

TITLE OF THE INVENTION

TITLE

**METHODS FOR REDUCING HEPATITIS B VIRUS SURFACE ANTIGEN (HBsAg)
AND THE HEPATITIS B DRUGS USED IN THE METHODS THEREOF**

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application is related to, and claims priority to, U.S. Provisional Application No. 63/506,582, filed June 6, 2023, the contents of which are hereby incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under contract no. 75N930220C00042 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0003] The present invention relates to methods for reducing cellular and serum hepatitis B virus surface antigen (HBsAg) levels in chronic hepatitis B virus (HBV) infections by maintaining high anti-HBs antibody levels and blocking de novo infection mediated cccDNA replenishment.

DESCRIPTION OF THE RELATED ART

[0004] Hepatitis B virus (HBV) has chronically infected 316 million people worldwide and nearly one million die of HBV related diseases each year. Current HBV drugs rarely deliver durable suppression of HBV replication after years of medication, let alone HBV functional cure.

[0005] Currently, both serum HBsAg and HBV DNA markers are required to become undetectable in establishing HBV functional cure, that is, HBV functional cure is defined as undetectable serum HBsAg and HBV DNA after a finite period of HBV treatment (Alter et

al., *Hepatology* 67, 1127-1131 (2018)). Serum HBV DNA can be inhibited and reduced to undetectable level after long-term treatment with the approved HBV drug of nucleos/tide analogues (NA) but the most difficult challenge is how to effectively reduce serum HBsAg to undetectable level.

[0006] The current strategy to reduce serum HBsAg level is to directly inhibit intracellular HBsAg synthesis with siRNA or antisense oligos (ASO) drugs. However, the efficacy of this strategy and siRNA/ASO based therapies is limited, showing average <2 log reduction in serum HBsAg demonstrated by both preclinical and clinical evaluations and serum HBsAg level bounces back after stopping the therapy.

[0007] A method that demonstrates more efficient reduction of HBsAg level is needed for establishing an effective HBV functional cure.

SUMMARY OF THE INVENTION

[0008] This invention discloses how to effectively reduce cellular and serum HBsAg level and it comprises following elements:

[0009] 1. In contrast to the current strategy to reduce serum HBsAg through direct inhibition of intracellular HBsAg synthesis, the present invention method does not require a direct inhibition of intracellular HBsAg synthesis.

[0010] 2. cccDNA is the major HBsAg transcription template but is frequently spontaneously lost in infected cells. HBV infected cells keep secreting HBsAg into blood, leading to emptying HBsAg in the infected cells if the lost cccDNA pool does not get replenished. Thus, the present invention method of reducing cellular and serum HBsAg aims to block de novo infection mediated cccDNA replenishment using sustained high level of anti-HBs antibody in the presence or absence of other anti-HBV drugs.

[0011] 3. The present invention provides the method for using AAV-anti-HBs vectors to express sustained high levels of anti-HBs antibody after a single injection and can be used to block de novo infection mediated cccDNA replenishment.

[0012] The advantages of the present invention, either alone or in combinations thereof, may be satisfied by method of curing chronic hepatitis B infection in humans, comprising:

administering to a subject in need thereof, an effective amount of one or both of an exogenous anti-HBs antibody or an anti-HBs antibody producing vector to reduce cellular and blood hepatitis B surface antigen (HBsAg), by providing a sustained elevated level of anti-HBs antibody in the subject, wherein the reduction of cellular and blood HBsAg occurs

(i) with blocking of de novo infection mediated cccDNA replenishment or (ii) without direct inhibition of HBsAg synthesis, or both (i) and (ii).

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

[0014] FIGS. 1A-1D show kinetic HBsAg levels in blood of HBV infected uPA/SCID chimeric mice with humanized livers (from day 14 to day 162 post infection), wherein Figure 1A shows Group 1 (G1) without treatment; Figure 1B-1D represent where Groups 2 and 3 (G2-G4) were treated with HBVZ10 and 12-weeks of entecavir but started at different timepoints to allow HBsAg to reach different levels. HBVZ10 was given at day 11 (d11) at a dose of 2.5×10^{11} copies and day 58 at a dose of 4×10^{11} copies, respectively in G2 (Figure 1B), given at day 22 at dose of 1.8×10^{12} copies in G3 (Figure 1C), and given at day 44 at dose of 7.2×10^{12} copies in G4 (Figure 1D). Timepoints were truncated among mice who deceased prior to day 162. Lower limit of qualification for HBsAg is 0.05IU/ml (HBsAg EIA kit, Bio-rad).

[0015] FIG. 2 shows copies of HBsAg per cell and numbers of input cells for assaying HBsAg in liver lysates from HBV infected uPA/SCID chimeric mice with humanized livers. Cellular HBsAg levels were titrated by serial dilutions from 1/1000, 1/100, 1/10, 1/2, 1/3.3, and 1/2.5. The inhibitory effect was detected at 1/2.5 dilution and the allowable dilution was 1/3.3. A total of 4 lysates from each of animals 825, 838, and 831 were analyzed and numbered as .1, .2, .3, and .4, respectively.

[0016] FIG. 3 shows numeral relation of cellular HBsAg and rcDNA per cell among mice in Figure 2. Figure 3A provides a graphical representation showing the copies of HBsAg and rcDNA per cells in 2 mice with undetectable serum HBsAg and one untreated mouse control. Figure 3B provides the ratios of cellular HBsAg and rcDNA in the mice of Figure 3A.

[0017] FIGS. 4A-4C provide graphical representations showing the serum HBsAg levels, serum HBV DNA levels, and intracellular HBsAg, rcDNA and cccDNA levels during HBV infection phases.

[0018] FIGS. 5A-5C provide graphical representations showing cccDNA levels in analyzed mouse liver samples.

[0019] FIG. 6 provides a graphical representation showing the correlation between average cccDNA and HBV RNA levels in HBV infection.

[0020] FIGS. 7A-7C provide graphical representations showing the effect on average cccDNA and rcDNA levels in mice after treatment with AAV-anti-HBs vector HBVZ10 of one embodiment of the present invention.

[0021] FIGS. 8A-8H provide graphical representations showing the effects on average cccDNA level, kinetic serum HBsAg level, kinetic serum HBeAg, evidencing progressive clearance of cccDNA after treatment with embodiments of the present invention.

[0022] FIGS. 9A-9B provide graphical representations showing that subjects treated using embodiments of the present invention have no significant differences in average Ki67 RNA levels, while having significant differences in average cccDNA levels compared to untreated subjects.

[0023] FIGS. 10A-10J provide graphical representations showing kinetic serum HBsAg levels and intracellular HBsAg levels, which were confirmed by the Western Blot analyses also shown in these figures.

[0024] FIGS. 11A-11B provide schematic representations for replication-driven cccDNA loss via either spontaneous clearance or cell destruction pathways.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0025] Embodiments identified herein as exemplary are intended to be illustrative and not limiting. Table 1 outlines some main features of the present invention compared to current conventional therapies to reduce HBsAg, and they are further elaborated in the description below.

[0026] **Table 1.**

	Present invention	Current therapies to reduce HBsAg
Method	Blocking de novo infection mediated cccDNA replenishment without direct inhibition of cellular HBsAg synthesis	Direct inhibition of cellular HBsAg synthesis
Drug modality	AAV vector-based gene therapy	siRNA or anti-sense oligos

Regimens	A single injection	Multi-injection
Average efficacy	1-5 log reduction	<2 log
Achieving undetectable HBsAg	Yes frequently	No or rarely
Durability	Durable	Non-durable

[0027] Hepatitis B virus (HBV) is a hepatotropic DNA virus that may cause persistent and largely noncytopathic infection if caught in infancy [See, e.g., Summers J., *Hepatology*, 1981;1(2):179-83; and Seeger C, et al., *The Liver: Biology and Pathobiology*, 2020:793-820]. Establishing and maintaining HBV infection in hepatocytes requires the formation of episomal covalently closed circular DNA (cccDNA) molecules, which function as templates for viral transcription in the nucleus [See, e.g., Summers J, et al., *Cell*, 1982;29(2):403-15; and Tuttleman JS, et al., *Cell*, 1986;47(3):451-60]. Therefore, a single copy of cccDNA in an infected cell is minimally required. The cccDNA molecules are assumed to be long-lived [Alter H, et al. *Hepatology*. 2018;67(3):1127-31] because a chronic HBV infection usually lasts for years or decades [Seto W-K, et al., *The Lancet*, 2018;392(10161):2313-24]. Chronic HBV infection is thought to result from the failure of the host's immune system to clear the established infection [Guidotti LG, et al., *Annual Review of Pathology*. 2006;1:23-61]; therefore, it is conventionally viewed as a continuation of the established initial infection. Conventional HBV cure strategies aim to directly eliminate or permanently silence cccDNA [Alter H, et al. *Hepatology*. 2018;67(3):1127-31], or clear infected cells from the livers [Fanning GC, et al., *Nature Reviews Drug discovery*, 2019;18(11):827-44].

[0028] HBV replicates robustly in infected human hepatocytes, as evidenced by high serum hepatitis surface B antigen (HBsAg), HBV DNA levels [See, e.g., Keating SM, et al., *The Journal of Infectious Diseases*, 2014;209(6):845-54; Jaroszewicz J, et al., *Journal of Hepatology*, 2010;52(4):514-22; and Nguyen T, et al., *Journal of Hepatology*, 2010;52(4):508-13], and accumulated HBsAg and hepatitis B core antigen (HBcAg) proteins in infected cells during chronic HBV infection [See, e.g., Naoumov NV, et al., *Gastroenterology*, 1990;99(4):1248-5312; and Chu CM, et al., *Journal of Clinical Pathology*, 1995;48(5):470-3] or in vitro infection [See, e.g., Ko C, et al., *Journal of Hepatology*, 2018;69(6):1231-41; and König A, et al., *Journal of Hepatology*, 2019;71(2):289-300]. The

intracellular accumulation of viral products may indicate that the secretion of viral particles by infected cells lags behind HBV replication capacity, which may cause cytopathic changes if not stopped. The retention of L protein in hepatocytes of HBV transgenic mice causes a spectrum of pathologies, including necrosis and persistently elevated ALT levels, and the severity of pathology is related to the concentration of intracellular envelope proteins [Chisari FV, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 1987;84(19):6909-13]. The intracellular HBsAg accumulation within smooth endoplasmic reticulum (ER) causes ER hyperplasia and displaces other organelles to the cell periphery, giving an appearance of “ground-glass” in some hepatocytes in chronic infection [Hadziyannis S, et al., *Arch Pathol*, 1973;96(5):327-30], [Gerber MA, et al., *The American Journal of Pathology*, 1974;75(3):489]. However, most HBsAg-positive cells in liver sections do not show a ground-glass appearance [Deodhar K, et al., *Journal of Clinical Pathology*, 1975;28(1):66-70]. In the early 1990s, Summers et al. discussed the following principles on the replication and persistent infection of Hepadnaviruses: i. a persistent infection of Hepadnaviruses is contingent upon the inhibition of replication at late phase of infection; ii. control of cccDNA copy numbers is required to maintain persistent noncytopathic infection because high levels of cccDNA were always associated with cytopathic effects in infected hepatocytes; and iii. intracellularly accumulated L protein acts as an overall suppressor of replication and permits persistent infection [See, e.g., Summers J, et al., *Journal of Virology*, 1991;65(3):1310-7; and Lenhoff RJ, et al., *Journal of Virology*, 1994;68(9):5706-13].

[0029] Clinical evidence has shown the dynamic evolution of the cccDNA population. The wild-type viral population in serum or cccDNA in the liver can be cleared and replaced with mutant populations during chronic hepatitis B infection [See, e.g., Brunetto MR, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 1991;88(10):4186-90; Carman WF, et al., *Lancet*, 1989;2(8663):588-91; Jiang B, et al., *Alimentary Pharmacology & Therapeutics*, 2019;50(8):940-54; Chen Q-Y, et al., *Infection, Genetics and Evolution*, 2021:105184; and Huang Q, et al., *Hepatology* (Baltimore, Md), 2020]. Complete cccDNA turnover in patients with chronic hepatitis B receiving nucleotide analog (NA) treatment occurs in a duration as short as 24 weeks [Huang Q, et al., *Hepatology* (Baltimore, Md), 2020].

[0030] The loss of pre-existing cccDNA is supported by the quantitative detection of cccDNA levels in serial liver tissues, which showed that cccDNA levels were progressively reduced by 20- to 100-fold during NA treatment of woodchucks chronically infected with

woodchuck hepatitis virus [Zhu Y, et al., *Journal of Virology*, 2001;75(1):311-22], an animal model closely resembling chronic HBV infection in humans. A 1–2.9 log reduction in cccDNA levels has also been reported in human patients treated with NA [See, e.g., Werle-Lapostolle B, et al., *Gastroenterology*, 2004;126(7):1750-8; Wong DK, et al., *Antiviral Therapy*, 2006;11(7):909-16; Wursthorn K, et al., *Hepatology*, 2006;44(3):675-84; Lutgehetmann M, et al., *Antiviral Therapy*, 2008;13(1):57-66; Boyd A, et al., *Journal of Hepatology*, 2016;65(4):683-91; and Lai C-L, et al., *Journal of Hepatology*, 2017;66(2):275-81]. The detected cccDNA reduction raises the possibility that cccDNA molecules can be spontaneously cleared from infected cells.

[0031] Taken together, the present inventor hypothesized that in vivo HBV-infected cells spontaneously clear cccDNA. Testing this hypothesis in uPA/SCID chimeric mice with humanized livers that support a robust persistent HBV infection in the absence of functional T- and B-cell immunity [Tateno C, et al., *PloS ONE*, 2015;10(11):e0142145], the present invention provides evidence supporting the concept of spontaneous cccDNA loss in HBV-infected cells and an cccDNA elimination strategy that transforms spontaneous cccDNA clearance into progressive cccDNA elimination by blocking cccDNA replenishment. The present invention thus relates to the treatment of persistent HBV infection by blocking cccDNA replenishment and providing an efficient cccDNA elimination therapy.

[0032] The Anti-hepatitis B Drugs Used in This Invention

[0033] Any existing or newly developed anti-hepatitis B drugs which can block the replenishment and/or synthesis of cccDNA and/or rcDNA can be used in the methods of treatment described above. Such drugs include, but are not limited to, antibody drugs that are exogenous or endogenously expressed, small molecules drugs against any of HBV infection and replication steps, peptide drugs and vector drugs. The following AAV-anti-HBs vectors-based HBV drugs that endogenously express anti-HBs antibodies after a single injection, were designed and tested in the present invention, and were found to be effective against chronic hepatitis infections by blocking cccDNA replenishment in cells, when used in the treatment methods described above. A total of nine AAV-anti-HBs vectors comprise the following sequences:

[0034] Nucleic acid sequence ID NO: 1 encodes the amino acid sequence of sequence ID NO: 2. Sequence ID NO:2 is the variable region of heavy chain of HBVZ10 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0035] Nucleic acid sequence ID NO: 3 encodes the amino acid sequence of sequence ID NO: 4. Sequence ID NO:4 is the variable region of light chain of HBVZ10 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0036] Nucleic acid sequence ID NO: 5 encodes the amino acid sequence of sequence ID NO: 6. Sequence ID NO:6 is the variable region of heavy chain of HBVZ20 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0037] Nucleic acid sequence ID NO: 7 encodes the amino acid sequence of sequence ID NO: 8. Sequence ID NO:8 is the variable region of light chain of HBVZ20 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0038] Nucleic acid sequence ID NO: 9 encodes the amino acid sequence of sequence ID NO: 10. Sequence ID NO: 10 is the variable region of heavy chain of HBVZ30 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0039] Nucleic acid sequence ID NO: 11 encodes the amino acid sequence of sequence ID NO: 12. Sequence ID NO: 12 is the variable region of light chain of HBVZ30 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0040] Nucleic acid sequence ID NO: 13 encodes the amino acid sequence of sequence ID NO: 14. Sequence ID NO: 14 is the variable region of heavy chain of HBVZ40 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0041] Nucleic acid sequence ID NO: 15 encodes the amino acid sequence of sequence ID NO: 16. Sequence ID NO: 16 is the variable region of light chain of HBVZ40 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0042] Nucleic acid sequence ID NO: 17 encodes the amino acid sequence of sequence ID NO: 18. Sequence ID NO: 18 is the variable region of heavy chain of HBVZ50 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0043] Nucleic acid sequence ID NO: 19 encodes the amino acid sequence of sequence ID NO: 20. Sequence ID NO:20 is the variable region of light chain of HBVZ50 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0044] Nucleic acid sequence ID NO: 21 encodes the amino acid sequence of sequence ID NO: 22. Sequence ID NO:22 is the variable region of heavy chain of HBVZ60 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0045] Nucleic acid sequence ID NO:23 encodes the amino acid sequence of sequence ID NO: 24. Sequence ID NO:24 is the variable region of light chain of HBVZ60 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0046] Nucleic acid sequence ID NO: 25 encodes the amino acid sequence of sequence ID NO: 26. Sequence ID NO:26 is the variable region of heavy chain of HBVZ70 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0047] Nucleic acid sequence ID NO: 27 encodes the amino acid sequence of sequence ID NO: 28. Sequence ID NO:28 is the variable region of light chain of HBVZ70 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0048] Nucleic acid sequence ID NO: 29 encodes the amino acid sequence of sequence ID NO: 30. Sequence ID NO:30 is the variable region of heavy chain of HBVZ80 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0049] Nucleic acid sequence ID NO:31 encodes the amino acid sequence of sequence ID NO: 32. Sequence ID NO: 32 is the variable region of light chain of HBVZ80 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0050] Nucleic acid sequence ID NO: 33 encodes the amino acid sequence of sequence ID NO: 34. Sequence ID NO:34 is the variable region of heavy chain of HBVZ90 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0051] Nucleic acid sequence ID NO:35 encodes the amino acid sequence of sequence ID NO: 36. Sequence ID NO: 36 is the variable region of light chain of HBVZ90 human anti-HBs monoclonal IgG1 antibody against 4 serotypes of HBsAg.

[0052] Nucleic acid sequence ID NO: 37 is nucleic acid sequence of AAV vector which consists of 3758bp including two ITRs (inverted Terminal Repeat from AAV), chicken Beta-actin promoter, constant regions of human IgG1 heavy and light chains, WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), and SV40 polyadenylation signal. This AAV vector (sequence ID NO: 37) allows cloning two variable regions of both heavy and light chains to express a full human IgG1 monoclonal anti-HBs antibodies.

[0053] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 2 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 4, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0054] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg

subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 6 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 8, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0055] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 10 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 12, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0056] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 14 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 16, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0057] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 18 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 20, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0058] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 22 and an antibody VL, wherein the VL comprises the amino acid

sequences of SEQ ID NO: 24, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0059] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 26 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 28, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0060] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 30 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 32, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0061] In one embodiment, the present invention is directed to an isolated binding molecule or antigen-binding fragment thereof which specifically binds to HBV virions and/or HBsAg subviral particles comprising an antibody VH, wherein the VH comprises the amino acid sequences of SEQ ID NO: 34 and an antibody VL, wherein the VL comprises the amino acid sequences of SEQ ID NO: 36, or a variant thereof with at least 95% sequence homology. In one embodiment, the present invention is directed to a nucleic acid molecule encoding the above isolated binding molecule or antigen-binding fragment. In one embodiment, the present invention is directed to a vector comprising the above nucleic acid molecule.

[0062] In one embodiment, the present invention is directed to a method of curing chronic hepatitis B infection in humans, comprising administering to a subject in need thereof, an effective amount of an anti-HBs antibody producing vector to reduce cellular and blood hepatitis B surface antigen (HBsAg), by providing a sustained elevated level of anti-HBs antibody in the subject. In certain embodiments, the sustained elevated level of anti-HBs antibody 100mIU/ml, 1000mIU/ml, 10,000mIU/ml, 100,000mIU/ml or higher for a period of

3-months or longer. In preferred embodiments, these levels of anti-HBs antibody are produced for the stated time period by a single administration of the anti-HBs antibody producing vector.

[0063] In preferred embodiments, the single dose administration of the anti-HBs antibody producing vector is an amount of $1E11$ copies or more, more preferably $2E11$ copies or more, still more preferably $1E12$ copies or more, most preferably $3E12$ copies or more.

[0064] If the concept of spontaneous cccDNA clearance from infected cells is valid, then continuous cccDNA replenishment is required to maintain cccDNA levels. The present invention method provides therapeutic interventions aimed at blocking cccDNA replenishment.

[0065] Two known pathways contribute to cccDNA replenishment: intracellular recycling and de novo infection. The intracellular recycling pathway involves the delivery of newly synthesized rcDNA molecules into the nucleus for cccDNA conversion [See, e.g., Tuttleman JS, et al., *Cell*, 1986;47(3):451-60; and Nassal M., *Gut*, 2015;64(12):1972-84] However, this pathway primarily operates during the early phase of replication and is impeded by the accumulation of envelope proteins during the late phase of replication [Lenhoff RJ, et al., *Journal of Virology*, 1994;68(9):5706-13]. Previous studies have suggested that de novo infection is the primary pathway of cccDNA replenishment [See, e.g., König A, et al., *Journal of Hepatology*, 2019;71(2):289-300; Allweiss L, et al., *Gut*, 2018;67(3):542-52; and Volz T, et al., *Journal of Hepatology*, 2013;58(5):861-7].

[0066] The present invention rethinks the cccDNA elimination strategy. Directly targeting cccDNA or killing infected cells is conventionally recommended for cccDNA elimination and complete cure of chronic HBV infection. Such a strategy may not be required based on the present invention. Instead, the present invention provides an unconventional cccDNA elimination strategy that does not require direct targeting of cccDNA molecules but aims to transform spontaneous cccDNA loss into progressive cccDNA elimination by blocking cccDNA replenishment.

[0067] In certain embodiments of the method of the present invention, the reduction of cellular and blood HBsAg occurs (i) with blocking of de novo infection mediated cccDNA replenishment or (ii) without direct inhibition of HBsAg synthesis, or both (i) and (ii).

[0068] In certain embodiments, the anti-HBs antibody provides blocking of de novo infection mediated cccDNA replenishment. In certain embodiments, the anti-HBs antibody blocks de novo infection through blocking HBV particles (virions and subviral particles) from attaching to human hepatocytes. In certain embodiments, the anti-HBs antibody has

specificity against the “a” determinant of HBsAg, and preferably has specificity against human hepatocyte attachment site in the “a” determinant of HBsAg.

[0069] In certain embodiments, the anti-HBs antibody is provided by one or more administrations, wherein the administration is performed by a procedure selected from injection, infusion, oral administration, or transdermal administration. In embodiments of the invention, the sustained high level of anti-HBs antibody is provided either endogenously or exogenously. In certain embodiments, the anti-HBs antibody can be expressed through administering viral, non-viral vectors or nano particles that deliver human anti-HBs antibody genes or mRNA to express anti-HBs antibody or vaccines that express or directly deliver HBsAg proteins or peptides to elicit anti-HBs antibody response in receipts.

[0070] In certain embodiments, anti-HBs antibody is expressed through administering viral vectors, including, but not limited to, AAV vector-based therapy to express a sustained high level of anti-HBs antibody. In preferred embodiments, the AAV vector-based therapy comprises administration of one or more members selected from the group consisting of HBVZ10, HBVZ20, HBVZ30, HBVZ40, HBVZ50, HBVZ60, HBVZ70, HBVZ80, and HBVZ90. In certain embodiments, the exogenous anti-HBs antibody is infused or injected with human anti-HBs antibody, nanoantibody or antibody fragments.

[0071] In the method of the present invention, the reduction of HBsAg in the blood can be progressive or precipitous, both having blood HBsAg reduced by 1-5 log, preferably 3-5 log, or to an undetectable level or <0.05IU/ml.

[0072] In one embodiment, the treating of chronic HBV infection comprises treating newborns/children who have been infected by HBV.

[0073] In one embodiment, the treating of chronic HBV infection comprises treating adults who have been infected by HBV.

[0074] In one embodiment, the HBV infected human patient is a chronic HBV infected individual who has been HBsAg positive for more than 6 months and have normal or elevated alanine aminotransferase (ALT) level.

[0075] In one embodiment, the HBV infected human patient is an HBV positive pregnant woman, or an organ transplant recipient who is HBsAg positive or HBsAg negative/anti hepatitis B core antibody (anti-HBc) positive, and is prone to recurrent HBV infection after transplant.

[0076] In one embodiment, the HBV neutralizing antibodies or antibody fragments are produced by HBV therapeutic vectors.

[0077] In one embodiment, the HBV therapeutic vectors comprise a mixed population of vectors each of which encodes one specific anti-HBs antibody or antibody fragment binding to one or more epitopes of HBV envelope proteins, or a single vector which encodes one HBV neutralizing antibody or antibody fragment binding to one or more epitopes of HBV envelope proteins.

[0078] In certain embodiments, the method is a monotherapy. In other embodiments, the method is a combinational therapy with one or more additional HBV drugs that inhibit intracellular HBV DNA, HBV RNA or/and viral proteins synthesis. In such combinational therapy embodiments, the one or more additional HBV drugs are preferably one or more members selected from the group consisting of reverse transcription inhibitors, capsid inhibitors, cccDNA inhibitors, RNA transcription inhibitors, viral protein synthesis inhibitors, entry inhibitors, interferons, therapeutic vaccines, immune checkpoint inhibitors, and immunoregulatory drugs

[0079] In an important embodiment, this invention utilizes the finding that cccDNA molecules, the main transcriptional template directing cellular HBsAg synthesis, is frequently spontaneously lost from infected cells. HBsAg is being continuously secreted into blood from infected cells. cccDNA loss and ongoing HBsAg secretion will lead to emptying HBsAg in infected cells. Thus, HBsAg can be cleared from infected cells through secretion alone if the replenishment of depleted cccDNA pool is prevented. This understanding forms the foundation of this invention.

[0080] In a preferred embodiment, this invention recognizes that HBsAg can't be cleared if cccDNA pool in infected cells keeps replenished despite HBsAg secretion or the inhibition of HBsAg synthesis because the inhibition of HBsAg synthesis alone does not address the root cause for maintaining HBsAg production in infected cells, as demonstrated by preclinical evaluations and clinical trials.

[0081] In a further embodiment, this invention utilizes the function of HBsAg secretion, a natural process of HBV infected cells and a property of HBsAg proteins for clearing HBsAg from infected cells.

[0082] There are two pathways to replenish cccDNA pool, one is recycling pathway that transports the de novo synthesized rcDNA molecules to nuclei for cccDNA conversion, and the other is do novo infection in which virions circulated in blood attach and enter hepatocytes to initiate new rounds of infection through establishing cccDNA pool in the nuclei of infected hepatocytes. However, these two cccDNA replenishment pathways are not utilized equally. This inventor found that the recycling pathway is generally restricted, and de

novo infection is the main pathway to replenish cccDNA pool. Thus, an expanding embodiment of this invention aims to block de novo infection to effectively prevent cccDNA replenishment.

[0083] To further demonstrate this invention, a durable blocking of de novo infection can be realized through using AAV-anti-HBs vectors that express sustained high level of anti-HBs antibody after a single intramuscular injection, which not only deliver durable efficacy without HBsAg relapse, but also simplifies the therapies for HBsAg reduction.

[0084] In a combined embodiment, in contrast to the current method of reducing serum HBsAg level through direct inhibition of intracellular HBsAg synthesis, the present invention reduces cellular and serum HBsAg through blocking cccDNA replenishment that eliminate the root cause for HBsAg production. In addition, this method does not require the direct inhibition of cellular HBsAg synthesis.

EXAMPLES

[0085] Example 1. Experimental procedures using uPA/SCID chimeric mice for HBV infection and treatment.

[0086] A total of 49 HBV infected uPA/SCID chimeric mice were divided into 7 groups as illustrated in table 2 below.

[0087] **Table 2.** Animal experiment design

Group	Starting Treatment	Doses	Duration
1. AAV malaria antibody	Day 15	2.5E11 genome copies IM	30W infection
2. HBVZ10 monotherapy	Day 15	2.5E11 genome copies IM	27W HBVZ10 treatment
3. HBVZ10+ETV	Day 15	2.5E11 AAV vector + ETV 0.3mg/kg 3 times a week for 16 weeks	12W ETV + 27W HBVZ10 treatment
4. HBVZ10 monotherapy	Day 29	1.8E12 genome copies IM	25 W HBVZ10 treatment
5. HBVZ10+ETV	Day 29	1.8E12 AAV vector + ETV 0.3mg/kg 3 times a week for 16 weeks	12W ETV + 25W HBVZ10 treatment
6. HBVZ10 monotherapy	Day 50	5.7E12 genome copies IM	22W HBVZ10 treatment
7. HBZZ10+ETV	Day 50	5.7E12 AAV vector + ETV 0.3mg/kg 3 times a week for 16 weeks	12W ETV + 22W HBVZ10 treatment

[0088] Serial blood samples were drawn biweekly or triweekly until termination upon which liver tissues were collected and snap-frozen.

[0089] Example 2. Analyzing HBV infection in blood and liver samples

[0090] HBsAg, HBeAg, HBV DNA, anti-HBs antibody, and human albumin levels in serial blood samples were quantitatively analyzed.

[0091] Average intracellular HBsAg, rcDNA, and cccDNA were determined by quantitative assays of 20 samplings of each liver.

[0092] Western blot of HBsAg in liver lysates and immunohistochemical staining of HBsAg and HBcAg in liver sections were also performed.

[0093] Figures 1A-1D provide graphical representations of kinetic HBsAg levels in blood of HBV infected uPA/SCID chimeric mice with humanized livers (from day 14 to day 162 post infection), wherein Figure 1A shows Group 1 (G1) without treatment; and Figures 1B-1D represent where Groups 2 and 4 (G2-G4) were treated with HBVZ10 and 12-weeks of entecavir but started at different timepoints to allow HBsAg to reach different levels. HBVZ10 was given at day 11 (d11) at a dose of 2.5E11 copies and day 58 at a dose of 4E11 copies, respectively in G2 (Figure 1B), given at day 22 at dose of 1.8E12 copies in G3 (Figure 1C), and given at day 44 at dose of 7.2E12 copies in G4 (Figure 1D). Timepoints were truncated among mice who deceased prior to day 162. Lower limit of qualification for HBsAg is 0.05IU/ml (HBsAg EIA kit, Bio-rad).

[0094] Figure 2 provides a graphical representation of copies of HBsAg per cell and numbers of input cells for assaying HBsAg in liver lysates from HBV infected uPA/SCID chimeric mice with humanized livers. Cellular HBsAg levels were titrated by serial dilutions from 1/1000, 1/100, 1/10, 1/2, 1/3.3, and 1/2.5. The inhibitory effect was detected at 1/2.5 dilution and the allowable dilution was 1/3.3. A total of 4 lysates from each of animals 825, 838, and 831 were analyzed and numbered as .1, .2, .3, and .4, respectively.

[0095] Figures 3A-3B shows numeral relation of cellular HBsAg and rcDNA per cell among mice in Figure 2. Figure 3A provides a graphical representation showing the copies of HBsAg and rcDNA per cells in 2 mice with undetectable serum HBsAg and one untreated mouse control. Figure 3B provides the ratios of cellular HBsAg and rcDNA in the mice of Figure 3A.

[0096] Example 3. The present invention method showed more effective serum HBsAg reduction than the reported efficacy with siRNA or ASO drugs that directly inhibit intracellular HBsAg synthesis, specifically.

[0097] 1. All treated mice with this invented method responded with serum HBsAg reduction. Furthermore, 3-5 log progressive reduction of serum HBsAg was observed and serum HBsAg became undetectable in 8 of 11 mice as of day 162 post inoculation. Note: the

direct inhibition of intracellular HBsAg synthesis showed average <2log reduction in both preclinical evaluations and clinical trials.

[0098] 2. Progressive serum HBsAg reduction was closely paralleled with progressive reduction of serum HBeAg levels, implying that the observed progressive serum HBsAg reduction reflects progressive HBV clearance in the livers.

[0099] 3. Intrahepatic HBsAg, rcDNA, and cccDNA levels were lowered by 3-4 logs in mice with undetectable serum HBsAg compared to untreated control mice.

Materials and Methods

Animals and HBV infection

[00100] All animal experiments were performed at Noble Life Sciences Inc (Sykesville, MD), a preclinical research contract service provider. The selection of Noble Life Sciences Inc as the subcontractor to perform animal experiments was approved by the NIAID contract office at NIH (NIH approved Animal Welfare Assurance number, A4633-01). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Noble Life Sciences protocol NLS-614. All animals received humane care.

[00101] Immunocompetent female mice (CD1) were purchased from Charles River Laboratories (Boston, MA, USA), and immunodeficient male mice (uPA/SCID chimeric mice) were supplied by PhoenixBio USA (New York, NY, USA). All mice were kept in housing cages (TP107, One Corporation, Osaka, Japan) in an BSL-2 room with controlled temperature at 23°C and 12 hour-light/dark cycle. All animals fed with γ -radiated CRF1 food and autoclaved water *ad libitum*. An HBV inoculum (HBsAg ADR subtype/genotype C), prepared from mouse serum (project no H01-108 animal 4) by diluting viremia of 5E9 HBV DNA copies/mL to 2E7 HBV DNA copies with PBS in 100 μ l volume, was administered intravenously (tail vein) to each chimeric mouse.

AAV-anti-HBs vector (HBVZ10)

[00102] The present invention provides new HBV therapy candidates called AAV-anti-HBs vectors, which utilize an optimized adeno-associated virus (AAV) vector [See, e.g., Balazs A, et al., *Nature Medicine*, 2014;20(3):296-300; Balazs A, et al., *Nature Biotechnology*, 2013;31(7):647-52; de Jong YP, et al., *Science Translational Medicine*, 2014;6(254):254ra129; and Deal C, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 2014;111(34):12528-32] to deliver human anti-hepatitis B surface antigen (anti-HBs) antibody genes. The AAV-anti-HBs vectors of preferred

embodiments of the present invention express sustained high levels of anti-HBs antibody after a single injection. By using these AAV-anti-HBs vectors as a new HBV therapy candidate, the method of the present invention remedies the deficiency in anti-HBs antibody production in chronic HBV infection. A most preferred AAV-anti-HBs vector is HBVZ10. Administration of HBVZ10 intramuscularly in chimeric mice expresses human anti-HBs antibody endogenously and blocks de novo infection in the absence or presence of entecavir.

Production of AAV-anti-HBs or anti-malaria antibody vectors

[00103] Briefly, 293 cells were co-transfected with an AAV vector encoding anti-HBs or anti-malaria antibody and the plasmid pDP8.ape (PlasmidFactory, Bielefeld, Germany), which provides pHELP plasmid function and encodes AAV2 rep and AAV8 cap proteins in trans for packaging AAV vectors. The resultant AAV vector does not contain any viral open reading frame (ORF). AAV was purified via PEG precipitation and cesium chloride ultracentrifugation. The infectivity of AAV aliquots was confirmed *in vitro* by transducing 293 cells and quantifying the antibody concentration in the medium using ELISA. A total of 1E14 genome copies were obtained for each vector after production, purification, and concentration.

HBVZ10 administration doses

[00104] A small dose of HBVZ10 at 1E11 genomic copies was intramuscularly administered in animal experiments 1 and 2, and the higher doses of HBVZ10 at 2.5E11, 1.8E12 or 7E12 genomic copies were administered for animal experiment 3.

Monitoring HBV infection and human albumin level in blood

[00105] Blood was collected tri-weekly for quantification of serum HBV DNA (qPCR see below), HBeAg (CSB-E13557h, CUSABIO), HBsAg (GS HBsAg EIA 32591, Bio-Rad), anti-HBs antibody (MONOLISA Anti-HBs EIA 25200, Bio-Rad) with calibrators (MONOLISA Anti-HBs 20-Calibrator kit 25219, Bio-Rad) and human albumin (Human albumin ELISA kit E-80AL, Immunology Consultants Laboratory) levels by ELISA per instructions.

In addition, serum HBsAg in the selected samples was analyzed by western blot.

Alanine transaminase (ALT) activity in serum

[00106] Serum ALT activity was assessed using the alanine transaminase colorimetric assay kit (Cayman Chemical item no 700260) according to the detection manual. Absorbance

values were measured at 340 nm once per minute for 10 min, and the resulting 10 absorbance values were plotted against time. Due to limited serum volumes, a modification was made: the 20 µl serum sample was adjusted to 10 µl and compensated with 10 µl of H₂O. Consequently, in the calculation formula, the 0.02 ml of serum sample was adjusted to 0.01 ml accordingly.

Analysis of intrahepatic HBV DNA

[00107] Each liver was randomly sampled 20-40 times by cutting 20-40 mg liver tissue (weighed and recorded) and placed in a disposable micro-homogenizer (BioMasher, Takara cat no: 9790B) in 500 µl of an isotonic buffer (154 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.05% TritonX-100) with 10 strokes. The homogenized tissue suspension was spun for 2 min at 14,000 rpm and 100 µl of lysate were saved for western blot or ELISA of intracellular HBsAg and the remaining 400 µl transferred to a new microtube for isolation of replicative intermediates (RI) while nucleic pellet remained in the tube for cccDNA isolation.

[00108] Two negative controls were included for each round of extraction, one placed in the 1st sample position and the other in the last position to monitor any contamination during extraction.

Extraction of rcDNA from the 400 µl supernatant is performed by the following procedure: [Zhang YY, et al., *Journal of Virology*, 2004;78(3):1195-201]

[00109]

1. Add 110 µl of proteinase K (final 0.5 mg/mL) with 1% SDS and incubate at 50°C for an hour.
2. Add 500 µl phenol, vortexed and chilled on ice for 3 min and centrifuge at 14,000 rpm for 2 min.
3. Transfer the supernatant to a new tube and add 1000 µl of 100% ethanol for precipitation and centrifuge samples at 14,000 rpm for 15 min.
4. Wash pellets with 1000 µl of 100% ethanol at 14,000 rpm for 10 min.
5. Remove residual ethanol and air dry for 5 min.
6. Dissolve the pellet in 200 µl of 10:1 TE buffer, pH 7.4, and then the rcDNA is ready for qPCR

Extraction of cccDNA from nucleic pellets is performed by the following procedure: [Zhang YY, et al., Proceedings of the National Academy of Sciences of the United States of America, 2003;100(21):12372-7]

[00110]

1. Suspend pellet with 200 μ l of 10:1 TE with 0.05% Triton-X100 pH7.4.
2. Add 200 μ l of 6% SDS-0.1 M NaOH solution and incubated at 37°C for 15 min.
3. Add 100 μ l of 3 M KAc pH 5.07 and mix thoroughly. Chill on ice for 5 min and then microfuge to remove KSDS-protein-ssDNA complexes (pellet) at 14,000 rpm for 2 min.
4. Transfer supernatant to a new tube, add 500 μ l of phenol, and centrifuge at 14,000 rpm for 2 min.
5. Recover supernatant and add 5 μ l of glycogen (4 μ g/ μ l for total 20 μ g).
6. Add 1000 μ l ethanol and centrifuge at 14,000 rpm for 15 min.
7. Wash with 1000 μ l ethanol and centrifuge at 14,000 rpm for 10 min.
8. Dissolve in 50 μ l EcoR I buffer at 37°C for 15 min and inactivate at 80°C for 20 min.

The cccDNA samples are ready for qPCR.

RT-qPCR detection of total HBV RNA in cccDNA and rcDNA samples

[00111] Total HBV RNA levels were determined in each of 120 cccDNA and 120 rcDNA samples prepared from untreated mice 842 and 836 (representing the amplification phase), 831 and 987 (representing the maintenance phase), and 38 and 813 (treated who achieved progressive reduction of serum HBsAg to undetectable levels accompanied by >100-fold reduction in cccDNA). Specifically, 20 cccDNA and 20 rcDNA samples from each liver were tested. Average RNA concentrations in cccDNA samples were approximately 1.5 μ g/ μ l and 0.5 μ g/ μ l in rcDNA samples. A260/280 ratios varied narrowly between 1.98 and 2.08. All RNA samples were 10-fold diluted and then 2 μ l were used for RT-qPCR with TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher 4444432) and primers/probe located in S gene (rcDNA for qPCR in Table 3). rcDNA was also detected in the same cccDNA and rcDNA samples without RT in the same plates with the same primers/probe for RNA detection, in addition to cccDNA detection in the same cccDNA samples. The detected HBV RNA levels were approximately 10 times higher than those of rcDNA in the same samples. The net RNA copies are plotted after subtracting rcDNA copies in the same samples. The ratios of RNA copies/cell to cccDNA copies/cell were calculated using the total net RNA copies (nuclear RNA copies + cytoplasmic RNA copies).

RT-qPCR detection of human Ki67 RNA levels in 840 cccDNA samples

[00112] One set of pre-stocked human Ki67 RNA primers/probe system (FAM-MGB, Hs01032435_g1) was purchased from ThermoFisher Scientific. This detection system generates a 179 bp-long amplicon that was isolated for the preparation of qPCR standards. Two microliters of cccDNA containing total nuclear RNAs were used for RT-qPCR detection of human Ki67 RNA with the same TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher 4444432). To first establish a broad picture of Ki67 RNA expression in humanized livers of chimeric mice, a total of 840 cccDNA samples from 42 livers were analyzed, including 15 untreated, 11 treated with either anti-HBs antibody or a combination of HBVZ10 with entecavir but with detectable serum HBsAg (HBsAg+), and 16 treated with progressive reduction of serum HBsAg to undetectable levels (HBsAg-). Subsequently, correlation analysis between Ki67 RNA and cccDNA in the same samples was conducted using scatter plots, generating correlation trendlines and R^2 values.

The cccDNA samples were consistently stored at -20°C, and all handling procedures were conducted in a biosafety cabinet with an air blower on. All tips, plates, tubes, and solutions used were nuclease-free. Analysis of differences in Ki67 RNA levels between the existing cccDNA and newly isolated cccDNA samples from 5 livers (mice 907, 471, 987, 831, and 805) revealed unremarkable results (data not shown), indicating no noticeable degradation of RNA samples in the existing cccDNA samples.

qPCR of serum HBV DNA and intrahepatic rcDNA and cccDNA

[00113] Primers and probe sequences for detection of serum HBV DNA and intracellular rcDNA by qPCR are listed in Table 3 while primers sequences for detection of cccDNA flank the gap region and the probe is placed immediately after DR1 sequence (Table 3).

[00114] **Table 3.** Positions and sequences of cccDNA and rcDNA primers and probes

Target	Primer ID	Nucleotide position	Sequence
rcDNA in qPCR	Pan122F	520-537	ccagcacgggaccatgc (SEQ ID No. 38)
	Pan160R	655-637	tgaggccactcccatagg (SEQ ID No. 39)
	S probe	563-583	FAM'tgttgctgtacaaaaccttcg (SEQ ID No. 40)
rcDNA in dPCR	rcDNA forward	1345-1370	gtcctctctcggaaatacacctcctt (SEQ ID No. 41)
	rcDNA reverse	1454-1438	tccgcgggattcagcgc (SEQ ID No. 42)
	rcDNA probe	1372-1405	Cy5' ccatggctgctcgggtgtgctgccaactggatcc (SEQ ID No. 43)

cccDNA in qPCR and dPCR	HBVcccF	1550-1570	cgtctgtgccttctcatctgc (SEQ ID No. 44)
	HBVcccR	1885-1868	aaggcacagcttggaggc (SEQ ID No. 45)
	cccDNA probe	1835-1862	FAM [*] ctaatcatctctgtacatgtcccactg (SEQ ID No. 46)

[00115] The specificity of the listed cccDNA primers and probe can discriminate against rcDNA amplification by 300-6000-fold. qPCR was performed using Taqman fast advanced master mix (ThermoFisher cat no:4444558) in a QuantStudio 3 instrument (ThermoFisher cat no: A28136) that accommodates 0.1 ml 96-well hard-shell plate.

[00116] All standards used for qPCR were calibrated with the Absolute Q digital PCR.

Absolute Q (ABQ) Digital PCR of cccDNA

[00117] cccDNA copies/cell were computed initially based on qPCR, then retested with the Absolute Q digital PCR (ThermoFisher cat no: A52864). Briefly, the procedures included the following steps:

1. Prepare 9.1 µl reaction mix consisting of 1.8 µl of 5x DNA dPCR mix (ThermoFisher cat no: A52490), 0.5 µl of 20x primers/probe mix (final concentration 900nM each primer and 250 nM probe), 1 µl cccDNA sample, and 5.8 µl of DNase and RNase free H₂O
2. Load 9 µl reaction mix into one well of microfluidic array plate (MAP, ThermoFisher cat no: A53301)
3. Run dPCR consisting of 10 min preheat at 96°C and 40 cycles of 5 s at 96°C and 15 s at 60°C
4. Generate data reports using QuantStudio Absolute Q Digital PCR software.

[00118] The sensitivity of dPCR is a single copy per microchamber and the result deemed valid if Rox fluorescent signal was read in >19,000 of 20,480 microchambers for each sample.

Procedures and principles of simultaneous detection of cccDNA and rcDNA by ABQ duplexing Digital PCR in the same nuclei

[00119] The main procedures for the detection of both cccDNA and rcDNA in the same nuclei are as follows:

1. Homogenizing 20-30mg liver tissues in 500µl of homogenization buffer (10 mM Tris_HCl (pH 7.5), 3 mM MgCl₂, 0.25 M sucrose, and 0.05% Triton X-100). The nuclei were pelleted by centrifugation and resuspended in homogenization buffer containing 2µg/ml ethidium bromide.
2. Individual nuclei were singly sorted and deposited in wells of 96-well plates.
3. The deposited nuclei were digested by proteinase K at concentration of 0.5mg/ml for 60 min, then inactivated for 15 min at 80 C.
4. The released HBV DNA was linearized with NcoI digestion.
5. The linearized HBV DNA was subjected to ABQ dPCR detection of both cccDNA and rcDNA.

Principles of simultaneous detection of cccDNA and rcDNA by ABQ duplexing Digital PCR in the same nuclei

[00120] The ABQ digital PCR instrument can simultaneously detect 4 fluorescent signals of FAM, VIC, ABY, and JUN/Cy5, which allows to detect 4 different targets in the same reaction (Multiplexing). The emission wavelengths of FAM and Cy5 are 517 and 670 nm, respectively, and there is no overlapping in their wavelength spectrum. Thus, FAM was selected for labeling cccDNA probe and Cy5 for labeling rcDNA probe to detect both molecules in the same reaction, called duplexing dPCR.

[00121] The specificity of cccDNA detection is provided through cccDNA specific primers that flank the gap region in HBV genome and the cccDNA-specific probe that is placed immediately after DR I sequence (Table 3).

Linearized cccDNA template cannot generate fluorescent signal with rcDNA primers/probe detection system

[00122] HBV DNA released from each of the deposited nuclei will be subjected to NcoI digestion to exclude cccDNA from detection with rcDNA primers/probe. Plus strand in rcDNA is only partially synthesized, containing a single-stranded gap of 600-2100 nucleotides at 3' end [Summers J, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 1975;72(11):4597-601]. Since NcoI is located between nt1372 and 1376, close to the 3' end of plus strand. Therefore it is most likely present in a single-strand sequence in rcDNA molecules [Summers J, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 1975;72(11):4597-601]. Thus, NcoI will linearize cccDNA but cannot cut rcDNA.

[00123] The NcoI linearized cccDNA sequence starts with C at nt1373 (5') and ends with C at nt1372 (3'). rcDNA forward primer will bind the 3' end of the linearized cccDNA, but the rcDNA probe binds its 5' end. The Taq DNA polymerase that binds the forward primer at 3' end cannot reach the probe that is located at the 5' end, thus cannot cut off the 1st base C with Cy5 dye through its 5'-3' exonuclease activity, and the Cy5 fluorescent signal cannot be generated. Cy5 fluorescent signal will be generated if both rcDNA forward primer and probe bind a continuous template comprising nt1345 to nt1454 sequentially, that is, F primer binds upstream of the probe binding position, which only occurs in rcDNA after NcoI cut. Thus, rcDNA, but not cccDNA, will be specifically detected with rcDNA primers/probe following NcoI cut.

[00124] An HBV DNA plasmid (an ADW subtype monomer cloned into the Psp65 vector) was used that was linearized by NcoI as a surrogate cccDNA molecule to validate that cccDNA was not detected by the rcDNA probe/primers. The NcoI-digested plasmid was serially diluted and tested using ABQ-duplexing dPCR containing both cccDNA and rcDNA probe/primers. Serially diluted cccDNA molecules were detected using the FAM labeled cccDNA probe; however, no positive signal was detected using the Cy5 labeled rcDNA probe, which detects the extracted rcDNA.

Verification of simultaneous detection of both cccDNA and rcDNA.

[00125] The cccDNA samples extracted with the modified Hirt method [Zhang YY, et al., *Journal of Virology*, 2005;79(15):9896-903] contain rcDNA molecules. Deproteinized rcDNA molecules were detected in the extracted cccDNA samples using Southern blot [See, e.g., Tuttleman JS, et al., *Cell*, 1986;47(3):451-60; Gao W, et al., *Journal of Virology*, 2007;81(12):6164-74; and Blondot M-L, et al., *Journal of Hepatology*, 2016;64(1):S49-S59]. Therefore, the extracted cccDNA samples were used to evaluate the ability of duplexing dPCR to detect cccDNA and rcDNA. The rcDNA was detected by a regular qPCR in the extracted cccDNA samples using a FAM labelled rcDNA probe. Both cccDNA and rcDNA were detected in the extracted cccDNA samples by ABQ-duplexing dPCR. These results not only demonstrated the ability of duplex dPCR to detect both cccDNA and rcDNA molecules, but also support the idea that nuclear rcDNA molecules can be used as HBV infection markers.

[00126] The sorted nucleus in each well is subjected to NcoI digestion. The DNA sample from a single nucleus will be mixed with dPCR solution containing both ccc and rcDNA primers/probes and loaded into microfluidic array plate (MAP) for dPCR detection. The dPCR results will be generated using QuantStudio absolute Q digital PCR software 6.0.

Threshold for FAM and Cy5 positive fluorescence

[00127] After extensively evaluating fluorescent intensity and distribution pattern among cccDNA positive and uninfected samples, the 500 value of both FAM and Cy5 fluorescent intensity was set as threshold for positive signal. However, about 5% of positive FAM signal and 1% Cy5 positive signal was detected among 576 nuclei prepared from 3 uninfected human livers (2 purchased from PheonixBio and one collected before infection) because there were some free fluorescence molecules remained in each probe despite the standard HPLC based purification of FAM and Cy5 labeled probes. Free fluorescence molecules are not associated with the quencher and can be detected at high intensity without amplification if many free fluorescence molecules are distributed in a microchamber. For example, ROX fluorescence (unlabeled and in free form) is included in ABQ dPCR master mix and distributed to each microchamber for quality control. However, the number of ROX molecules distributed to each of 20480 microchambers varied and resulted in high intensity if many molecules are distributed in one microchamber. Using the findings from the 3 uninfected livers as reference, that false positive rates for cccDNA and rcDNA by duplexing dPCR were assumed at about 5% and 1%, respectively, implying that the true number of cccDNA positive nuclei in each of 3 infected liver samples could be 5% lower than the number detected. However, they do not significantly impact the main findings of cccDNA-/rcDNA+ nuclei.

Detected rcDNA molecules in the nuclei were not non-specifically bound to nuclei

[00128] To evaluate the possibility that detected rcDNA in sorted individual nuclei was non-specifically bound to nuclear membrane during preparing nuclei suspension through homogenization that released virions and capsids into lysate, nuclei suspensions were prepared from two livers of two uninfected chimeric mice (animal ID HKB-043-020 or B20 and HKB-043-046 or B46 purchased from PheonixBio). Each nuclei suspension was divided into two vials, one was directly used for sorting, the other was mixed with lysate (containing 0.05% triton-X100) from mouse 987 who was an untreated control with average 870 copies of rcDNA/cell for 20 minutes, then removed the lysate and dissolved in isotonic buffer (154:1 TE with 0.05% triton-X100) for sorting. The sorted nuclei from 4 vials were subjected to duplexing ABQ dPCR. Table 4 shows no significant differences in detecting nuclei with Cy5 intensity ≥ 500 between two nuclei suspensions mixed with mouse 987 lysate and the two nuclei suspensions without mixing, suggesting that detected rcDNA molecules in sorted nuclei unlikely derived from released virions and capsids that non-specifically bound nuclei,

which is consistent with the concept that HBV capsid mainly utilizes cellular transport machineries, but not diffusion or passive trapping to reach nuclear membrane where interaction between nuclear localization signal on capsid and nuclear import receptors occurs [See, e.g., Blondot M-L, et al., *Journal of Hepatology*, 2016;64(1):S49-S59; and Gallucci L, et al., *Viruses*, 2017;9(1):21].

Western blot of HBsAg in serum and HBsAg and HBc proteins in liver lysates

[00129] Serum and liver samples were resolved using SDS-PAGE and HBV surface proteins were detected by western blot analysis using a rabbit polyclonal anti-HBs antibody (Virostat) [See, e.g., Hong X, et al., *Journal of Virology*, 2021;95(3):10.1128/jvi. 01695-20; and Xi J, et al., *Journal of Virology*, 2022;96(1):e01305-21].

Immunohistochemical staining of HBsAg on sections

[00130] Briefly, formalin-fixed paraffine-embedded liver sections were cut at thickness of 5µm and used for HBsAg staining after deparaffinization, proteinase K digestion, and inactivation of endogenous peroxidase with 3% hydrogen peroxide. Rabbit anti-HBs antibody (LS-C683282, LSBio) and goat anti-rabbit IgG conjugated with HRP (LS-C316062, LSBio) were used as primary and secondary antibody, respectively. DAB chromogen kit (ACH500-IFU, CP Lab Chemicals) was used for color development.

Statistical analysis

[00131] HBsAg (IU/ml) and HBV DNA (copies/ml) and antibody levels (µg/mL) are expressed as mean ± standard deviation (SD). Average intracellular rcDNA and cccDNA levels are expressed as copies/cell. The number of cells per sampling was calculated using sample weight (mg) multiplied by 1.39E5 cells per mg liver tissue [Sohlenius-Sternbeck A-K, *Toxicology in vitro*, 2006;20(8):1582-6], then normalized by factor 0.7 by considering 70% of human liver cells are hepatocytes [Mason WS, et al., *Journal of Virology*, 2010;84(16):8308-15. Epub 2010/06/04]. The formula to calculate copies/cell is listed below:

$$\text{Copies/cell} = \frac{\text{Total rcDNA or cccDNA copies in a liver sample}}{\text{Sample weight (mg)} * 1.39e5 * 0.7}$$

[00132] The procedure to demonstrate the method of the present invention included three components:

1. Assessing the impact of efficient HBV replication on the presence of cccDNA in infected cells

2. Analyzing cccDNA levels at the bulk-cell and single-nucleus level
3. Evaluating the therapeutic impact on cccDNA levels by blocking cccDNA replenishment

In vivo replication kinetics suggested that the inhibition of HBV replication was likely mediated through cccDNA clearance

[00133] To understand the kinetics of in vivo HBV replication, HBV-infected, untreated chimeric mice were euthanized on days 18, 45, 50, 52, 82, 99, 141, and 212 post inoculation (pi) for assaying kinetic serum HBsAg and HBV DNA levels and intrahepatic HBV markers. There were two infection phases (Figures 4A and 4B). The first was the phase of spread of infection to all infectible cells in which both serum HBsAg and HBV DNA levels rapidly increased after inoculation and peaked at around day 82 pi. The second was the persistent infection phase where HBV infection was maintained at a steady level. The observed kinetics of serum HBsAg and HBV DNA in this model recaptures the typical acute HBV infection that becomes persistent in humans [Keating SM, et al., *The Journal of Infectious Diseases*, 2014;209(6):845-54].

[00134] The kinetics of intracellular accumulation of viral products also comprises two phases as shown in Figure 4C. The first was the phase of a progressive increase in the accumulation of viral products. For instance, intrahepatic HBsAg levels increased from 230 copies per cell on day 18 to 110,000 copies on day 82 pi, which reflects a robust HBV replication and that the secretion of virions and subviral particles lags behind unrestricted HBV replication during the accumulation phase. The second was the phase of stopping the increase in the accumulation after the peak on day 82 pi. For instance, intracellular HBsAg levels remained at approximately 100,000 copies/cell after the peak. Average cccDNA levels fluctuated 2-fold but also ceased to rise over the next 130 days. Serum HBsAg and HBV DNA levels remained steady (Figures 4A and 4B), indicating the arrest in the accumulation was unlikely caused by an increase in the secretion of viral particles, rather by the inhibition of replication that is required to establish persistent noncytopathic infection [See, e.g., Summers J, et al., *Journal of Virology*, 1991;65(3):1310-7; and Lenhoff RJ, et al., *Journal of Virology*, 1994;68(9):5706-13].

[00135] Direct cytopathic effects of HBV infection in this model were reported [35]. Severely confluent liver necrosis with numerous infiltrates that involved up to 50% of the parenchyma on sections was observed in 4 of 18 HBV-infected livers but was absent in the remaining 14 liver sections.

[00136] As noted above, in vivo cccDNA kinetics includes two phases (Figure 4C), and cccDNA can be lost in both phases:

- i. **Amplification phase.** The total cccDNA level in the liver was amplified mainly by expanding the infection in the liver. The cccDNA pool in individual infected cells is amplified through the intracellular recycling pathway in the early phase of infection [See, e.g., Tuttleman JS, et al., *Cell*, 1986;47(3):451-60; Ko C, et al., *Journal of Hepatology*, 2018;69(6):1231-41; Summers J, et al., *Journal of Virology*, 1990;64(6):2819-24; and Tuttleman JS, et al., *Journal of Virology*, 1986;58(1):17-25]. The cccDNA level was increased from 0.00001 copies/cell (day 18 pi) to 0.35 copies/cell on day 82 pi. This was not the only event that occurred. Reaching peak infection means that all infectible cells must have been infected, as evidenced by the detection of HBsAg and HBcAg in almost all hepatocytes. **The cccDNA level is expected to be ≥ 1 copy/cell because a minimum of one copy of cccDNA is required in each infected cell.** However, the average cccDNA level at peak infection (day 82 pi) was 0.35 copies/cell, that is, approximately 1 copy of cccDNA per 3 infected cells, suggesting that cccDNA after the initial establishment may have been lost in a portion of infected cells.
- ii. **Maintenance phase.** cccDNA was maintained at a steady level (0.35–0.6 copies/cell) to maintain HBV infection at a steady level upon reaching the peak. With an average cccDNA level of <1 copy/cell, the Poisson distribution predicted that some cells may contain >1 copy/cell and other cells may contain no cccDNA. This suggests that cccDNA was spontaneously cleared from some cells during the maintenance phase. Therefore, a steady HBV infection level in the persistent infection phase is likely reached by establishing an equilibrium between the number of infected cells with cccDNA that maintain HBV replication and those that have lost cccDNA and ceased viral replication. This implies that the infected cells regulate HBV replication mainly by clearing cccDNA.

Average cccDNA levels after peak infection were <1 copies/cell

[00137] The analysis of cccDNA levels in untreated mice can be extended to obtain the range of cccDNA levels in livers. To avoid non-representative findings by a single sampling or a few samplings, each liver was routinely sampled 20 times, resulting in 220 cccDNA samples from 11 livers collected between days 82 and 253 pi. The highest average cccDNA

level was 2.5 copies/cell while the lowest was 0.003 copies/cell among the 220 cccDNA samples (Figure 5A).

[00138] The average cccDNA levels in 28 (12.7%) of the 220 cccDNA samples were >1 copies/cell, whereas there were <1 copies/cell in the remaining 192 (87.3%) cccDNA samples (Figure 5C), indicating that some cells may not contain cccDNA molecules at different time points.

[00139] The detected cccDNA level per liver varied considerably from 1.2 to 0.16 copies/cell among 11 livers (Figure 5B). An average cccDNA level > 1 copies/cell (1.2 copies/cell) was detected in only 1 of the 11 livers.

[00140] Total HBV RNA levels were assayed in four untreated livers to compare the relative RNA transcription efficiency between two phases of infection. Mice 842 and 836 were sacrificed on day 50pi and 52pi, respectively, representing the amplification phase. Mice 831 and 987 were sacrificed on day 141pi and 218 pi, representing the maintenance phase. A RT-qPCR assay was used to determine the total HBV RNA levels in 20 cccDNA and 20 rcDNA samples from each liver.

[00141] HBV RNAs were detected in both cccDNA and rcDNA samples and were more abundantly located in the cytoplasm ranging from a few hundred to eight thousand copies/cell than the nuclei ranging from a few copies to several hundred copies/cell. Generally, total HBV RNA copies in cytoplasm were 10-20 times higher than the rcDNA copies/cell. The reported frequency of HBV DNA integration in chronically infected human livers is approximately one integration per 100 cells [Mason WS, et al., *Gastroenterology*, 2016;151(5):986-98. e4] and HBV DNA integration in this model was detected but appeared transcriptionally silent [Allweiss L, et al., *Gut*, 2018;67(3):542-52], thus, the detected HBV RNAs were likely mainly transcribed from the cccDNA. The ratio of RNA copies/cell to cccDNA copies/cell was used to measure the relative efficiency of RNA transcription from cccDNA. The high ratios of average RNA copies per cell to average cccDNA copies per cell in the same samples were notable, ranging from 6107 to 7518 in mice 842 and 836 representing the amplification phase), and ranging from 4837-fold in mouse 831 to 9187-fold in mouse 987 representing the maintenance phase. These findings suggest that a single copy of cccDNA can undergo transcription over 1000 times, indicating efficient RNA transcription through repeated utilization of a single or few copies of cccDNA in HBV-infected cells. Notably, no discernible differences in relative transcription efficiency were observed between the two phases of infection.

[00142] To investigate if RNA transcription could be affected by reduced cccDNA levels, the total HBV RNA levels were examined in two additional livers (mice 813 and 838) that only contained residual cccDNA after >100-fold of cccDNA reduction in response to treatment of blocking cccDNA replenishment. Average HBV RNA levels were 5 copies/cell, >100 -fold lower than those observed in the four untreated mice, indicating the reduced amount of HBV RNA is proportional to the extent of cccDNA reduction. However, the ratios of RNA copies per cell to cccDNA copies per cell were still over 1000 (averaging from 7000 to 10000), indicating that the relative transcription efficiency remained high even in residual infected cells. In addition, there was a positive correlation between average cccDNA and HBV RNA levels (copies/cell) among 6 mice ($R^2=0.93$, Figure 6), suggesting that total HBV RNA level is largely determined by cccDNA level in the infected cells of this model. Unlike the latent phase of HIV infection at which viral RNA transcription in reservoir cells is inhibited, the efficient RNA transcription from cccDNA suggests that the suppression of RNA transcription is an unlikely mechanism to stop HBV replication and stay noncytopathic by HBV-infected cells.

cccDNA loss detected at a single nucleus level

[00143] One of the criteria used by the vendor PhoenixBio for selecting uPA/SCID chimeric mice with human livers is a liver replacement index (RI) of >70% [Tateno C, et al., *PloS ONE*, 2015;10(11):e0142145]. Among the 57 mice received, only four had RI between 76–78% and ranged between 80–93% in the remaining 53 mice, approximately 76–93% of liver cells being human liver cells. As bulk cells are routinely used for cccDNA quantification in our assay, an average of 30% non-human (mouse) liver cells was used to normalize the calculated cccDNA copies/cell. The actual number of non-human liver cells varied in each sample, which may have impacted the calculated copies/cell. Quantitative detection of cccDNA copies at the single nucleus level [Zhang YY, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 2003;100(21):12372-7] was sought to corroborate the absence of cccDNA in some infected cells.

[00144] HBV rcDNA is 100–1000-fold more abundant than cccDNA and is also expected to be delivered to the nucleus for cccDNA conversion [Tuttleman JS, et al., *Journal of Virology*, 1986;58(1):17-25]. cccDNA and rcDNA were simultaneously detected in each nucleus using Absolute Q duplexing digital PCR (ABQ-duplexing dPCR), and the detected rcDNA was used as an HBV infection marker. The strategy, principle, and controls of the simultaneous detection of cccDNA and rcDNA are described in detail herein.

[00145] Nuclei from three livers, harvested on day 141, 218 or 253pi from untreated mice 831, 987, and 907, respectively, were deposited at one nucleus per well in a 96-well plate using a BD FACS Aria II. The total number of analyses performed is listed in Table 4.

[00146] **Table 4.** Percentages of HBV-positive nuclei determined by duplexing dPCR

Mouse ID	No. wells analyzed	No. wells failed in dPCR	No. successful wells in dPCR	No. HBV positive wells (%)
831	208	4	204	66 (32)
987	192	0	192	104 (54)
907	192	11	181	61 (33)

Detected cccDNA and rcDNA at the single nucleus level

[00147] cccDNA was detected as cccDNA only or coexisting with rcDNA, and rcDNA was detected coexisting with cccDNA or rcDNA only.

cccDNA copies per nucleus

[00148] The cccDNA was detected as a single copy in most of the cccDNA-positive nuclei. Twenty (66.7%) of the 30 cccDNA-positive nuclei in mouse 831 contained only a single copy, while the remaining 10 nuclei had >1 copy, ranging from two to eight. In mouse 987, 41 (75%) of the 55 cccDNA-positive nuclei contained a single copy of cccDNA, while 14 (25%) nuclei had > 1 copy. In mouse 907, the cccDNA was detected as a single copy in 34 (77%) of the 44 cccDNA-positive nuclei, while the remaining 10 nuclei contained >1 copy ranging between 2–6 copies/nucleus. Thus, $\geq 2/3$ of the detected cccDNA-positive nuclei contained only a single cccDNA copy.

rcDNA copies per nucleus

[00149] A single copy of rcDNA was detected in 24 (57%) of the 42 rcDNA-positive nuclei in mouse 831, and the remaining 18 (43%) nuclei had 2–11 copies/nucleus. In mouse 987, a single copy of rcDNA was detected in 41 (60%) of the 66 rcDNA-positive nuclei, while the remaining 25 rcDNA-positive nuclei contained 2–8 copies/cell. In mouse 907, rcDNA was detected as a single copy in 13 (52%) of the 25 rcDNA-positive nuclei, and the remaining 12 (48%) nuclei had >1 copy of rcDNA, ranging from 2 to 19 copies/nucleus.

cccDNA-/rcDNA+ nuclei

[00150] A portion of infected cells from 27%, 47%, to 55% of the nuclei among the three livers (Table 5) had no detectable cccDNA, whereas rcDNA was detectable in the same nuclei. Infection kinetics data (Figure 4A and 4B) and the published data on HBV infection kinetics in this model [Ishida Y, et al., *Hepatology*, 2018;68(2):473-84] show that peak infection can be reached on days 82–90 pi, implying that all infectible human liver cells are likely already infected at approximately 90 days pi. HBsAg and HBcAg staining showed that most cells were positive in mice 831, 987, and 907 sections. Thus, cccDNA-/rcDNA+ cells likely represent either a loss of cccDNA from infected cells or cccDNA is yet to be formed with the rcDNA delivered from de novo infection in recently generated uninfected cells after cccDNA loss.

[00151] The detection of cccDNA-/rcDNA+ nuclei corroborated the bulk cell-based finding that cccDNA may have been spontaneously lost from a portion of the infected cells.

[00152] **Table 5.** Percentages of cccDNA and rcDNA-positive nuclei

Animal ID	Bulk cells	Positive percentage at single nucleus				
		ccc+ only	ccc + rc	Total ccc +	rc+ only	Total rc+
831	0.3	36	9	45	55	64
987	0.7	37	16	53	47	64
907	0.8	59	14	73	27	41

Average cccDNA levels lowered by >100 fold upon blocking two cccDNA replenishment pathways

[00153] The therapeutic effect of blocking de novo infection with anti-HBs antibodies on cccDNA levels was evaluated.

[00154] Two sources of anti-HBs antibody both of which are against “ad and ay” subtypes were used: exogenous mouse anti-HBs antibody (AM31509 PU-N OriGene) and endogenous anti-HBs antibody expressed by an AAV-anti-HBs vector HBVZ10 (described in detail in the Methods section). HBVZ10 can express high levels (up to 500 µg/mL) of anti-HBs antibody and was sustained at >100 µg/mL for at least 252 days in both immunocompetent and immunodeficient mice after a single injection or reached >100,000 mIU/mL if measured using WHO referenced calibrators, which are the standards for clinical report.

[00155] A total of 15 mice received anti-HBs treatment, 13 of which were injected with the AAV-anti-HBs vector HBVZ10 at a dose of 1E11 genomic copies in week 7 pi, and the remaining two with mouse anti-HBs antibody triweekly at a dose of 250 µg per injection

started in day 74 pi for consecutive 9 times. Anti-HBs antibodies were detectable in all 15 chimeric mice after treatment. However, serum HBsAg remained positive, suggesting that not all viral particles were neutralized, and de novo infection was only partially blocked.

[00156] The first set, consisting of six livers, and the second set, consisting of nine livers, were collected on days 204 and 253 pi, respectively. Each of the 15 livers was randomly sampled 20 times, and a second round of 20 samplings was performed in seven of the 15 livers, resulting in a total of 440 cccDNA samples. The average cccDNA levels in 440 samples are shown in Figure 7A. The average cccDNA levels per liver or per 20 samples are presented in Figure 5B (middle samples). The cccDNA levels >1 copies/cell were only detected in five (1.1%) of the 440 samples, which was significantly lower than the 13% of cccDNA samples in untreated mice. The mean cccDNA level in 15 mice with partially blocked de novo infection was 0.2 copies/cell, which is significantly lower ($p=0.012$) than 0.5 copies/cell in untreated mice (Figure 7B). Lower cccDNA levels in this group were supported by proportionally lower rcDNA levels (Figure 7C). These results suggest that cccDNA levels are sensitive to partial blocking of de novo infection.

[00157] The impact of completely blocking de novo infection or blocking both cccDNA replenishment pathways on cccDNA levels was then evaluated.

[00158] A complete blocking of de novo infection is marked by conversion of serum HBsAg from positive to HBsAg negative/anti-HBs positive (HBsAg-/anti-HBs+). Blocking of both cccDNA replenishment pathways was accomplished using a combination of anti-HB antibodies to block new rounds of infection with 9–12 weeks of entecavir therapy to decrease rcDNA synthesis and intracellular recycling of cccDNA. A cccDNA analysis was conducted on 17 mice that achieved HBsAg negative/anti-HBs positive status. Among these the two mice (mouse 970, which received nine injections of mouse anti-HBs antibody at triweekly interval and mouse 819, which received a single dose of 1.8×10^{12} copies of HBVZ10) underwent anti-HBs antibody monotherapy, while the remaining 15 mice were treated with a combination of anti-HBs antibody and entecavir.

[00159] Each of the 17 livers that were collected after the peak infection between days 123 and 253 pi, were randomly sampled 20 times and a second round of 20 samplings was performed for the two livers, resulting in a total of 380 cccDNA samples (right-most group in Figure 5B). Among these, 140 cccDNA samples from 7 mice were analyzed using qPCR and ABQ dPCR. All cccDNA levels were <1 copies/cell, and most were <0.01 copies/cell (Figure 8A). The mean cccDNA level in 17 mice was 0.0028 copies/cell, which is >100-fold lower ($p=0.0001$) than 0.5 copies/cell in untreated mice and significantly lower ($p=4 \times 10^{-4}$) than 0.2

copies/cell in mice with partially blocked de novo infection (Figure 5B and 8B). In addition, cccDNA was not detected in two mice after 20 samplings of each liver. This demonstrates that complete blocking of de novo infection is critical for cccDNA elimination. The addition of entecavir to anti-HBs antibody blocks the recycling pathway and makes blocking of de novo infection by anti-HBs antibody more effective because the reduced virion production lowers the potential of de novo infection. These results further support the hypothesis that cccDNA replenishment is required to maintain cccDNA levels, underlining the spontaneous clearance of cccDNA from infected cells.

[00160] Kinetic human albumin levels were similar between untreated and treated mice, suggesting that cccDNA elimination mainly resulted from the blocking of cccDNA replenishment and not from the loss of human hepatocytes in humanized livers.

cccDNA was progressively cleared upon blocking replenishment pathways in both infection phases

[00161] Experiments were further run to show the effect of progressive cccDNA clearance upon blocking cccDNA replenishment pathways.

[00162] In HBV-infected chimeric mice, rising serum HBsAg levels paralleled rising viremia following inoculation (Figure 4A and 4B), suggesting that HBV replication is mainly cccDNA-driven. Entecavir treatment is known to reduce serum HBV DNA levels but does not have a parallel impact on serum HBsAg levels, especially over a short duration [Chang TT, et al., *Hepatology*, 2010;52(3):886-93]. Thus, serum HBsAg levels, but not HBV DNA levels, in entecavir-treated mice can be used as a surrogate for intrahepatic cccDNA levels. Serum HBsAg in all 16 mice (Figure 8C and 8D) experienced a 3–5 log progressive reduction and became undetectable upon blocking the two cccDNA replenishment pathways. The blocking either started before peak infection, that is, during the cccDNA amplification phase (Figure 8C) or the cccDNA maintenance phase (Figure 8D). Additional doses of mouse anti-HBs antibody were administered to boost the levels of anti-HBs antibodies, transitioning from partial blocking to complete blocking after the peak infection. The progressive serum HBsAg reduction correlated with and likely resulted from the progressive elimination of cccDNA. This was further supported by the progressive decrease of serum HBeAg in 16 mice (green in Figure 8E and 8F). HBeAg is synthesized from pre-core mRNA, which is transcribed from cccDNA molecules [Yuh CH, et al., *Journal of Virology*, 1992;66(7):4073-84]. The cccDNA level is generally reduced by 10–100-fold when HBV infection transitions from the HBeAg-positive to HBeAg-negative phase in chronic HBV infection [See, e.g.,

Werle-Lapostolle B, et al., *Gastroenterology*, 2004;126(7):1750-8; and Laras A, et al., *Hepatology*, 2006;44(3):694-702]. Thus, a progressive serum HBeAg reduction (Figure 8E and 8F) reflects the progressive reduction of cccDNA in the liver. Both anti-HBs antibodies and entecavir mainly block cccDNA replenishment without directly eliminating cccDNA molecules, therefore, the observed cccDNA elimination was likely mediated through spontaneous clearance (non-treatment-mediated) that occurred in both phases.

>100-fold reduction in cccDNA level observed in 80 days

[00163] uPA/SCID chimeric mice with human livers are fragile and cannot withstand stressful procedures, such as serial liver resections, posing a challenge in establishing baseline cccDNA levels before treatment. Therefore, cccDNA levels from different mice with comparable serum HBsAg levels were used as a reference. Figure 5F shows that baseline HBsAg levels on days 54 and 82 reached approximately 5000IU/mL before a progressive decline in mouse 838, which was treated with anti-HBs antibody expressed by HBVZ10 and entecavir. This is comparable to that in mouse 833 who received HBVZ10 monotherapy on day 44. Mouse 833 displayed dual positivity for serum HBsAg (Figure 8G) and anti-HBs antibodies after treatment; thus, de novo infection in mouse 833 was considered partially blocked. Serum HBsAg levels remained steady at approximately 5000IU/mL from day 82 and 162 (termination day) in mouse 833. The cccDNA levels in mouse 833 was used as a reference for baseline cccDNA levels before HBsAg clearance in mouse 838. Both the intracellular HBsAg and cccDNA levels in mouse 838 were reduced by >100-fold during 80 days from day 82 to day 162 pi compared with those in mouse 833 (Figure 8H). The average reduction in cellular HBsAg levels was 423 copies/day. The cccDNA results further support that kinetic serum HBsAg levels can be used as a surrogate for cccDNA levels, and that the observed spontaneous cccDNA clearance was efficient and could be transformed into progressive cccDNA elimination upon blocking both cccDNA replenishment pathways.

Human Ki67 RNA levels in 840 cccDNA samples

[00164] Human Ki67 was chosen as a marker to examine the proliferation of human liver cells and its association with observed cccDNA loss in the humanized livers of chimeric mice. This selection was made based on the previous utilization of human Ki67 for proliferation studies within the same model [Allweiss L, et al., *Gut*, 2018;67(3):542-52]. Ki67 RNA levels were measured using RT-qPCR in existing cccDNA samples containing nuclear RNAs. Ki67 RNA levels were determined in 840 cccDNA samples isolated from 42 livers, comprising 15 untreated, 11 treated with detectable serum HBsAg, and 16 treated with progressive reduction of serum HBsAg to undetectable levels accompanied by >100-fold

reduction in cccDNA levels. In most samples (76%, 639 out of 840), Ki67 RNA levels were <0.01 copies per cell. This implies that only one copy of Ki67 RNA was detected in approximately >100 cells, or less than 1% of cells likely expressed Ki67 RNA in those samples (Figure 9A). The highest Ki67 expression levels, ranging from 0.1 to 0.28 copies per cell, were detected in 9 (1.1%) out of 840 samples. This indicates that 10 to 28 copies of Ki67 RNA were detected in 100 cells, or 10-28% of cells possibly expressed Ki67 RNA, though some cells may contain more than one copy of Ki67 RNA. The actual percentages of cells expressing Ki67 RNA may thus be lower than 10-28%.

[00165] No significant differences in average Ki67 RNA level per liver were observed among three groups of untreated, treated with detectable HBsAg, and treated with undetectable HBsAg while there were significant differences in average cccDNA levels among the three groups (Figure 9B).

[00166] If cell proliferation were a primary factor driving cccDNA loss, one would anticipate an inverse correlation between Ki67 RNA and cccDNA levels. However, no correlations were detected between Ki67 RNA and cccDNA kinetics during both the cccDNA amplification and maintenance phases, among treated and untreated mice, or across three distinct experiments utilizing different batches of chimeric mice. The maximum Ki67 RNA level per liver reached 0.08 copies per cell, indicating that approximately 8% of cells expressed detectable Ki67 RNA in one mouse (ID:823) treated with combination therapy. Ki67 RNA levels ranging from 0.1 to 0.21 copies per cell were detected in 6 out of 20 samples. However, no correlations were observed between Ki67 RNA and cccDNA levels in this particular mouse.

Serum alanine transaminase (ALT) activity

[00167] ALT activity was assessed in 9 serial serum samples, comprising 3 from untreated mice and 6 from treated mice who achieved a progressive reduction of serum HBsAg to undetectable levels alongside a >100-fold reduction of cccDNA compared to untreated mice. ALT activity exhibited fluctuations over a 6-month duration, spanning from day 21 to day 207 pi, and remained below 40 U/L in 84 out of 88 samples. In the remaining 4 samples from the treated group, collected on day 35 pi before entecavir (ETV) treatment commenced, ALT levels were borderline or slightly elevated, ranging from 40 to 54 U/L. However, these levels subsequently decreased and remained below 40 U/L during the treatment and follow-up period, spanning from day 60 to day 207 pi. The slight elevation in ALT levels at a single timepoint may simply reflect the upper limit of ALT fluctuation ranges and was deemed insignificant.

Confirmation of HBsAg reduction in both serum and liver with ELISA, western blot, and immunohistochemical staining

[00168] Two serial serum samples from untreated mice and two from treated mice, whose serum HBsAg underwent progressive reduction and became undetectable (Figure 10A and 10B), were subjected to western blot analysis. This showed similar kinetics as detected by ELISA, confirming the progressive reduction in serum HBsAg in two serial serum samples with anti-HBs treatment (Figure 10C, 10D, and 10E).

[00169] Intrahepatic HBsAg levels in seven mice was also analyzed with progressive serum HBsAg reduction, respectively. Intrahepatic HBsAg was either not detectable or was detectable at low levels by ELISA (Figure 10F), which was confirmed by western blot analysis (Figure 10G). In addition, intrahepatic HBcAg levels were analyzed by western blot in 20 liver lysates of five mice (n=4 lysates for each liver) with progressive serum HBsAg reduction. Cellular HBcAg was not detected in all 20 liver lysates (Figure 10H and 10J), supporting the concept that the progressive serum HBsAg reduction reflects intracellular HBV clearance upon blocking cccDNA replenishment.

[00170] Consistent with ELISA and western blot data, immunohistochemical staining of HBsAg in sections showed that intracellular HBsAg was reduced to undetectable or barely detectable levels among 7 mice that achieved HBsAg-/anti-HBs positive status.

In contrast to the dominant view that HBV cccDNA molecules are stable in infected cells [Alter H, et al. *Hepatology*. 2018;67(3):1127-31], spontaneous cccDNA loss was observed during both the spread of infection and persistent HBV infection phases in this study using chimeric mice with humanized livers. Moreover, cccDNA replenishment was required to maintain cccDNA levels and persistence.

[00171] The analysis of cccDNA copies at the single nucleus level shows that most infected cells contain a single copy of cccDNA; nonetheless, average intracellular HBsAg levels are accumulated at approximately 100,000 copies/cell upon reaching the peak, highlighting an extraordinary efficiency of both RNA transcription and viral protein synthesis. There are two likely scenarios. In scenario 1, the continuous presence of cccDNA after reaching the peak in infected cells is expected to continuously drive-up transcription and further increase the accumulation of viral products to an intolerable level, leading to cytopathic destruction through which cccDNA will be lost. Furthermore, unlike HIV, HBV is not known to have a latent infection phase, implying the absence of the noticeable inhibition of RNA transcription, which is also supported by high serum HBsAg levels in both HBeAg positive and negative

phases [See, e.g., Jaroszewicz J, et al., *Journal of Hepatology*, 2010;52(4):514-22; and Nguyen T, et al., *Journal of Hepatology*, 2010;52(4):508-13]. Thus, the long-term presence of cccDNA in infected cells may not be feasible under such circumstances of highly efficient RNA transcription and viral protein synthesis.

[00172] However, HBV is largely noncytopathic. In scenario 2, infected cells may respond well to the stress caused by accumulating high levels of intracellular viral products. As reported during the second phase of hepadnaviral replication, viral-envelope proteins accumulate in the infected cells. The accumulated L protein alone or in combination with M and/or S proteins inhibits further cccDNA amplification or decreases cccDNA levels [See, e.g., Lenhoff RJ, et al., *Journal of Virology*, 1994;68(7):4565-71; Gao W, et al., *Journal of Virology*, 2007;81(12):6164-74; and Lentz TB, et al., *Journal of Virology*, 2011;85(22):11916-27]. Because the highly efficient RNA transcription and viral-protein synthesis suggest the absence of efficient inhibition of these steps, clearing cccDNA appears to be the only effective option to stop HBV replication and protect cells from destruction.

[00173] Therefore, such replication-driven cccDNA loss can occur either through spontaneous clearance (Figure 11A) or cell destruction (Figure 11B). Cytopathic effects in infected primary hepatocytes and acute liver injury were also observed during in vivo infection with the duck hepatitis B virus (DHBV, a member of the Hepadnaviridae family) L protein mutant G133E, which caused a defect in enveloped virus production and increased intracellular levels of cccDNA, RNA, capsid, and rcDNA [See, e.g., Lenhoff RJ, et al., *Journal of Virology*, 1994;68(9):5706-13; and Lenhoff RJ, et al., *Hepatology*, 1999;29(2):563-71].

[00174] The timeframe from cccDNA establishment to its loss in infected cells represents one cycle of infection. The duration of cccDNA presence in infected cells is likely influenced by both HBV replication efficiency and the infected cells' ability to secrete viral particles. Slower replication or more efficient viral particle secretion likely prolongs cccDNA presence in infected cells, whereas more efficient HBV replication or inefficient viral particle secretion may accelerate cccDNA loss. A new cycle of infection commences when cccDNA is re-established through the de novo infection of cccDNA-negative cells. This cyclic feature of cccDNA indicates that persistent HBV infection is maintained by multiple cycles of de novo infection (Figure 11A).

[00175] Published studies on kinetic intracellular viral product levels showed that intracellular HBcAg reached a peak level at approximately 2–4-week in HBV infected HepG2-NTCP cells and was dependent upon the efficiency of viral particle secretion from

infected cells [See, e.g., Ko C, et al., *Journal of Hepatology*, 2018;69(6):1231-41; and König A, et al., *Journal of Hepatology*, 2019;71(2):289-300]. In in vivo DHBV infection, the intracellular L protein peaked around day 5 pi [Zhang YY, et al., *Journal of Virology*, 2004;78(3):1195-201], suggesting that intracellular hepadnaviral infection may peak at 1–4 weeks pi on average. In this timeframe, after which cccDNA clearance may occur, each cycle of HBV infection likely ends with cccDNA loss within a few weeks at the individual cell level. However, HBV infection at the liver level without sufficient anti-HBs antibodies may last for years or decades because there are always new cycles of infection, whereas early cycles of infection end following cccDNA loss.

[00176] Previous studies have suggested that cccDNA molecules may be lost during cell division [See, e.g., Allweiss L, et al., *Gut*, 2018;67(3):542-52; and Zhang YY, et al., *Proceedings of the National Academy of Sciences of the United States of America*, 2003;100(21):12372-7]. Thus, spontaneous cccDNA loss could also be caused by random human liver-cell proliferation. However, our findings indicate infrequent expression of human Ki67 RNA, with levels < 0.01 copies per cell detected in 76% of 840 samples. Moreover, no correlations were observed between Ki67 RNA and cccDNA levels. These results suggest minimal human hepatocyte turnover. These findings regarding Ki67 RNA levels align with the observations of the mouse provider (PhoenixBio, personal communication) that the proliferation of human liver cells is negligible after the completion of human liver growth and are consistent with Ki67 immunostaining results, which revealed 3% Ki67-positive cells in liver sections prepared 100 days after hepatocyte transplantation in the same uPA/SCID mouse model [Allweiss L, et al., *Gut*, 2018;67(3):542-52]. The Ki67 RNA data align with normal serum ALT levels among both treated and untreated mice and the steady levels of serum human albumin. Consequently, the low-level proliferation of human liver cells observed in humanized mice is unlikely to be a significant driver of cccDNA loss in these data.

[00177] The following are exemplary embodiments of the present invention:

[00178] Embodiment 1. A method of curing chronic hepatitis B infection in humans, comprising:

administering to a subject in need thereof, an effective amount of one or both of an exogenous anti-HBs antibody or an anti-HBs antibody producing vector to reduce cellular and blood hepatitis B surface antigen (HBsAg), by providing a sustained elevated level of anti-HBs antibody in the subject.

- [00179] Embodiment 2. The method of Embodiment 1, wherein the reduction of cellular and blood HBsAg occurs (i) with blocking of de novo infection mediated cccDNA replenishment or (ii) without direct inhibition of HBsAg synthesis, or both (i) and (ii).
- [00180] Embodiment 3. The method of one of Embodiments 1 or 2, wherein the anti-HBs antibody has specificity against the “a” determinant of HBsAg.
- [00181] Embodiment 4. The method of Embodiment 3, wherein the anti-HBs antibody has specificity against human hepatocyte attachment site in the “a” determinant of HBsAg.
- [00182] Embodiment 5. The method of Embodiment 4, wherein the anti-HBs antibody blocks de novo infection through blocking HBV particles (virions and subviral particles) from attaching to human hepatocytes.
- [00183] Embodiment 6. The method of Embodiment 1, wherein the sustained elevated level of anti-HBs antibody is an amount of 100mIU/ml or higher for a period of 3 months or longer.
- [00184] Embodiment 7. The method of Embodiment 6, wherein the sustained elevated level of anti-HBs antibody is an amount of 1,000mIU/ml or higher for a period of 3 months or longer.
- [00185] Embodiment 8. The method of Embodiment 7, wherein the sustained elevated level of anti-HBs antibody is an amount of 10,000mIU/ml or higher for a period of 3 months or longer.
- [00186] Embodiment 9. The method of Embodiment 8, wherein the sustained elevated level of anti-HBs antibody is an amount of 100,000 mIU/ml or higher for a period of 3 months or longer.
- [00187] Embodiment 10. The method of any one of Embodiments 1 to 9, wherein the administration is a single or multi-administration of the exogenous anti-HBs antibody or a single administration of the anti-HBs antibody producing vector.
- [00188] Embodiment 11. The method of any one of Embodiments 1 to 10, wherein cellular and blood HBsAg is reduced through blocking of de novo infection mediated cccDNA replenishment.
- [00189] Embodiment 12. The method of Embodiment 12, wherein the effective amount of exogenous anti-HBs antibody or an anti-HBs antibody producing vector is an amount to maintain a level of anti-HBs antibody sufficient to effectively and completely block de novo infection mediated cccDNA replenishment in the presence of or absence of other anti-HBV drugs.

- [00190] Embodiment 13. The method of any one of Embodiments 1 to 12, wherein the administration is administration of an effective amount of the anti-HBs antibody.
- [00191] Embodiment 14. The method of any one of Embodiments 1 to 12, wherein the administration is administration of an effective amount of the anti-HBs antibody producing vector, which provides an endogenous production of anti-HBs antibody in the subject.
- [00192] Embodiment 15. The method of Embodiment 14, wherein the administration is a single dose administration of the anti-HBs antibody producing vector, in an amount of 1E11 copies or more.
- [00193] Embodiment 16. The method of Embodiment 15, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 2E11 copies or more.
- [00194] Embodiment 17. The method of Embodiment 16, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 1E12 copies or more.
- [00195] Embodiment 18. The method of Embodiment 16, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 3E12 copies or more.
- [00196] Embodiment 19. The method of any one of Embodiments 14 to 18, wherein the anti-HBs antibody producing vector is an AAV vector selected from the group consisting of HBVZ10, HBVZ20, HBVZ30, HBVZ40, HBVZ50, HBVZ60, HBVZ70, HBVZ80, and HBVZ90.
- [00197] Embodiment 20. The method of any one of Embodiments 14 to 18, wherein the anti-HBs antibody producing vector is a viral vector, a non-viral vector, or a nanoparticle.
- [00198] Embodiment 21. The method of Embodiment 20, wherein the anti-HBs antibody producing vector can be administered singly or in combinations thereof.
- [00199] Embodiment 22. The method of Embodiment 1, wherein the exogenous anti-HBs antibody is co-administered with a human anti-HBs antibody, nanoantibody or antibody fragments.
- [00200] Embodiment 23. The method of Embodiment 1, wherein the administration produces a reduction in blood HBsAg which can be progressive or precipitous, in either case having blood HBsAg reduced by at least 1-5 log.
- [00201] Embodiment 24. The method of Embodiment 23, wherein the administration produces a reduction in blood HBsAg which can be progressive or precipitous, in either case having blood HBsAg reduced to a level of <0.05IU/ml.

[00202] Embodiment 25. The method of any one of Embodiments 1 to 24, wherein the method is a monotherapy.

[00203] Embodiment 26. The method of any one of Embodiments 1 to 24, wherein the method is a combinational therapy with one or more additional HBV drugs that inhibit intracellular HBV DNA, HBV RNA or/and viral proteins synthesis.

[00204] Embodiment 27. The method of Embodiment 26, wherein the one or more additional HBV drugs are one or more members selected from the group consisting of reverse transcription inhibitors, capsid inhibitors, cccDNA inhibitors, RNA transcription inhibitors, viral protein synthesis inhibitors, entry inhibitors, interferons, therapeutic vaccines, immune checkpoint inhibitors, and immunoregulatory drugs.

[00205]

[00206] Numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

CLAIMS:

1. A method of curing chronic hepatitis B infection in humans, comprising:
administering to a subject in need thereof, an effective amount of one or both of
an exogenous anti-HBs antibody or an anti-HBs antibody producing vector to reduce
cellular and blood hepatitis B surface antigen (HBsAg), by providing a sustained
elevated level of anti-HBs antibody in the subject.
2. The method of claim 1, wherein the reduction of cellular and blood HBsAg occurs (i)
with blocking of de novo infection mediated cccDNA replenishment or (ii) without
direct inhibition of HBsAg synthesis, or both (i) and (ii).
3. The method of one of claims 1 or 2, wherein the anti-HBs antibody has specificity
against the “a” determinant of HBsAg.
4. The method of claim 3, wherein the anti-HBs antibody has specificity against human
hepatocyte attachment site in the “a” determinant of HBsAg.
5. The method of claim 4, wherein the anti-HBs antibody blocks de novo infection through
blocking HBV particles (virions and subviral particles) from attaching to human
hepatocytes.
6. The method of claim 1, wherein the sustained elevated level of anti-HBs antibody is an
amount of 100mIU/ml or higher for a period of 3 months or longer.
7. The method of claim 6, wherein the sustained elevated level of anti-HBs antibody is an
amount of 1,000mIU/ml or higher for a period of 3 months or longer.
8. The method of claim 7, wherein the sustained elevated level of anti-HBs antibody is an
amount of 10,000mIU/ml or higher for a period of 3 months or longer.
9. The method of claim 8, wherein the sustained elevated level of anti-HBs antibody is an
amount of 100,000 mIU/ml or higher for a period of 3 months or longer.
10. The method of any one of claims 1 to 9, wherein the administration is a single or multi-
administration of the exogenous anti-HBs antibody or a single administration of the
anti-HBs antibody producing vector.
11. The method of any one of claims 1 to 10, wherein cellular and blood HBsAg is reduced
through blocking of de novo infection mediated cccDNA replenishment.
12. The method of claim 12, wherein the effective amount of exogenous anti-HBs antibody
or an anti-HBs antibody producing vector is an amount to maintain a level of anti-HBs

antibody sufficient to effectively and completely block de novo infection mediated cccDNA replenishment in the presence of or absence of other anti-HBV drugs.

13. The method of any one of claims 1 to 12, wherein the administration is administration of an effective amount of the anti-HBs antibody.
14. The method of any one of claims 1 to 12, wherein the administration is administration of an effective amount of the anti-HBs antibody producing vector, which provides an endogenous production of anti-HBs antibody in the subject.
15. The method of claim 14, wherein the administration is a single dose administration of the anti-HBs antibody producing vector, in an amount of 1×10^{11} copies or more.
16. The method of claim 15, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 2×10^{11} copies or more.
17. The method of claim 16, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 1×10^{12} copies or more.
18. The method of claim 16, wherein the single dose administration of the anti-HBs antibody producing vector is in an amount of 3×10^{12} copies or more.
19. The method of any one of claims 14 to 18, wherein the anti-HBs antibody producing vector is an AAV vector selected from the group consisting of HBVZ10, HBVZ20, HBVZ30, HBVZ40, HBVZ50, HBVZ60, HBVZ70, HBVZ80, and HBVZ90.
20. The method of any one of claims 14 to 18, wherein the anti-HBs antibody producing vector is a viral vector, a non-viral vector, or a nanoparticle.
21. The method of claim 20, wherein the anti-HBs antibody producing vector can be administered singly or in combinations thereof.
22. The method of claim 1, wherein the exogenous anti-HBs antibody is co-administered with a human anti-HBs antibody, nanoantibody or antibody fragments.
23. The method of claim 1, wherein the administration produces a reduction in blood HBsAg which can be progressive or precipitous, in either case having blood HBsAg reduced by at least 1-5 log.
24. The method of claim 23, wherein the administration produces a reduction in blood HBsAg which can be progressive or precipitous, in either case having blood HBsAg reduced to a level of $<0.05 \text{ IU/ml}$.
25. The method of any one of claims 1 to 24, wherein the method is a monotherapy.
26. The method of any one of claims 1 to 24, wherein the method is a combinational therapy with one or more additional HBV drugs that inhibit intracellular HBV DNA, HBV RNA or/and viral proteins synthesis.

27. The method of claim 26, wherein the one or more additional HBV drugs are one or more members selected from the group consisting of reverse transcription inhibitors, capsid inhibitors, cccDNA inhibitors, RNA transcription inhibitors, viral protein synthesis inhibitors, entry inhibitors, interferons, therapeutic vaccines, immune checkpoint inhibitors, and immunoregulatory drugs.