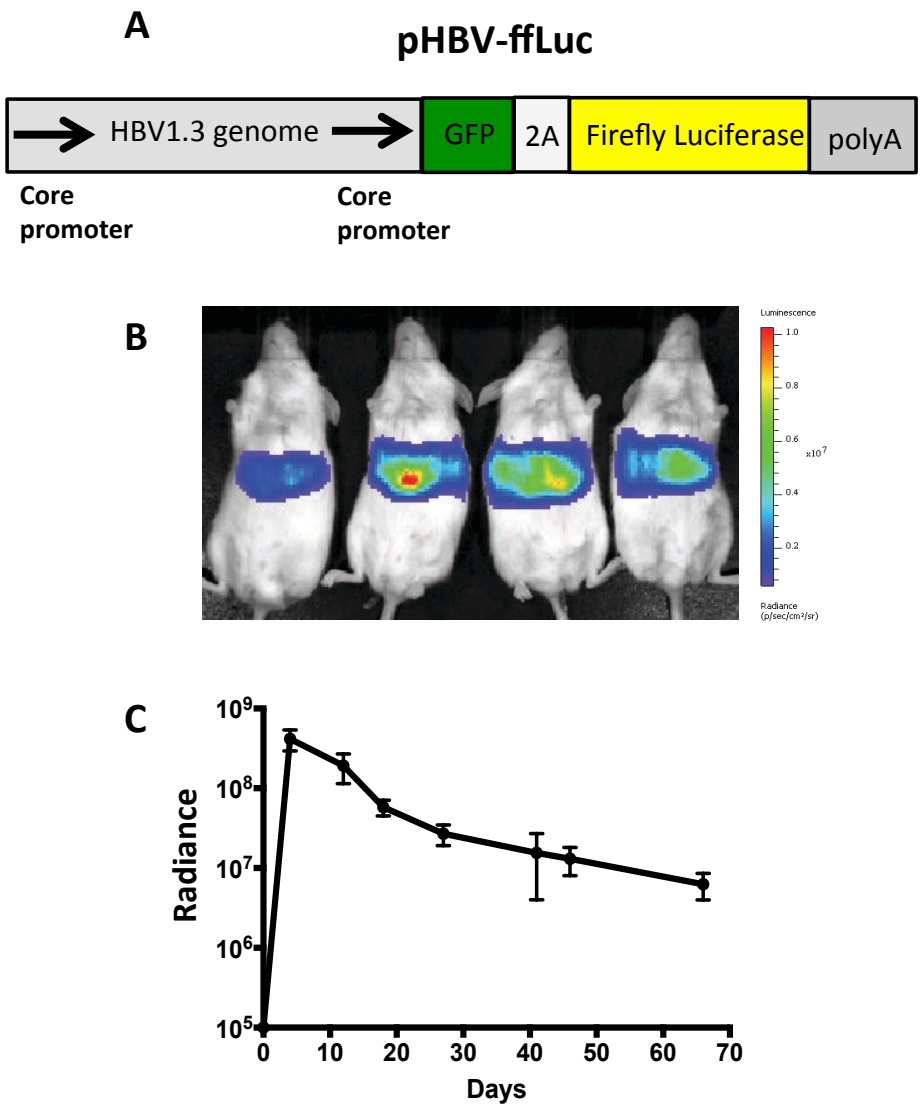
OMTM, Volume 7

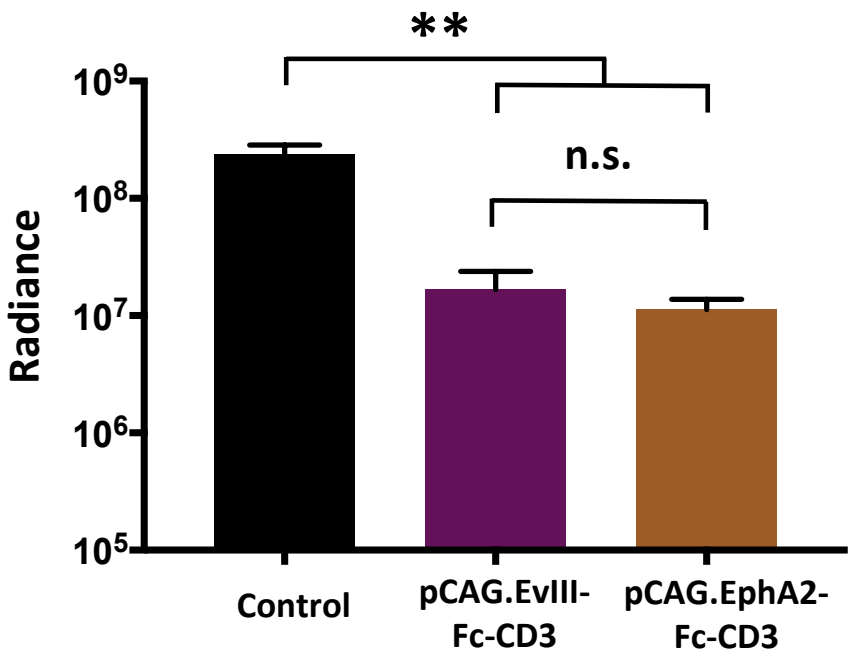
Supplemental Information

In Situ Liver Expression of HBsAg/CD3-Bispecific Antibodies for HBV Immunotherapy

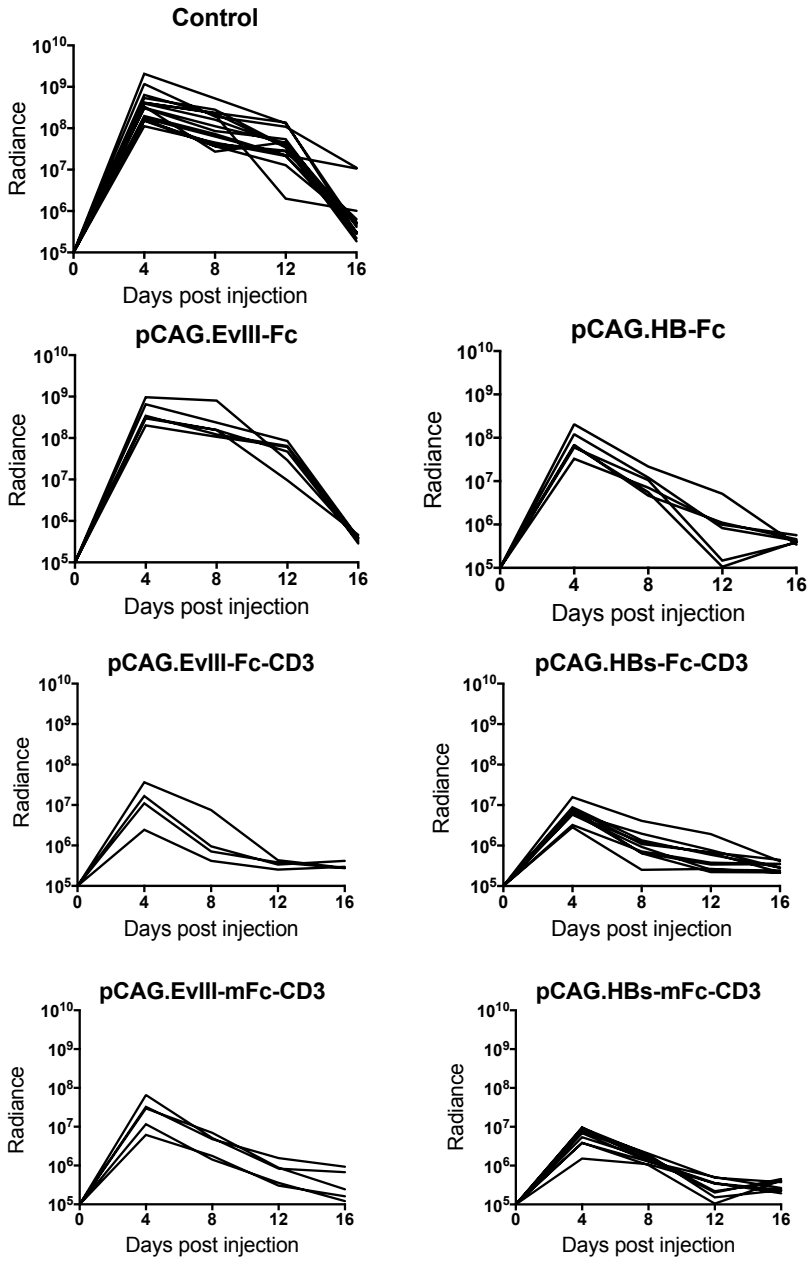
Robert L. Kruse, Thomas Shum, Xavier Legras, Mercedes Barzi, Frank P. Pankowicz, Stephen Gottschalk, and Karl-Dimiter Bissig



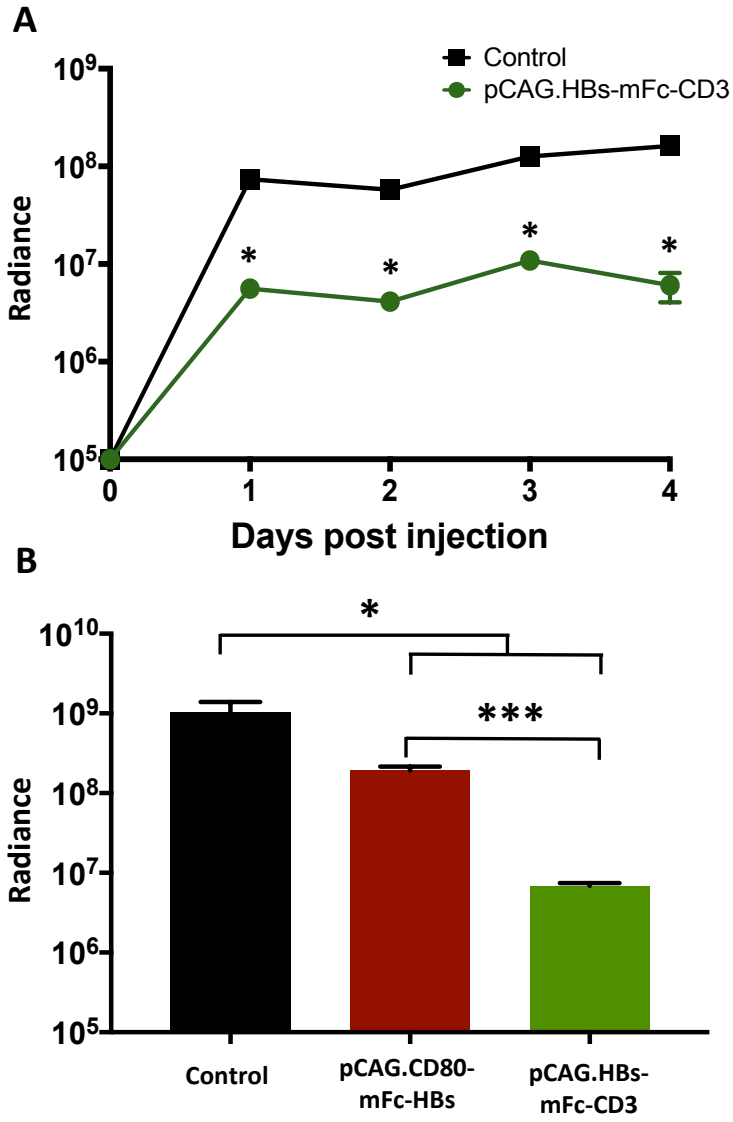
Supplementary Figure 1: Mouse model measures the clearance of HBV using non-invasive bioluminescence imaging. (**A**) Scheme of plasmid encoding the overlength (1.3-mer) HBV genome and a core protein fused GFP-2A-ffLuc cassette, both under the transcriptional control of identical HBV core promoters. (**B,C**) NSG mice were injected with 5 µg of pHBV-ffLuc by hydrodynamic tail vein injection, and ffLuc expression was monitored by bioluminescence imaging. (**B**) Bioluminescence images of mice on day 4 post-injection. (**C**) Quantitative bioluminescence imaging data (radiance = photons/sec/cm^2/sr) is shown over time (mean ± SEM, n=4).



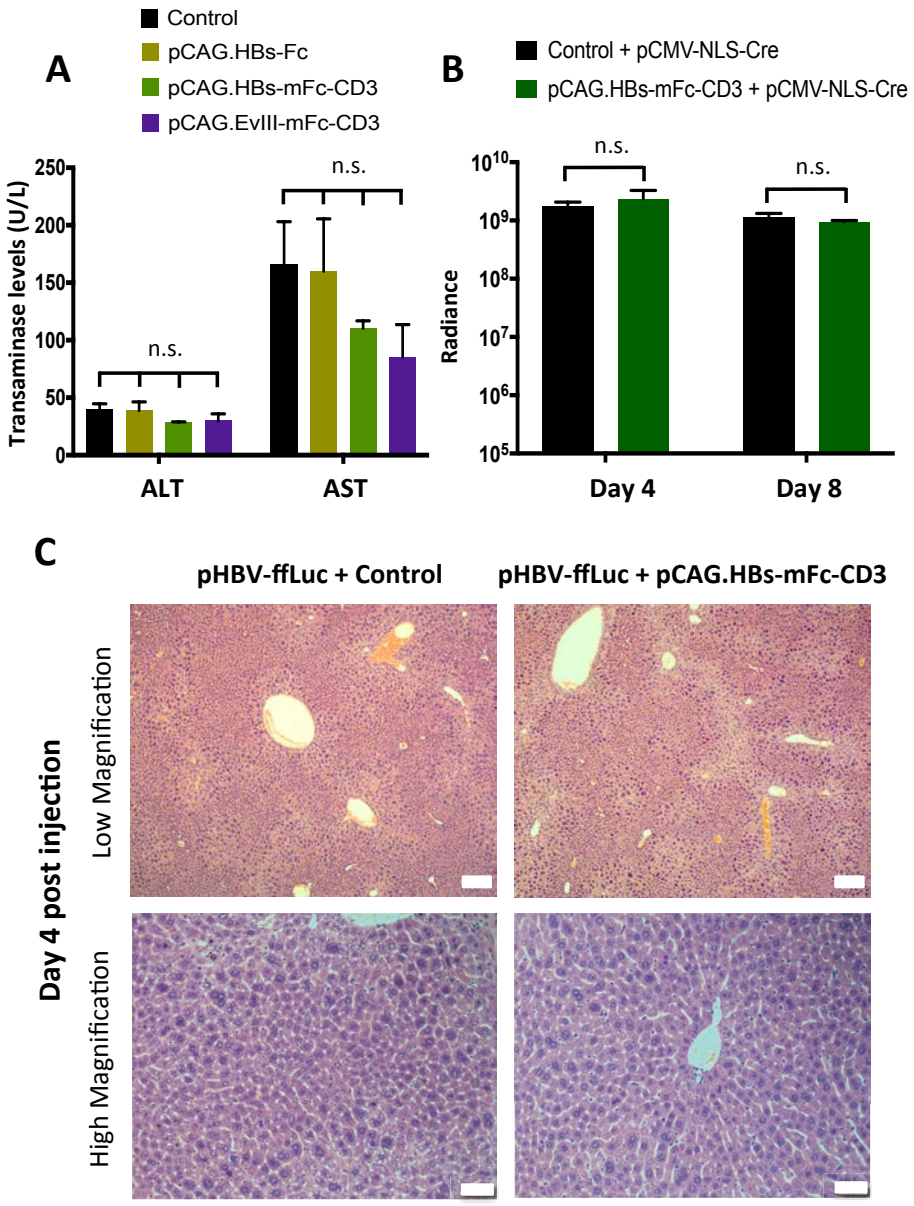
Supplementary Figure 2: *In vivo* expression of EphA2-Fc-CD3 has anti-HBV activity. To confirm the antigen-independent activity of EvIII-Fc-CD3, we replaced the EvIII-specific scFv in pCAG.EvIII-Fc-CD3 with an scFv derived from the EphA2-specific mAb 4H5 to form pCAG.EphA2-Fc-CD3. Immunocompetent mice were co-injected by hydrodynamic tail vein injection with 5 μg pHBV-ffLuc and 15 μg pCAG.EvIII-Fc-CD3, pCAG.Epha2-Fc-CD3, or control plasmid. Quantitative bioluminescence imaging data (radiance = photons/sec/cm^2/sr) for all mice are depicted at day 4 post-injection (mean ± SEM, n=4, n.s. = not significant, ** $p < 0.005$).



Supplementary Figure 3: The antiviral effects of *in vivo* expression of bispecific antibodies are consistent across multiple experiments. Collated bioluminescence data (radiance = photons/sec/cm^2/sr) of replicates across the different experiments for each construct are depicted demonstrating consistent effects. Control plasmid, n=16; pCAG.EvIII-Fc, n=6; pCAG.EvIII-Fc-CD3, n=4; pCAG.EvIII-mFc-CD3, n=5; pCAG.HBs-Fc, n=6; pCAG.HBs-Fc-CD3, n=10; pCAG.HBs-mFc-CD3, n=9.

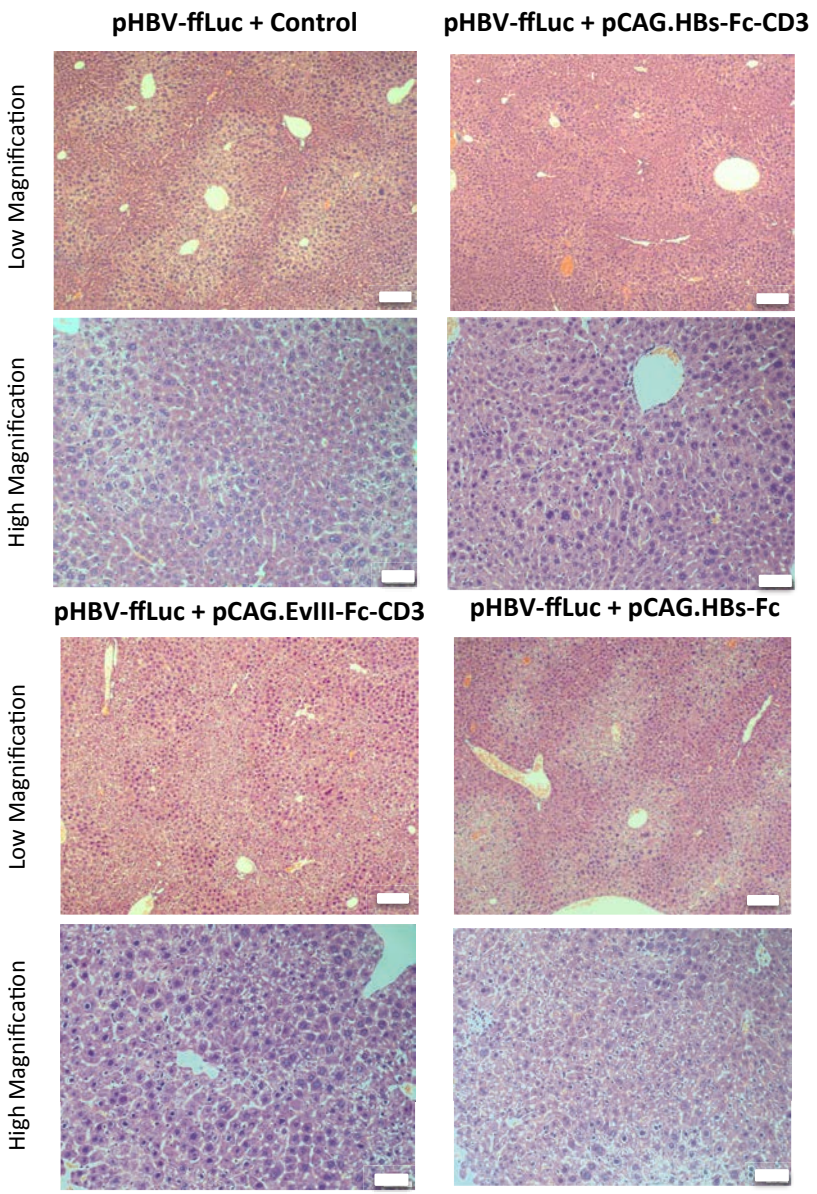


Supplementary Figure 4: Bispecific antibodies act early after injection and through CD3 engagement to mediate antiviral activity. (**A**) Bioluminescence was followed after co-injection of 15 μg pCAG.HBs-mFc-CD3 or control plasmid and 5 μg pHBV-ffLuc over the first 4 days post-injection in mice (mean ± SEM, n=3). (**B**) In a similar experiment, 15 μg pCAG.HBs-mFc-CD3, 15 μg pCAG.CD80-mFc-HBs, or control plasmid and 5 μg pHBV-ffLuc were injected into mice and measured at day 4 post-injection (mean ± SEM, n=4). Quantitative bioluminescence imaging data (radiance = photons/sec/cm^2/sr) for all mice are shown and significant differences denoted (* $p < 0.05$, *** $p < 0.0001$).

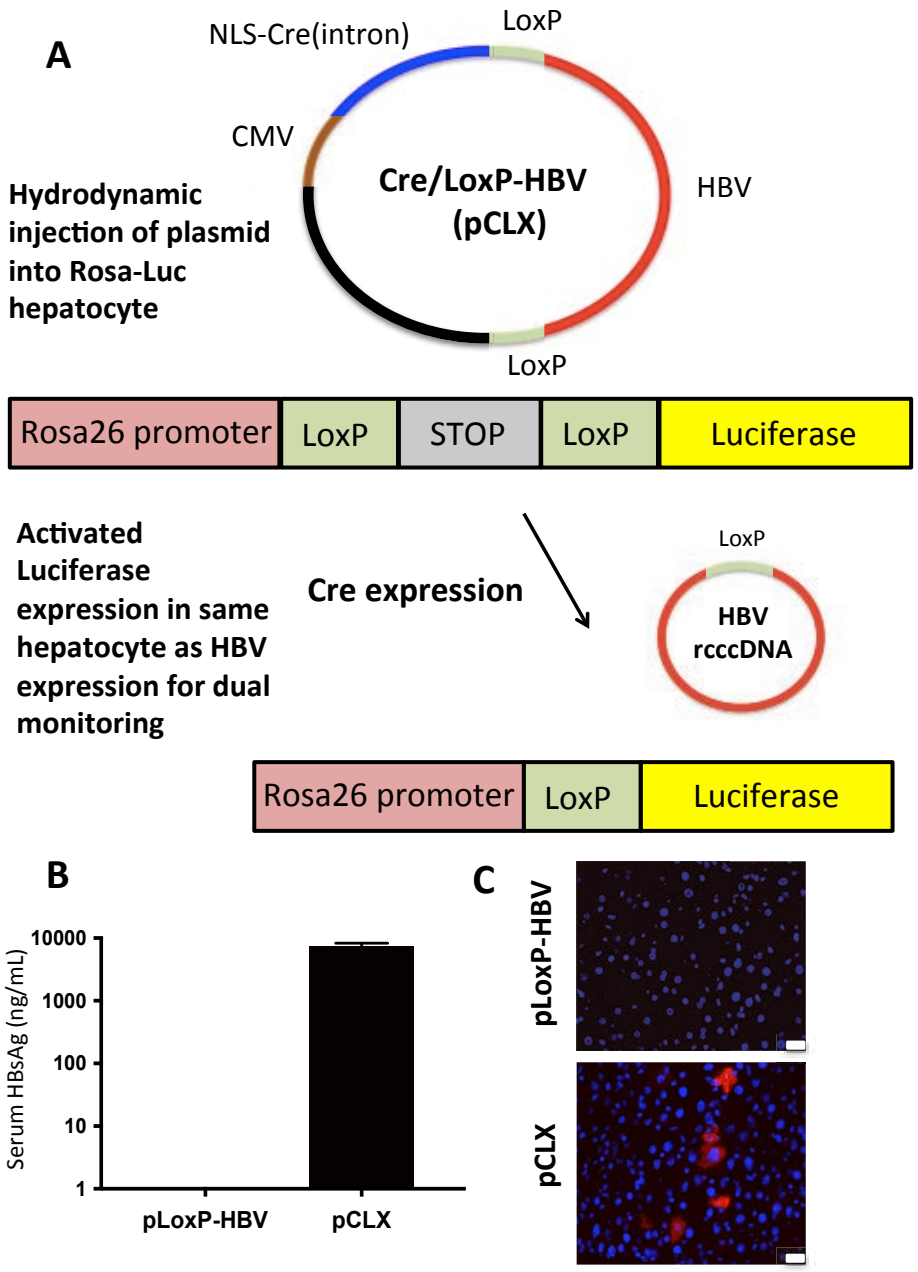


Supplementary Figure 5: *In vivo* expression of HBs-mFc-CD3 in hepatocytes is non-toxic. (**A**) Transaminase levels (ALT and AST) were measured at day 4 post-injection of 5 µg pHBV-ffLuc and 15 µg pCAG.HBs-Fc, pCAG.HBs-mFc-CD3, control plasmid (n=3), or pCAG.EvIII-mFc-CD3 (n=4), (mean ± SEM). There was no significant (n.s.) difference between any of the groups in either ALT or AST measurements. (**B**) Toxicity of HBs-mFc-CD3 expression was assessed by co-injecting pCMV-NLS-Cre with pCAG.HBs-mFc-CD3 or control plasmid into

Rosa-Luc mice containing a reporter LoxP-STOP-LoxP-ffLuc cassette inducing ffLuc expression in transduced, Cre recombinase-expressing hepatocytes. Quantitative bioluminescence imaging data (radiance = photons/sec/cm^2/sr) for all mice are shown at day 4 and day 8 post-injection (mean ± SEM, n=3). There was no significant (n.s.) difference between pCAG.HBs-mFc-CD3 and control plasmid injected groups. (**C**) Liver tissue of mice was harvested at day 4 post-injection in mice co-injected with 5 μg pHBV-ffLuc and 15 μg pCAG.HBs-mFc-CD3 or control plasmid, fixed in paraformaldehyde, and tissue stained with hematoxylin and eosin. No difference in tissue morphology was observed between mice (Low magnification scale bar = 100 μm, High magnification scale bar = 50 μm).



Supplementary Figure 6: *In vivo* expression of HBs-Fc, HBs-Fc-CD3, or EvIII-Fc-CD3 in hepatocytes is non-toxic. Liver tissue was harvested at day 4 post-hydrodynamic tail vein injection of mice co-injected with 5 μg pHBV-Luc and 15 μg pCAG.HBs-Fc-CD3, pCAG.HBs-Fc, pCAG.EvIII-Fc-CD3, or control plasmid. Tissues were fixed in paraformaldehyde, and sections were stained with hematoxylin and eosin (Low magnification scale bar = 100 μm, High magnification scale bar = 50 μm).



Supplementary Figure 7: Recombinant cccDNA HBV mouse model to monitor antiviral activity and hepatoxicity of antiviral agents. (**A**) Scheme of pCLX, which contains a CMV-NLS-Cre (intron) cassette and a LoxP-HBV flanked genome (derived from pLoxP-HBV), with the LoxP site inserted between amino acid 83 and 84 of the HBV X protein. When pCLX is injected by hydrodynamic tail vein injection into Rosa-Luc mice, which contain a LoxP-STOP-LoxP-ffLuc cassette driven by the *Rosa26* promoter, Cre recombinase expression will i) excise

and form a recombinant (r)cccDNA molecule, and ii) induce ffLuc expression in the same cell. Thus, every cell that contains HBV rcccDNA will also express ffLuc enabling toxicity monitoring of antiviral agents by non-invasive bioluminescence imaging. (**B**) 20 μg pCLX or pLoxP-HBV was injected by hydrodynamic tail vein injection into NSG mice. Serum was collected one week post-injection and HBsAg levels were measured by ELISA (mean ± SEM, n=4). (**C**) 5 μg pCLX or pLoxP-HBV were injected by hydrodynamic tail vein injection into mice and 4 days post-injection liver sections were stained for HBV core protein (red = HBV core, blue = DAPI, scale bar = 20 μm).

A

M D I D P Y K E F G
A T V E L L S F L P
S D F F P S V R D P
R A S S M V S K G E
E L F T G V V P I L
V E L D G D V N G H
K F S V

B

ATT GGT CTG CGC ACC AGC ACC ATG CAA CTT TTT CAC
CTC TGC CTA ATC ATC TCT TGT TCA TGT CCT ACT GTT
CAA GCC TCC AAG CTG TGC CTT GGG TGG CTT TGG GGC
ATG GAC ATC GAC CCT TAT AAA GAA TTT GGA GCT ACT
GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC TTC TTT
CCT TCA GTA CGA GAT CCC CGG GCG AGC TCG ATG GTG
AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG
CCC ATC CTG GTC GAG CTG GAC GGC GAC GTA AAC GGC
CAC AAG TTC AGC

Supplementary Figure 8: Sequence information for pHBV-ffLuc. (**A**) HBV core protein sequence (blue) fused to GFP reading frame (green) enables downstream expression of GFP-2A-ffLuc. (**B**) DNA sequence of core-GFP fusion, with the transcriptional start site for HBV core mRNA indicated in red (same as the canonical HBV pregenomic RNA), the start codon for the core protein indicated in blue, and the start codon of GFP indicated in green.