibility to HBV infection, although the reduction in HBV susceptibility was moderate compared with the case of the wild type AID (Fig. 5*B*).

To examine the relevance of endogenous AID in the anti-HBV activity of IL-1, we transduced a lentiviral vector carrying a short hairpin RNA (shRNA) against AID (sh-AID) or a non-relevant protein cyclophilin A (Fig. 5*C*), and we observed the anti-HBV activity of IL-1β in these cells. IL-1β decreased HBV infection in the control and sh-cyclophilin A -transduced cells by ~3.0-fold as determined by HBs secretion (Fig. 5*D*, *lanes 1* and *2*, *black bars*). In contrast, anti-HBV activity of IL-1β was limited to only 1.6–1.7-fold in the cells transduced with sh-AIDs (Fig. 5*D*, *lanes 3* and *4*, *black bars*). Such relieved anti-HBV activity following AID knockdown was not observed in the case for heparin treatment (Fig. 5*D*, *lanes 1–4*, *gray bars*). Similar results were obtained by monitoring intracellular HBV DNA after infection (data not shown). Although the anti-HBV effect of IL-1β was not completely blunted, these data suggest that AID plays a significant role in mediating the anti-HBV effect of IL-1β.

Similar observations were obtained in HBV-replicating cells overexpressing AID (Fig. 5, *E* and *F*). Core particle-associated HBV DNA in HepG2 cells transfected with an HBV-encoding plasmid was decreased by overexpression with AID as well as with A3G (Fig. 5*E*, *lanes 1* and *3*). Intriguingly, HBV DNA in core particles was also decreased by expression of an AID mutant AID(H56Y), which contains a mutation in the cytidine deaminase motif and is derived from a class switch deficiency patient (Fig. 5*E*, *lane 2*) (48). Southern blot also showed that the HBV rcDNA level in HepG2.2.15 cells was reduced by transduction with AID and another mutant AID(M139V), with diminished activity to support class switching (Fig. 5*F*) (48). These data suggest that AID could suppress HBV replication, and this restriction activity can be still observed with reduced enzymatic activity. In addition, AID was shown to interact with HBV core protein by coimmunoprecipitation assay (Fig. 5*G*). Moreover, overexpression of AID reduced the levels for nucleocapsid-associated HBV RNA (Fig. 5*H*). These results further suggest an antiviral activity of AID against HBV replication.

AID Could Induce Hypermutation of HBV DNA—Major enzymatic activity for APOBEC family proteins is the introduction of hypermutation in target DNA/RNA, and hypermutation accounts for antiviral activity for A3G against HIV-1 to some extent (2). Several groups reported that APOBEC family proteins could induce hypermutation in HBV DNA (27, 30, 32, 34). Next we asked whether AID could induce hypermutations in HBV DNA. In differential DNA denaturation PCR analysis, a high content of A/T bases introduced by hypermutation decreased denaturation temperatures (51). As shown in Fig. 6*A*, ectopic expression of AID decreased the denaturation temperature of HBV DNA as shown by that of A3G. Sequence analyses of the HBV DNA X region amplified at 83 °C by differential DNA denaturation PCR indicated a massive accumulation of

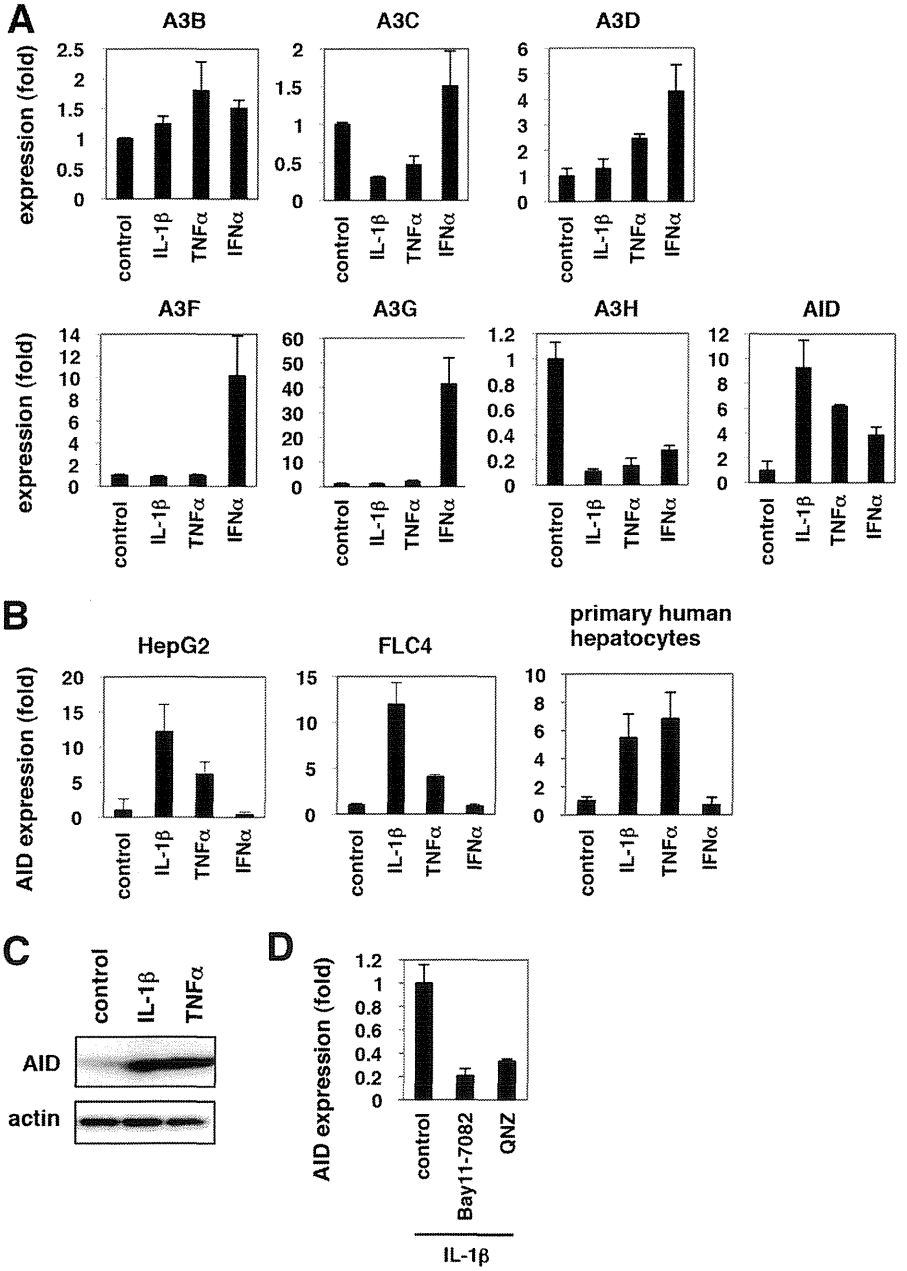

FIGURE 4. **AID expression was induced by IL-1β and TNFα.** *A*, mRNAs for *A3B*, *-C*, *-D*, *-F*, *-G*, *-H* and *AID* were quantified by real time RT-PCR analysis in HepaRG cells treated with 100 ng/ml IL-1β, 100 ng/ml TNFα, or 100 IU/ml IFNα for 12 h or left untreated. *Graphs* show the relative expression levels compared with the controls set at 1. *B*, *AID* mRNA was detected in HepG2, FLC4 cells, and PHH treated with IL-1β, TNFα, or IFNα or left untreated. Induction of AID by IL-1β and TNFα was observed in HepG2 and FLC4 cells and primary human hepatocytes. *C*, AID protein (*upper panel*) and actin levels as an internal control (*lower panel*) were examined by immunoblot of primary human hepatocytes treated with IL-1β or TNFα or left untreated. *D*, AID mRNA was detected in PHH treated with 100 ng/ml IL-1β in the presence or absence of NF-κB inhibitors, Bay11-7082, or QNZ for 12 h.

G-to-A mutations by AID (Fig. 6*B*). The frequency of G-to-A mutations was augmented by AID expression (Fig. 6*C*). In this experiment, AID(JP8Bdel), a hyper-active mutant of AID (62), further promoted the accumulation of the G-to-A and C-to-T mutations, although AID(H56Y) showed mutations in HBV DNA equivalent with mock GFP control sample (Fig. 6*C*). Thus, AID had the potential to introduce hypermutation in nucleocapsid-associated HBV DNA.

IL-1 Suppressed the Infection of Different HBV Genotypes but Not That of HCV—We examined whether the antiviral activity of IL-1β and TNFα could be generalized to other viruses or was specific to HBV. As shown in Fig. 7*A*, the production of infectious HCV and HCV core proteins in the medium was not significantly altered by treatment with these cytokines in HCV-infected cells, compared to when IFNα was used as a positive control (Fig. 7*A*). In contrast, IL-1 suppressed the infection of HBV genotype A and C into HepaRG cells (Fig. 7*B*) as well as genotype D (Fig. 1*C*). These data suggest that the antiviral activity of proinflammatory cytokines IL-1 and TNFα is specific to HBV.

DISCUSSION

In this study, cytokine screening revealed that IL-1 and TNFα decreased the host cell susceptibility to HBV infection.

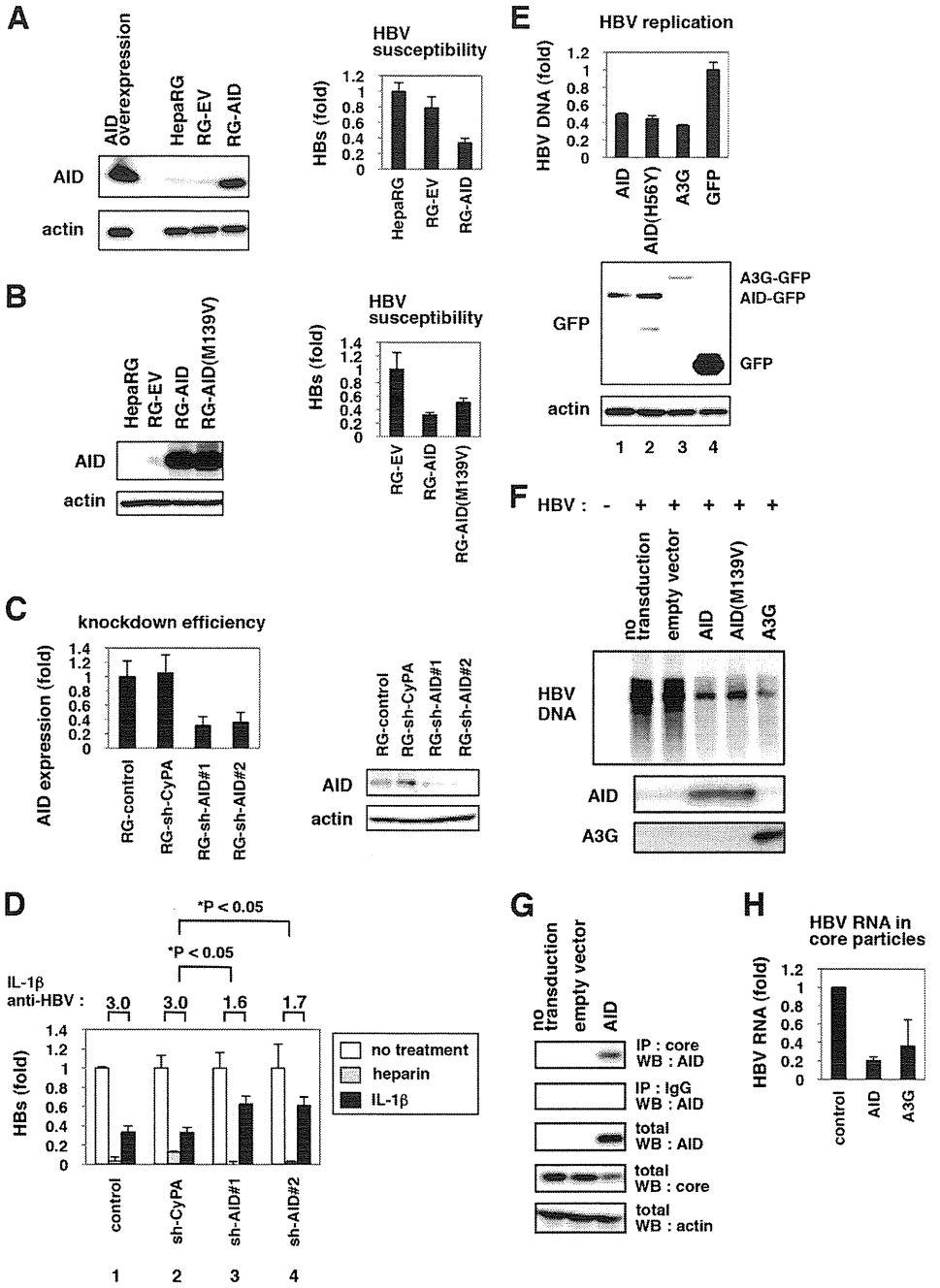

FIGURE 5. **AID played a significant role in IL-1-mediated anti-HBV activity.** *A* and *B*, *left panels*, HepaRG cells were transduced with a lentiviral vector carrying the expression plasmid for AID (*RG-AID*), AID(M139V) mutant (*RG-AID(M139V)*) (*B*), or the control vector (*RG-EV*). Protein expression for AID (*upper panel*) and actin (*lower panel*) in these cells, the parental HepaRG cells (*HepaRG*), and those transiently transfected with AID expression plasmid (*AID overexpression*) (*A*) was examined by immunoblot. *Right panels*, these cells were infected with HBV followed by detection of secreted HBs protein as Fig. 1*A*. AID-transduced cells were less susceptible to HBV infection. *C*, HepaRG cells were transduced with lentiviral vector carrying shRNAs for AID (*RG-shAID#1* and *RG-shAID#2*) or for cyclophilin A (*RG-shCyPA*) as a control. AID mRNA (*left panel*) and protein (*right panel*) were quantified by real time RT-PCR and immunoblot analysis. *D*, cells produced in *C* were infected with HBV in the absence or presence of IL-1β or heparin, and HBs was detected in the medium as in Fig. 1*A* to examine the anti-HBV effect of IL-1β and heparin. The fold reduction of HBV infection by IL-1β treatment is shown as *IL-1β anti-HBV* above the *graph*. The *white*, *gray*, and *black bars* indicate HBs value of the cells without treatment and with heparin and IL-1β treatment, respectively. The anti-HBV activity of IL-1β but not heparin was reduced in the AID-knockdown cells. *E*, AID and its mutant suppressed HBV replication. HepG2 cells were cotransfected with GFP-tagged AID, AID(H56Y), A3G, and GFP itself along with an HBV-encoding plasmid. Following 3 days, cytoplasmic nucleocapsid HBV DNA was quantified (*upper graph*), and the overexpressed proteins as well as actin were detected (*lower panels*). *F*, lentiviral vectors carrying AID, AID(M139V) mutant, A3G, or an empty vector (*empty vector*) were transduced or left untransduced (*no transduction*) into HepG2.2.15 cells. Nucleocapsid associated HBV DNA in these cells or in HepG2 cells (*HBV−*) was detected by Southern blot (*upper panel*). AID (*middle panel*) and A3G protein (*lower panel*) were also detected by immunoblot. *G*, HBV core interacted with AID. HepAD38 cells transduced without (*no transduction*) or with AID-expressing vector or the empty vector (*empty vector*) were lysed and treated with anti-core antibody (*1st panel*) or control normal IgG (*2nd panel*) for immunoprecipitation (*IP*). Total fraction without immunoprecipitation (*3rd* to *5th panels*) was also recovered to detect AID (*1st* to *3rd panels*), HBV core (*5th panel*), and actin (*5th panel*) by immunoblot. *WB*, Western blot. *H*, HBV RNA in core particles was extracted as shown under "Experimental Procedures" in HepG2 cells overexpressing HBV DNA together with or without AID or A3G.

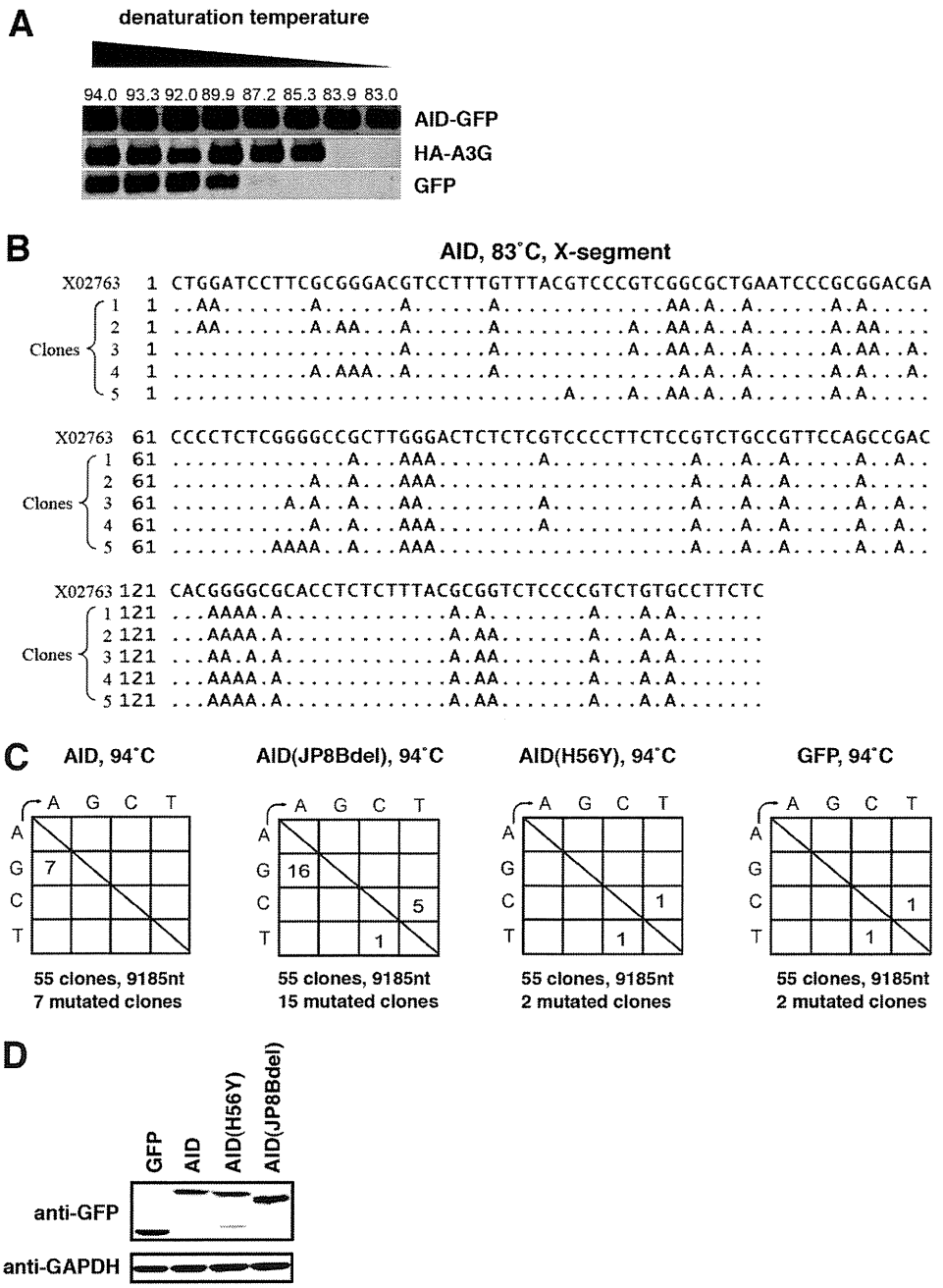

FIGURE 6. **AID could induce hypermutation of HBV DNA.** *A* and *B*, HepG2 cells were cotransfected with an expression vector for GFP-tagged AID, HA-tagged A3G, or GFP along with an HBV-encoding plasmid. 3 days after transfection, nucleocapsid-associated HBV DNA was extracted, and differential DNA denaturation PCR was performed to amplify the X gene segments. The *numbers above* the panels in *A* show denaturing temperatures. The X gene fragment amplified at 83 °C in the AID sample was cloned in to a T vector and sequenced in *B*. Alignment of independent five clones with reference sequence (X02763) is indicated. *C*, AID and its mutant (JP8Bdel) induced G-to-A and C-to-T hypermutations in HBV DNA. HepG2 cells were transfected with expression vectors of GFP-tagged AID, AID(H56Y), AID(JP8Bdel), or GFP itself together with HBV encoding plasmid. Three days after transfection, cells were harvested, and nucleocapsid-associated HBV DNA was extracted. X gene fragments were amplified at 94 °C and cloned in T vector. 55 clones were sequenced as described under "Experimental Procedures." The *numbers* indicate the clone numbers carrying the mutation. *D*, expression of GFP, GFP-tagged AID, AID(H56Y), and AID(JP8Bdel) is shown by immunoblot.

This antiviral mechanism is rather unique, given that the intracellular immune response against viruses is typically triggered by IFNs. So far, type I, II, and III IFNs are reported to suppress the replication step of the HBV life cycle (19, 20, 25, 26). In contrast, we suggest that IL-1 and TNFα inhibit the early phase of HBV infection as well as the replication. This is consistent with cumulative clinical evidence suggesting that these proinflammatory cytokines contribute to HBV elimination (63–65). IL-1 and TNFα are generally produced mainly in macrophages and also in other cell types, including T cells and endothelial cells (66). Although the main producer cells of these cytokines in hepatitis B patients are not defined, it has been reported that the secretion of IL-1 and TNFα in nonparenchymal cells were increased by HBV infection into hepatocytes (67). TNFα production in macrophages was augmented by addition of recombinant HBc (68). A number of clinical studies cumulatively

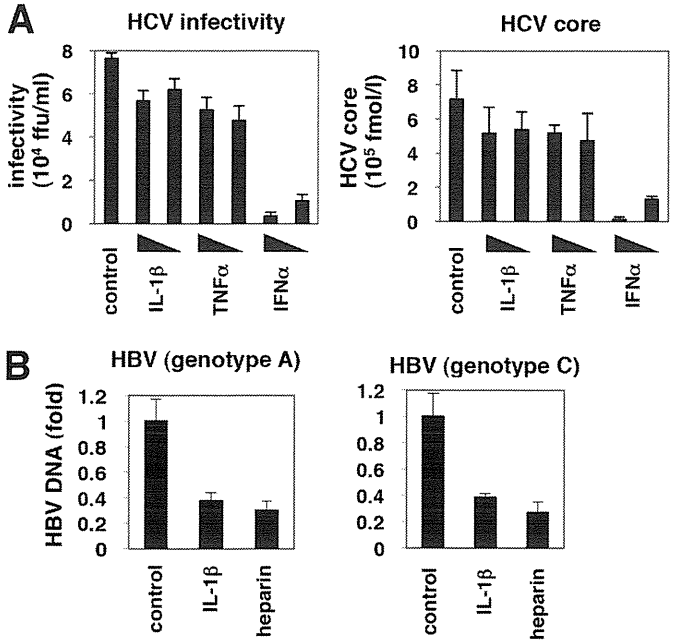

FIGURE 7. **Antiviral activity of AID was specific to HBV.** *A*, Huh-7.5.1 cells were pretreated with IL-1β, TNFα, or IFNα for 3 h or left untreated and then coincubated with HCV for 4 h. After washing HCV and cytokines and culturing the cells with normal medium for 72 h, the infectivity of HCV (*left panel*) as well as HCV core protein (*right panel*) in the medium was quantified. *B*, HepaRG cells were treated with IL-1β or heparin or left untreated for 3 h prior to and 16 h during infection of HBV genotype A (*left graph*) or C (*right graph*) as shown in Fig. 1*A*. HBV infection was monitored with cellular HBV DNA at 12 days after the infection as Fig. 1*C*.

show that serum levels of IL-1 and TNFα are increased in hepatitis B patients (12). Recently, it has been a significant clinical problem that HBV reactivates during the course of treatment with immunosuppressants such as anti-TNFα agents (64, 65). Taken together, it is proposed that acute or chronic HBV infection induces IL-1/TNFα from macrophages or other cells in the liver of infected patients, which can directly suppress HBV infection in hepatocytes, in addition to their immunomodulatory effects to the host immune cells. Although IL-1 level in HBV-infected patients varies between papers, Daniels *et al.* (63) reported that the peak IL-1β level in HBV-infected patients was 9–36 ng/ml under Toll-like receptor stimulation, at which concentration IL-1β showed significant anti-HBV effects in this study. In general, downstream genes of NF-κB include a number of antiviral factors such as *viperin*, *iNOS*, and *RANTES* (69). Although some of these genes may function cooperatively for IL-1- and TNFα-induced anti-HBV machinery, our data suggest that AID, at least in part, plays a role in the elimination of HBV that was potentiated by proinflammatory cytokines IL-1 and TNFα.

AID belongs to APOBEC family proteins that share enzyme activity to convert cytidine to uracil in mainly DNA, and occasionally RNA (51, 70, 71). Although AID was initially identified in B cells, chronic inflammation can trigger its expression in hepatocytes (60). The induction of AID was reportedly mediated by NF-κB (60), consistent with the results in this study. Although AID in B cells is essential for class switch recombination and somatic hypermutation of immunoglobulin genes (70, 72), the physiological role of AID in hepatocytes is unknown. Although expression of AID in hepatocytes is still lower than in B cells, AID is reportedly expressed in the liver both in cell culture and *in vivo* settings (34, 60). Our results raise the idea that AID plays a role in innate antiviral immunity. AID also has a role in virus-induced pathogenesis as it was reported to counteract oncogenesis induced by Abelson-murine leukemia virus (73). In addition, AID was reported to restrict L1 retrotransposition, which can predict the role of AID in innate immunity (74). This study is significant in that it revealed a biological function of AID in viral infection itself, linking it to the restriction of a pathogenic human virus. It will be interesting to analyze the role of AID in the infection process of other viruses in the future.

Although the mechanism for AID suppression of the HBV life cycle is the subject of future study, AID possibly targets the early phase of HBV infection, including entry as well as the replication stage, including assembly and reverse transcription (Fig. 3). It has been recently reported that chicken AID reduced cccDNA of duck HBV possibly through targeting cccDNA as well as nucleocapsid-associated HBV DNA (75). This study is likely to support the idea that AID may target cccDNA formed after HBV entry into hepatocytes, and also associates with nucleocapsid-associated HBV DNA during HBV replication, although it is not clear whether the innate immune machinery against HBV/duck HBV is conserved in human and chicken cells. A3G blocked HBV replication through the inhibition of reverse transcriptase (29), packaging of pregenomic RNA (33), and the destabilization of packaged pregenomic RNA (31) independently of its deaminase activity, and it also induced hypermutation of HBV DNA (27, 30, 32, 34). It was recently reported that AID was packaged into the HBV nucleocapsid (51). Moreover, AID induced C-to-T and G-to-A hypermutations in HBV DNA/RNA, although the anti-HBV activity has not been demonstrated so far (51). The hypermutation activity of AID was likely to be dispensable for its anti-HBV replication function (Figs. 5 and 6), as reported for APOBEC3G by several groups (29, 30, 33). Further analysis is required to elucidate the precise mechanisms for AID-mediated suppression of the HBV life cycle.

In conclusion, we have identified that host cell susceptibility to HBV infection is modulated by IL-1 and TNFα, and AID is involved in this machinery. This sheds new light on the link between proinflammatory cytokines and the development of the innate antiviral defense.

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