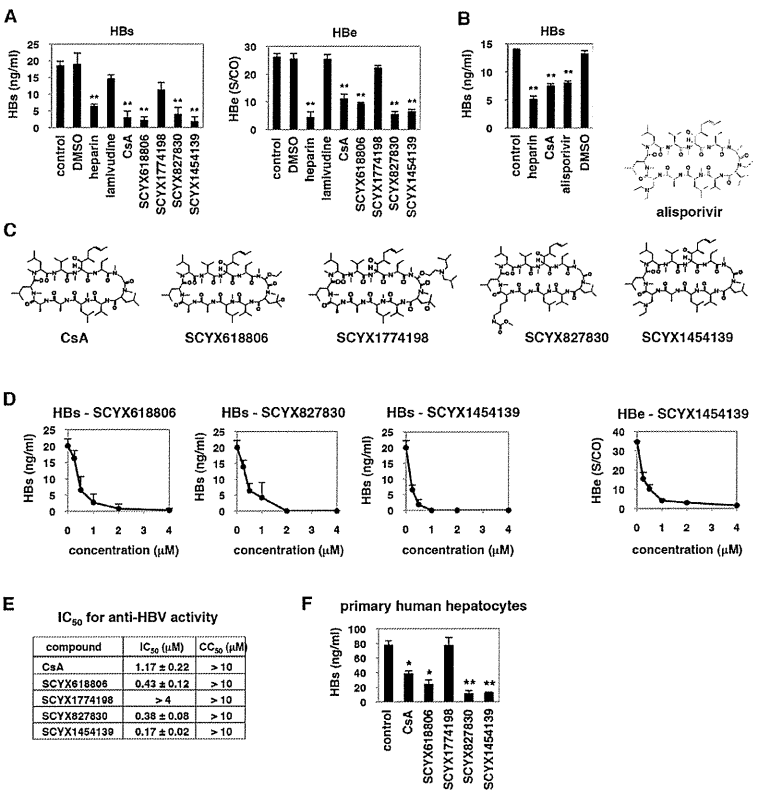
Fig. 6.



E

IC_{50} for anti-HBV activity

compound	IC_{50} (μM)	CC_{50} (μM)
CsA	1.17 ± 0.22	> 10
SCYX618806	0.43 ± 0.12	> 10
SCYX1774198	> 4	> 10
SCYX827830	0.38 ± 0.08	> 10
SCYX1454139	0.17 ± 0.02	> 10

197x262mm (300 x 300 DPI)

Supporting information

Supporting Experimental Procedures

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). HepAD38 cells (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center) (33) were cultured with DMEM/F-12+GlutaMax (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, and 5 μg/ml insulin in the presence of 400 μg/ml G418 (Nacalai) and 0.3 μg/ml tetracycline. Tetracycline was removed to induce HBV production. Huh-7.5.1 cells (kindly provided by Dr. Francis Chisari at Scripps Research Institute) were cultured as described previously (51). The isolation of and the culture medium for primary human hepatocytes (PHH) (Phoenixbio) were described previously (52).

HBV preparation and infection

Most experiments in this study utilized HBV derived from HepAD38 cells (33). To induce virus production, HepAD38 cells were cultured in the absence of tetracycline. Culture medium, collected every three days over the period 7-31 days post-induction, was passed through a 0.45 μm filter. Virus was precipitated using 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with medium at approximately 200-fold concentration. HBV DNA was quantified by real time PCR. For Fig. 3A, we recovered the media of HepG2 cells transfected with an expression plasmid for HBV genotype A, B, or D (HBV/Aeus, HBV/C-AT, or HBV/D-IND60) (53) at 3 and 6 days posttransfection. Virus was concentrated and quantified as described for HepAD38 cells.

HepaRG cells and PHH were infected with HBV at 2000-20000 (normally 6000) genome equivalents (GEq)/cell in the presence of 4% PEG8000 at 37°C for 16 h as previously described (23). Under these conditions, efficient infection of HepaRG cells using virus derived from HepAD38 or HepG2.2.15 cells requires an inoculum of $>10^4$ HBV GEq/cell (i.e. 1.25 - 40 x 10^4 GEq/cell) (54).

Indirect immunofluorescence analysis

Indirect immunofluorescence analysis was performed essentially as described previously (16). Briefly, after fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100, an anti-HBc antibody (DAKO, #B0586) was used as the primary antibody.

ELISA

To quantify HBs protein by ELISA, microwell antigen capture plates (Maxisorp nunc-immuno plate, Nunc #439454) were prepared by overnight incubation at 4°C with a sheep anti-HBs antibody at 1:5000 dilution, followed by coating with 0.2% BSA/0.02% NaN_3/1 x PBS at 4°C until use. For HBs detection, samples were incubated in individual wells of the capture plates for 2 h. After washing, horseradish peroxidase-labeled rabbit anti-HBs antibody was added for an additional 2 h incubation. The substrate solution (from the HCV core ELISA kit: Ortho) was reacted for 15-60 min before the OD_{450} values were measured.

Real time PCR and RT-PCR

HBV DNA was extracted from cells using a QIAamp mini kit (QIAGEN) according to the manufacturer's protocol. HBV DNA was quantified by real time PCR analysis using the primer set 5'-ACTCACCAACCTCCTGTCCT-3' and 5'-GACAAACGGGCAACATACCT-3' and probe 5'FAM-TATCGCTGGATGTGTCTGCGGCGT-TAMRA3'. Detection of cccDNA was achieved using 5'-CGTCTGTGCCTTCTCATCTGC-3' and 5'-GCACAGCTTGGAGGCTTGAA-3' as primers and 5'-CTGTAGGCATAAATTGGT (MGB)-3' as a probe. mRNAs for NTCP, CyPA, CyPB, and GAPDH were detected using a one step RNA PCR kit (Takara) following the manufacturer's protocol. Primers are 5'-CCGGCTGAAGAACATTGAGGCACTGG-3' and 5'-AGGGAGGAGGTGGCAATCAAGAGTGG-3' for NTCP, 5'-CTCCTTTGAGCTGTTTGCAGACAAGGTCCC -3' and 5'-CATTTGCCATGGACAAGATGCCAGGACCCG -3' for CyPA, 5'-AGACTGTTCCAAAAACAGTGGATAA-3' and 5'-AGTGCTTCAGTTTGAAGTTCTCATC-3' for CyPB, and 5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3' for GAPDH,

respectively.

Southern blot analysis

Southern blotting was performed as described previously (23).

HBV attachment and internalization assay

To allow HBV attachment to the cells without subsequent internalization, HBV was added to HepaRG cells at 4°C for 3 h. Free HBV was removed by washing the cells with PBS. Attached HBV was quantified by real time PCR analysis of DNA extracted from the washed cells. To allow virus internalization, cells treated with HBV as above were transferred to 37°C and incubated for an additional 16 h. The cells were then trypsinized to digest the cell surface HBV and extensively washed with PBS. Internalized HBV from the washed cells was quantified by real time PCR analysis of HBV DNA.

Pretreatment of HBV particles with compounds

HBV inoculum, prepared as described above, was preincubated with the indicated compounds (Fig. 2F) for 30 min at 37°C. Each compound-treated HBV inoculum was concentrated from 450 μl to 15 ul via ultrafiltration, followed by dilution to 450 μl with PBS and re-concentration to recover 15 ul of HBV-containing medium. This procedure is expected to result in a 900-fold reduction in compound concentration. The infectivity of the resultant HBV-containing concentrate (10 μl) was evaluated by inoculating HepaRG cells as described above.

MTT assay

The MTT cell viability assay was performed as described previously (23).

Reporter assay

Gene expression reporter assays were performed essentially as described (18). Briefly, the reporter plasmids pNF-AT-luc (promoter binding elements for NF-AT upstream of the firefly luciferase gene) and pRL-TK (herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene) were transfected into Jurkat cells using lipofectamine 2000. At 4

h posttransfection, cells were stimulated with the indicated compounds. After an additional 28 h incubation, the cells were stimulated with 1 μM PMA and 1 μM ionomycin for 16 h and the luciferase activities were measured.

HCV pseudoparticle assay

The HCV pseudoparticle (HCVpp) assay was essentially performed as previously described (55, 56). Plasmids used to produce HCVpp, were kindly provided by Dr. Francois-Loic Cosset at University of Lyon.

Cyclophilin binding assay

Binding to human cyclophilins A, B, and D by CsA, CsA analogs, and other compounds was measured in a fluorescence polarization ligand competition assay. The binding probe for this assay (FP-CsA ligand) was the carboxyfluorescein (CF)-labeled [Ser]8-CsA derivative [O-(CF-NH(CH2)2NHC(O) CH2-)D-Ser]8CsA, synthesized at Scynexis as described in (57). Compound stock solutions were serially diluted in DMSO, and added to assay buffer (20 mM HEPES pH 8.0, 0.01% Triton-X100) containing 20 nM FP-CsA ligand. Competitive ligand mixtures were then added to assay buffer containing a cyclophilin protein (1500 nM for CypA, 500 nM for CypB and CypD) in black 96-well microtiter plates. Final compound concentrations in the assay ranged from 10 to 0.15625 μM. Plates were incubated at room temperature on an orbital shaker for 30 min prior to reading fluorescence polarization. Fluorescence polarization measurements were performed on a PheraStar fluorescence reader (BMG Labtech, Cary, NC) equipped with a filter set for fluorescein polarization measurement (FP480/FP535). The anisotropy values (r) were calculated from the fluorescence intensity parallel (F_1) and perpendicular (F_2) to the excitation plane: $r = (F_1-F_2)/(F_1+2F_2)$. The anisotropy values obtained were plotted and IC_{50} values calculated using Excel Fit Software.

Isolation of HepG2 cells stably overexpressing NTCP (HepG2-NTCP cells)

HepG2 cells were transfected with an expression plasmid for NTCP (25) using TransIT LT1 transfection reagent (Mirus). The cells were plated into a 10 cm dish at 4 h posttransfection, and cultured in the presence of G418 1 mg/ml to select plasmid-bearing cells beginning on day 2 posttransfection. After 20 days of growth, individual cell colonies were isolated and

expanded. Each cell clone and parental HepG2 cells as a negative control were infected with HBV as described above for HepaRG cells, and cell clones highly susceptible to HBV infection were selected for use in experiments.

NTCP transporter assay

NTCP transporter activity (Fig. 5A) was assayed essentially as described (25, 58) using 293 cells permanently overexpressing human NTCP (Sekisui Medical). In this assay, we observed no cytotoxic effects at any of the compound concentrations tested. Cells were pre-incubated at 37°C for 15 min in Hanks' Balanced Salt Solution buffer (HBSS buffer, 142 mM NaCl, 23.8 mM $NaHCO_3$, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$, adjusted to pH 7.4) with or without CsA, FK506, rapamycin, or PSC833. After pre-incubation, the HBSS was removed completely, and the transport assay was initiated by adding [^{3}H]taurocholic acid (TCA) solution with or without compounds at 37°C for 5 min. The transport assay was terminated by adding ice-cold PBS containing 0.2% BSA (Sigma-Aldrich). Cells were washed twice with 1 mL of ice-cold PBS, then pelleted cells were solubilized by pipetting in 0.5mL of 0.1 N NaOH. Cell lysate (300 µL) was combined with 10 mL of scintillator (Hionic-Fluor) in a glass scintillation vial, and the radioactivity was measured using a liquid scintillation counter (TRI-CARB 2500TR, PerkinElmer). Protein concentration was determined with 20 µL of the remaining cell lysate using a BCA-protein assay kit (Thermo Fisher Scientific, Waltham, MA). Cleared volume (µl/mg protein) was calculated from the uptake amount (disintegrations/well), protein amount (mg protein/well) and initial concentration (disintegrations/µl). The percent of control (%) was calculated from the ratio of cleared volume in the presence of the compound to that in the absence of the compound. The IC_{50} value was calculated from the relationship between the concentration of the compounds and the percent of the control using eq.1: Percent of control (%) = IC_{50} /(IC_{50}+I) x 100, where I is concentration of the compound (µM).

For the HepG2-based transporter assay in Fig. 5B, the uptake experiments were performed as described (59, 60) by using HepG2 and HepG2-NTCP with modification. The uptake of [^{3}H]TCA was measured at 37°C for 5 min in HBSS containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 25 mM HEPES (pH 7.4) and 50 nM [^{3}H]TCA (370 GBq/mmol; American Radiolabeled Chemicals, Inc; St. Louis, MO, USA) in the presence or absence of 10 µM inhibitors as indicated in the figure. NTCP

specific TCA uptake values were obtained by subtracting background (HepG2 uptake value) from HepG2-NTCP uptake value.

Binding assay for CsA to NTCP-containing membranes

Crude membrane fractions of HepG2 and HepG2-NTCP cells were prepared as described (60). For the binding assay, 42.5 μg of crude membrane fraction was mixed with 100 μl of 30 μM [^{3}H]cyclosporin A (CsA) (1.23 GBq/mmol; Perkin Elmer) in buffer containing 20 mM KPi pH 7.0 and 150 mM NaCl. The reaction was incubated at 25°C for 5 min. Membrane–bound [^{3}H]CsA was captured using rapid filtration and quantified using a liquid scintillation counter (61).

Scintillation proximity assay for binding of [^{3}H]TCA to crude recombinant NTCP

A scintillation proximity assay (SPA) (62) was utilized to test substrate binding by in vitro synthesized NTCP. Three mg/ml of crude recombinant NTCP-mycHis or GST-mycHis was mixed with 100 μM [^{3}H]TCA (0.6 GBq/mmol), followed by 250 μg of YSi Copper HIS TAG SPA bead (Perkin Elmer) in binding buffer containing 50 mM KPi pH 7.0, 100 mM NaCl, 0.2% w/v bovine serum albumin (Cohn fraction V) and 0.05% n-dodecyl β-D-maltopyroside. The reaction was incubated at 4°C for 1 h. The radioactivity of bound [^{3}H]TCA was measured using a 1450 MicroBeta TriLux scintillation counter (Perkin Elmer), and corrected for the assay background that was measured following addition of 200 mM imidazole to each well. The total radioactivity (ccpm) in each well was obtained in the presence of OptiPhase Supermix scintillation cocktail, and used for calculation of ccpm-to-mol of substrate/well.

AlphaScreen assay for NTCP-HBs binding

Recombinant NTCP and HBs proteins were synthesized using a wheat cell-free protein system as described previously (63-65). The template for transcription was produced by split-primer PCR using the following primers: a target protein specific primer (5'-5'-CCACCCACCACCACCAatgnnnnnnnnnnnnnnnn-3'; lowercase indicates the 5'-coding region of the target gene) and the SV40_5-23 (5'- AAAACGATTCCGAAGCCC) or the pcDNA_Neo472 (5'- CTGATGCTCTTCGTCCAG). The second round of PCR was performed with a 1/100 volume of the first PCR product using 100 nM of SPu primer (5'-GCGTAGCATTTAGGTGACACT-3'), 100 nM of the SV40_2-21 (5'-AAACGATTCCGAAGCCCAA) or the pcDNA_Neo469 (5'- ATGCTCTTCGTCCAGATC)

and 1 nM of deSP6E01 primer (5'-GGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCACTAACCACCTCCACCCACCACCACCAATG-3') or deSP6E02-bls-S1 primer (5'-GGTGACACTATAGAACTCACCTATCTCTCTACACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAACTCCACCCACCACCACCAATG). In vitro transcription and wheat cell-free protein synthesis were performed in accordance with the manufacturer's instructions (CellFree Sciences, Yokohama, Japan).

The binding assay was carried out in a total volume of 15 µl consisting of 100 mM Tris-HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 0.1 µl crude recombinant NTCP-His and 0.1 µl crude recombinant Biotin-HBs at 26°C for 1 hr in a 384-well Optiplate (PerkinElmer, Boston, MA, USA). For the binding inhibitor assay, 0.1 µl recombinant NTCP-His was incubated with serially diluted inhibitors at 26°C for 10 min followed by addition of biotinylated HBs protein. Mixtures were then incubated at 26°C for 1 hr in a 384-well Alphaplate. In accordance with the AlphaScreen IgG (ProteinA) detection kit instruction manual (PerkinElmer), 10 µl of detection mixture containing 100 mM Tris-HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 5 µg/ml Anti-6xHis antibody (GeneTex Inc., Irvine, CA, USA), 0.1 µl streptavidin-coated donor beads and 0.1 µl anti-IgG (ProteinA) acceptor beads were added to each well of the 384 Alphaplate followed by incubation at 26°C for 1 hr. Luminescence was analyzed using the AlphaScreen detection program of an Envision spectrophotometer (PerkinElmer). The pre-S1 lipopeptide HBVpreS/2-48^{myr} was kindly provided by Dr. Stephan Urban at University Hospital Heidelberg. The mutant peptide for HBVpreS/2-48^{myr} (myr-SLNTGVQNAEPLPNPVWHDKNDPFFGPNFDWPDLQHDPNNSNAGFA), which was shown to be deficient for inhibiting HBV infection, was synthesized (CS Bio, Co.).

Statistical analyses

Statistical significance was determined using the Student's t-test.

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Supporting Table S1

Activity of CsA analogs for immunosuppression and CyP binding

		Binding IC_{50} (μM)		
Compound	IL-2 IC_{50} (nM) *	CyPA	CyPB	CyPD
CsA	0.065	0.36	0.73	1.25
SCYX618806	2366	0.76	2.6	1.7
SCYX1774198	5085	>10	>10	>10
SCYX827830	4	2.0	6.1	5.0
SCYX1454139	166	4.7	>10	>10

* *In vitro* immunosuppression assay measured inhibition of IL-2 secretion from activated Jurkat cells as described in (66).

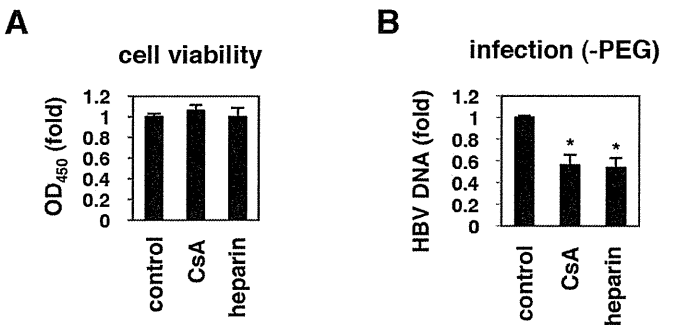
Supporting Figure Legends

Fig. S1. Cyclosporin A (CsA) blocked HBV infection. (A) Cells were treated with 4 μM CsA and 25 U/ml heparin according to the protocol shown in Fig. 1A. Cell viability was quantified using the MTT assay. (B) Primary human hepatocytes were pretreated with or without 4 μM CsA and 25 U/ml heparin and then inoculated with HBV in the absence of 4% PEG8000 using the protocol shown in Fig. 1A.

Fig. S2. Binding of [^{3}H]CsA to native membrane-bound NTCP protein. The binding of [^{3}H]CsA was assayed as described in the Supplemental Experimental Procedures using the membrane fraction of HepG2 and HepG2-NTCP cells. Membranes containing NTCP protein bound increased levels of CsA.

Fig. S3. Characterization of recombinant NTCP and LHBs proteins. (A) Binding capacity of recombinant NTCP protein for [^{3}H]TCA. Scintillation proximity assay was performed using in vitro synthesized NTCP protein as described in Experimental Procedures. Recombinant NTCP protein bound to TCA. (B) Neutralizing activity of recombinant LHBs in HBV infection. HepaRG cells were pretreated with or without BSA or recombinant 300 ng LHBs for 1 h, and then infected with HBV for 16 h. HBV infection was evaluated by quantifying HBs antigen in the medium at 12 days postinfection. Pretreatment with recombinant LHBs significantly reduced HBV infection. (C) An AlphaScreen protein-interaction assay was performed by incubating LHBs and GST-His or NTCP-His in the presence or absence of wild-type or mutant HBVpreS/2-48myr peptide. The assay results are shown normalized to the AlphaScreen signal from the interaction of LHBs and NTCP-His in the absence of peptide.

Fig. S1.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cell viability
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0.4
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control
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infection (-PEG)
HBV DNA (fold)
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0.8
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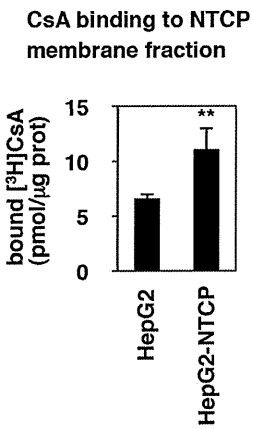
*
control
CsA
heparin

Fig. S2.


CsA binding to NTCP in membrane fraction
bound [3H]CsA (pmol/μg prot)
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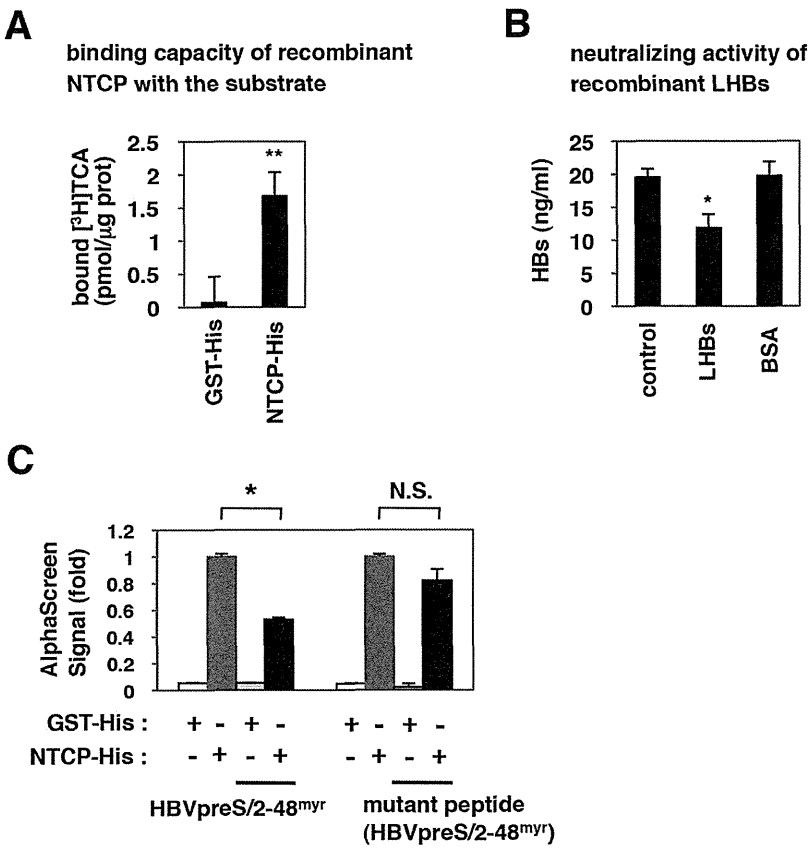
10
5
0
**
HepG2
HepG2-NTCP

Fig. S3.


A
binding capacity of recombinant NTCP with the substrate
bound [3H]TCA (pmol/μg prot)
2.5
2
1.5
1
0.5
0
**
GST-His
NTCP-His
B
neutralizing activity of recombinant LHBs
HBs (ng/ml)
25
20
15
10
5
0
*
control
LHBs
BSA
C
*
N.S.
AlphaScreen Signal (fold)
1.2
1
0.8
0.6
0.4
0.2
0
GST-His : + - + - + - + -
NTCP-His : - + - + - + - +
HBVpreS/2-48myr
mutant peptide (HBVpreS/2-48myr)

⌘ *Author's Choice*

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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′-CAGTTTTCCACCA-CAACAAA-3′ for *XIAP*; 5′-TAGCCAACATGTCCTCACA-GAC-3′ and 5′-TCTTCTACCACTGGTTTCATGC-3′ for *ISG56*; 5′-GCCTTTTCATCCAAATGGAATTC-3′ and 5′-GAAATCTGTTCTGGGCTCATG-3′ for *PKR*; and 5′-CCATG-GAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATG-ACC-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′-TCC-CTCGCCTCGCAGACG-3′ and 5′-GTTTCCCACCTTAT-GAGTC-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-

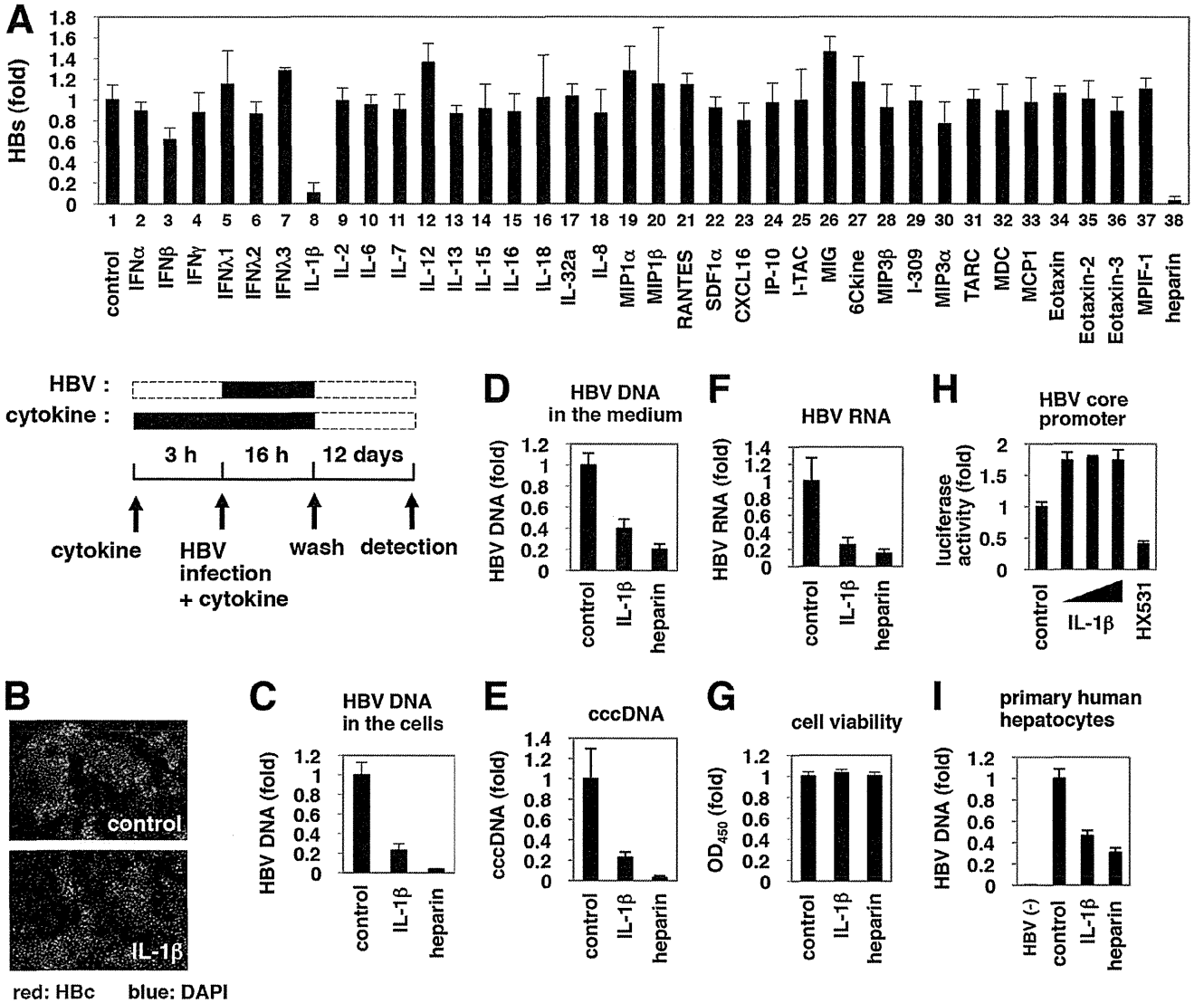

FIGURE 1. **Suppression of HBV infection by IL-1β.** *A, upper graph*, HepaRG cells were pretreated with cytokines at 100 ng/ml (except for IFNα and IFNβ at 100 IU/ml) or heparin at 25 units/ml as a positive control or were left untreated (*control*) for 3 h and then infected with HBV in the presence 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.

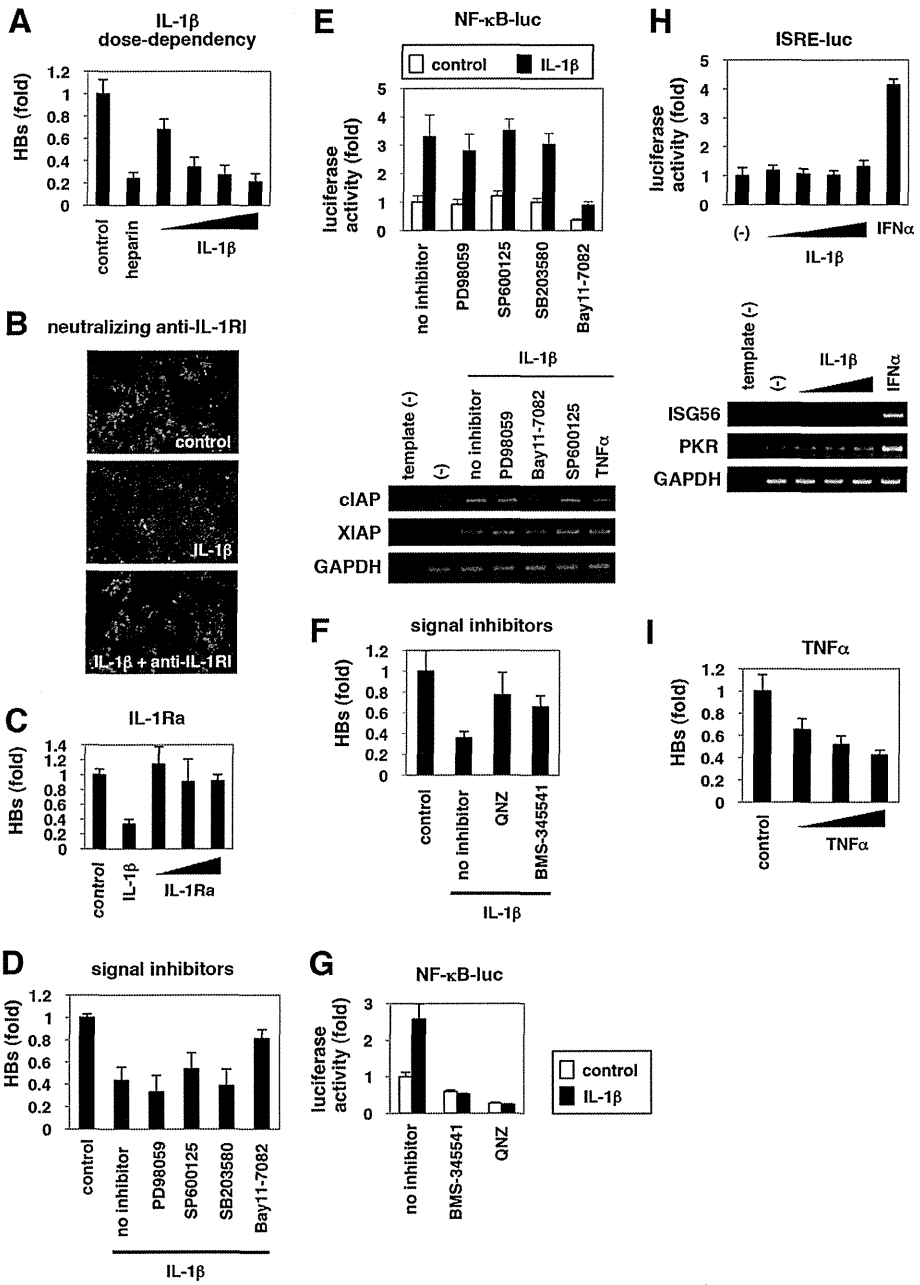

FIGURE 2. **NF-κB activation triggered by IL-1 and TNFα was critical for anti-HBV activity.** *A–D, F,* and *I,* HepaRG cells were left untreated (*control*) or treated with varying concentrations of IL-1β (1, 10, 30, and 100 ng/ml) or 25 units/ml heparin (*A*), with 30 ng/ml IL-1β together with or without 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-

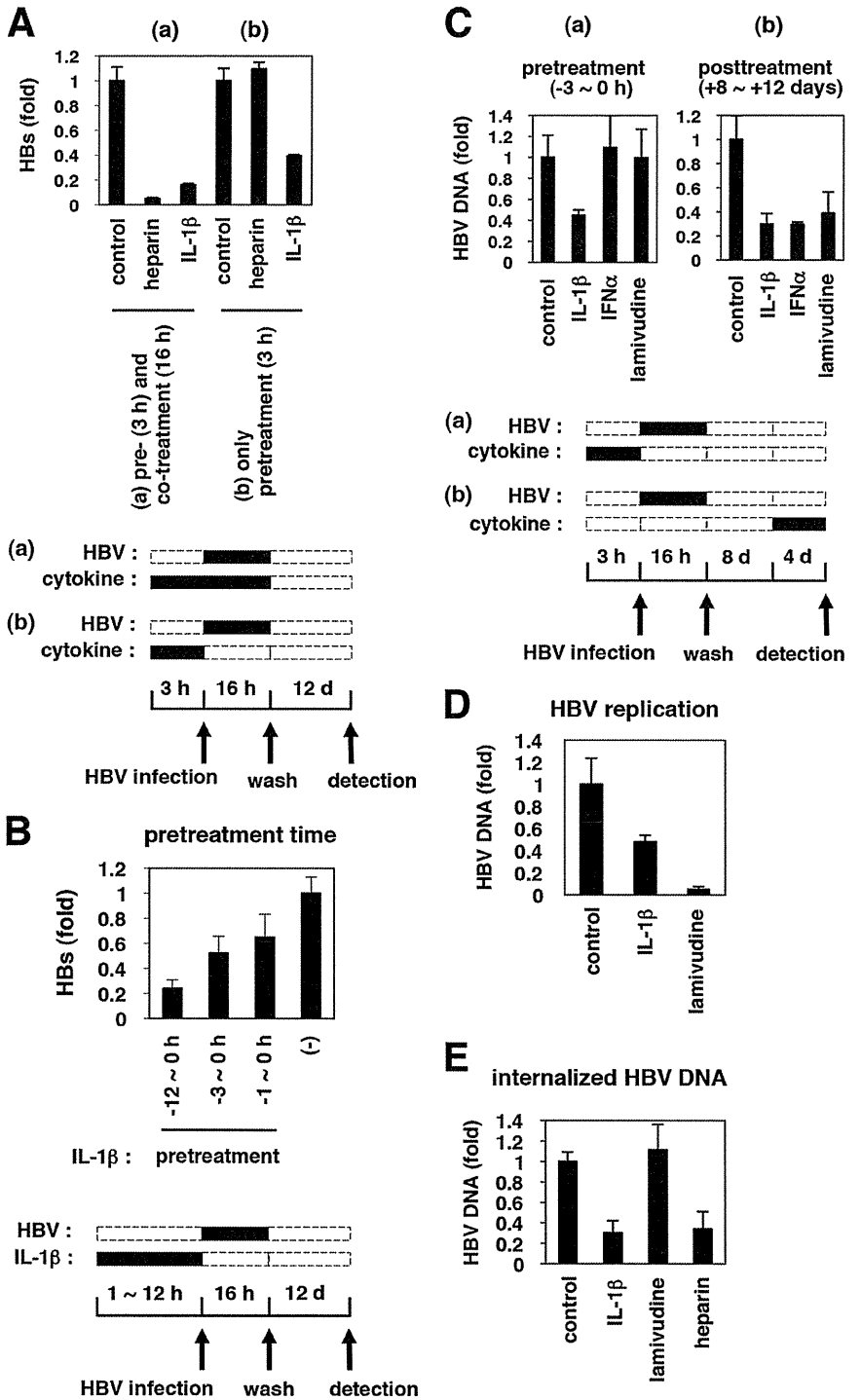

FIGURE 3. **Defining the steps of the HBV life cycle targeted by IL-1β.** *A*, HepaRG cells were pretreated with IL-1β or heparin for 3 h and then infected with HBV in the presence (*A, panel a*) or absence (*A, panel b*) of IL-1β or heparin for 16 h. HBV infection was monitored with HBs protein secretion 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.