Molecular Therapy
Methods & Clinical Development
Original Article

HBVZ10, an AAV8 vector-based new HBV therapy candidate for cccDNA elimination

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Eliminating hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) remains a major challenge, requiring innovative treatment strategies and drug candidates. Clinical studies reveal that wild-type HBV in the blood is often replaced by gradually rising mutant populations. This replacement reflects loss of the early cccDNA pool, then replenished predominantly through *de novo* infection. We proposed that blocking *de novo* infection is essential for cccDNA elimination and establishing a finite HBV treatment regimen. To achieve sustained inhibition of *de novo* cccDNA replenishment, we developed HBVZ10, a gene therapy candidate that utilizes an optimized adeno-associated virus (AAV) vector 8 to deliver human anti-HBs antibody genes into muscle cells for expanding endogenous anti-HBs antibody production capacity. HBVZ10 expression and therapeutic function were evaluated in uPA/SCID chimeric mice. HBVZ10 therapy achieved sustained antibody expression of ≥100,000 mIU/mL for at least 200 days following a single dose administration. Combining HBVZ10 with intracellular replication inhibitor entecavir resulted in >100-fold reductions in cccDNA within a few months, accompanied by progressive reductions in serum HBeAg and HBsAg to undetectable levels. These findings establish preclinical evidence of HBVZ10 as a novel gene therapy candidate and support a paradigm-shifting cccDNA elimination strategy.

INTRODUCTION

Hepatitis B virus (HBV) chronically infects 292 million people globally[1] and 1.1 million patients die of HBV-related diseases each year.[2]

Establishment and persistence of HBV infection require the formation and maintenance of covalently closed circular DNA (cccDNA) in the nuclei of infected cells.[3,4] HBV cccDNA functions as the template for transcription of viral mRNAs, including pregenomic RNA (pgRNA), precore RNA, S RNAs, and HBx RNA.[5] The pgRNA encodes the viral core and polymerase proteins and is also packaged into nucleocapsids, where it serves as the template for reverse transcription to generate the minus strand of relaxed circular DNA (rcDNA). The precore RNA encodes the hepatitis B e antigen (HBeAg),[6] which is secreted into blood and serves as an important HBV infection marker.[7] Clinically, chronic HBV infection (CHB) is often classified into HBeAg-positive and HBeAg-negative phases. Loss of detectable serum HBeAg generally indicates that ≥90% of intrahepatic rcDNA and cccDNA load has been cleared.[8,9]

The S RNAs (PreS1 and PreS2/S) encode the viral envelope proteins: large (L), middle (M), and small (S), with the small envelope protein being the most abundantly expressed.[10] These envelope proteins play multiple roles in the HBV life cycle: (1) they are required for virion production by envelopment of mature nucleocapsids[11]; (2) intracellular levels of envelope proteins regulate nucleocapsid recycling pathways for cccDNA replenishment[12–15]; and (3) the small envelope protein constitutes the major portion of hepatitis B surface antigen (HBsAg), which is secreted into the bloodstream.[16] Serum HBsAg not only serves as a key marker for HBV infection but also contributes to HBV persistence by depleting endogenous anti-HBs antibodies, thereby facilitating new rounds of infection and replenishment of the cccDNA pool.

Intracellular rcDNA levels reflect the extent of HBV replication and intrahepatic infection load.[17,18] The rcDNA is intracellularly supplied to replenish cccDNA pool, with higher rcDNA levels generally correlating with higher intrahepatic cccDNA levels,[4] although most rcDNA molecules are enveloped to form virions, serving extracellular cccDNA replenishment pathway. Thus, rcDNA, either intracellularly synthesized or released into the infected cells from new rounds of infection, is critical for maintaining cccDNA levels. Currently, approved intracellular HBV inhibitors such as nucleotide or nucleoside analogs (NAs) mainly target rcDNA synthesis.[19]

A major challenge to cure CHB is to eliminate HBV cccDNA in infected livers. The cccDNA is conventionally viewed as stable.[20] Current cccDNA elimination strategies primarily focus on directly targeting cccDNA[20] or killing infected cells.[21]

Received 9 June 2025; accepted 14 November 2025;
https://doi.org/10.1016/j.omtm.2025.101646.

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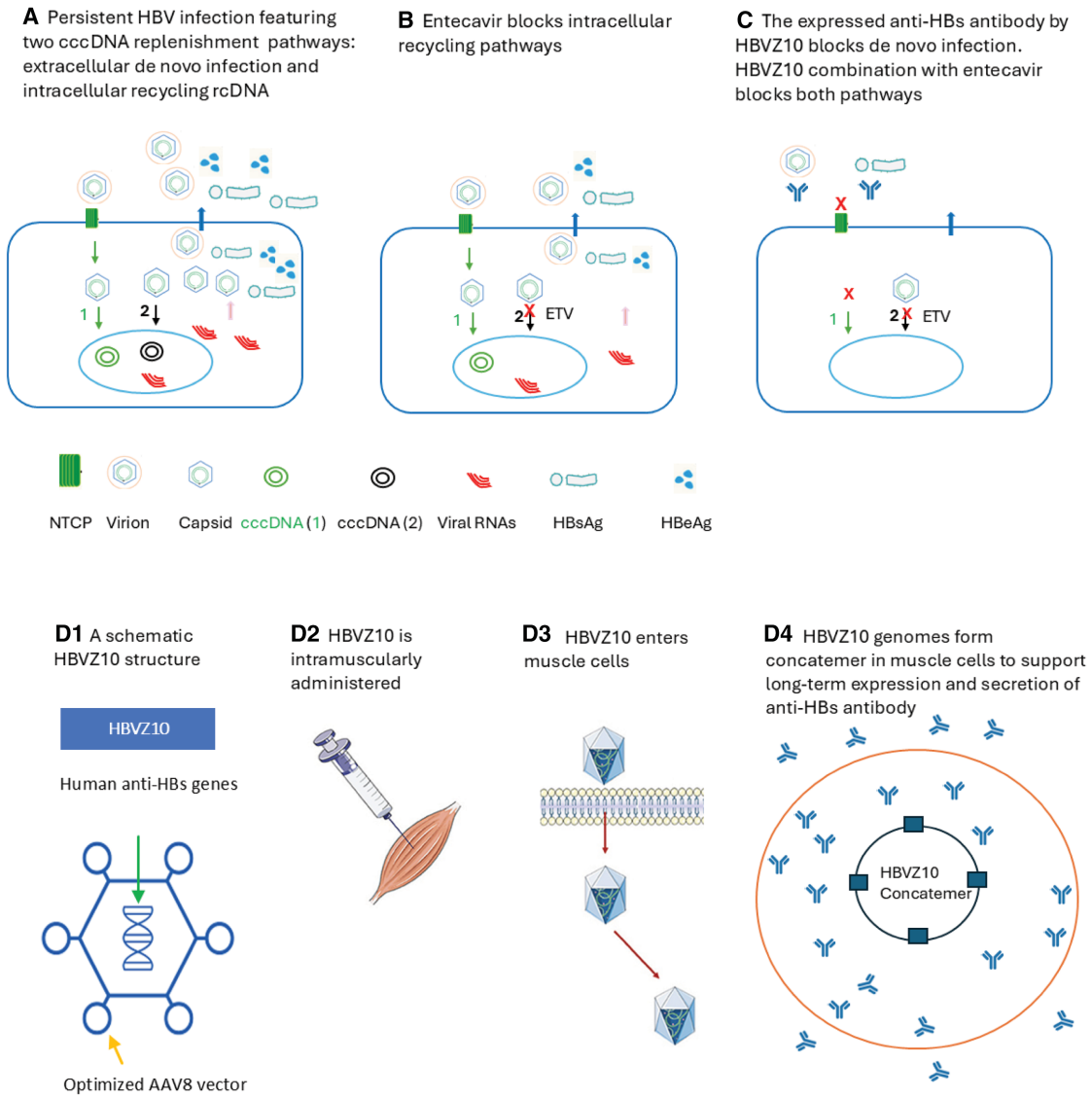


Figure 1. Schematic illustration of the mechanisms underlying cccDNA persistence in infected cells and sustained HBVZ10 expression in transduced cells
(A) The cccDNA pool in infected cells is maintained through two major replenishment pathways: intracellular recycling (black) and extracellular *de novo* infection (green). (B) The intracellular recycling pathway can be blocked by nucleos(t)ide analogs such as entecavir (ETV). (C) The anti-HBs antibody expressed by HBVZ10 blocks the extracellular *de novo* infection pathway. Combination therapy with HBVZ10 and ETV thus inhibits both cccDNA replenishment routes. (D1) Schematic representation of the HBVZ10 construct. (D2–D4) Following intramuscular injection, HBVZ10 enters and transduces skeletal muscle cells. Multiple copies of the HBVZ10 genome within the same nucleus form concatemers, enabling long-term and stable expression of anti-HBs antibody.

However, clinical evidence shows a dynamic evolution of the cccDNA population in CHB. For instance, wild-type (WT) viral population in serum or cccDNA in the liver can be cleared and replaced with mutant populations in both untreated and treated conditions.[22–26] Replacement of the initially dominant WT population suggests that cccDNA pool experiences loss and replenishment in infected livers.

Consistent with clinical observation, we also detected cccDNA loss in infected cells of humanized livers of chimeric mice through analyzing bulk cells, single nuclei, and single HBsAg positive cells. However, cccDNA is largely maintained at steady levels after peak infection despite cccDNA loss as the loss is continuously counterbalanced by ongoing replenishment.[27] The cccDNA replenishment is mainly mediated by two pathways: intracellular recycling and extracellular *de novo* infection (Figure 1A).[28] The recycling pathway is inhibited at a late phase of replication where the accumulated high levels of envelope protein promote the formation and secretion of virions utilizing the synthesized rcDNA.[13,29] The secreted virions, in the absence of sufficient amount of anti-HBs antibodies, in turn,

drive *de novo* infection, which serves as a dominant pathway for cccDNA replenishment. Consistent with these findings using duck hepatitis B virus (DHBV) model,[13,29] it has also been reported that *de novo* infection is the primary mechanism for cccDNA replenishment in the urokinase-type plasminogen activator/severe combined immunodeficient (uPA/SCID) chimeric mouse model.[30] Clearly, the HBV life cycle exploits intracellular envelope protein levels to shift cccDNA replenishment from an intracellular pathway to the dominant extracellular route as replication progresses. In addition, HBV promotes *de novo* infection by favoring the conversion of rcDNA into virions for secretion[12,13] and by producing and secreting large amount of HBsAg into the bloodstream that deplete endogenous anti-HBs antibodies. These observations and understanding suggest that blocking *de novo* infection is essential to effectively halt cccDNA replenishment.

Endogenous anti-HBs antibody is a natural extracellular blocker for HBV *de novo* infection but is largely undetectable in patients with CHB.[31] This suggests that the endogenous capacity for anti-HBs antibody production is insufficient to counteract the massive pool of HBsAg. Exogenous human anti-HBs monoclonal antibodies are currently under evaluation in clinical trials.[32–34] However, exogenous anti-HBs antibodies require repeated administration, and their efficiency wanes as antibody levels decline over time. A lack of durable efficacy may lead to resuming new rounds of infection and reversing the gain in HBV elimination.

Current HBV antiviral treatments require long-term medication and HBV relapses frequently occur, marked by accelerating spread of infection following treatment cessation.[35] This relapse can be prevented by maintaining sufficient levels of circulating anti-HBs antibodies, as demonstrated in HIV clinical trials where HIV viremia was suppressed through infusing sustained high levels of broad neutralizing antibodies after interruption of antiretroviral therapy.[36,37] Therefore, maintaining high and durable levels of anti-HBs antibodies may also enable to shorten infinite NA therapy to a finite treatment duration, potentially allowing medication withdrawal without risking relapse. Such a therapy strategy could benefit approximately 5 million CHB patients currently on long-term NA therapy.[1]

However, it is difficult to maintain durable therapeutic levels without continuous infusion of exogenous antibodies. Thus, supplementing current NA regimens with continuous infusion of exogenous anti-HBs antibodies is unlikely to shorten the duration of HBV therapy. A novel strategy that enables sustained, endogenous expression of anti-HBs antibodies could overcome this critical limit for shortening HBV therapy.

Adeno-associated virus (AAV) vector-based gene therapy offers a promising platform for delivering the genes required to expand and sustain endogenous production of anti-HBs antibody. For this purpose, we developed a new HBV therapy candidate that uses an optimized adeno-associated virus serotype 8 (AAV8) vector[38–41] to deliver human anti-HBs antibody genes, called AAV anti-HBs vector. The leading candidate is HBVZ10. When injected into muscle tissue, it can turn muscle cells into anti-HBs antibody producing cells (Figure 1D), bypassing the traditional reliance on B cells for antibody production.

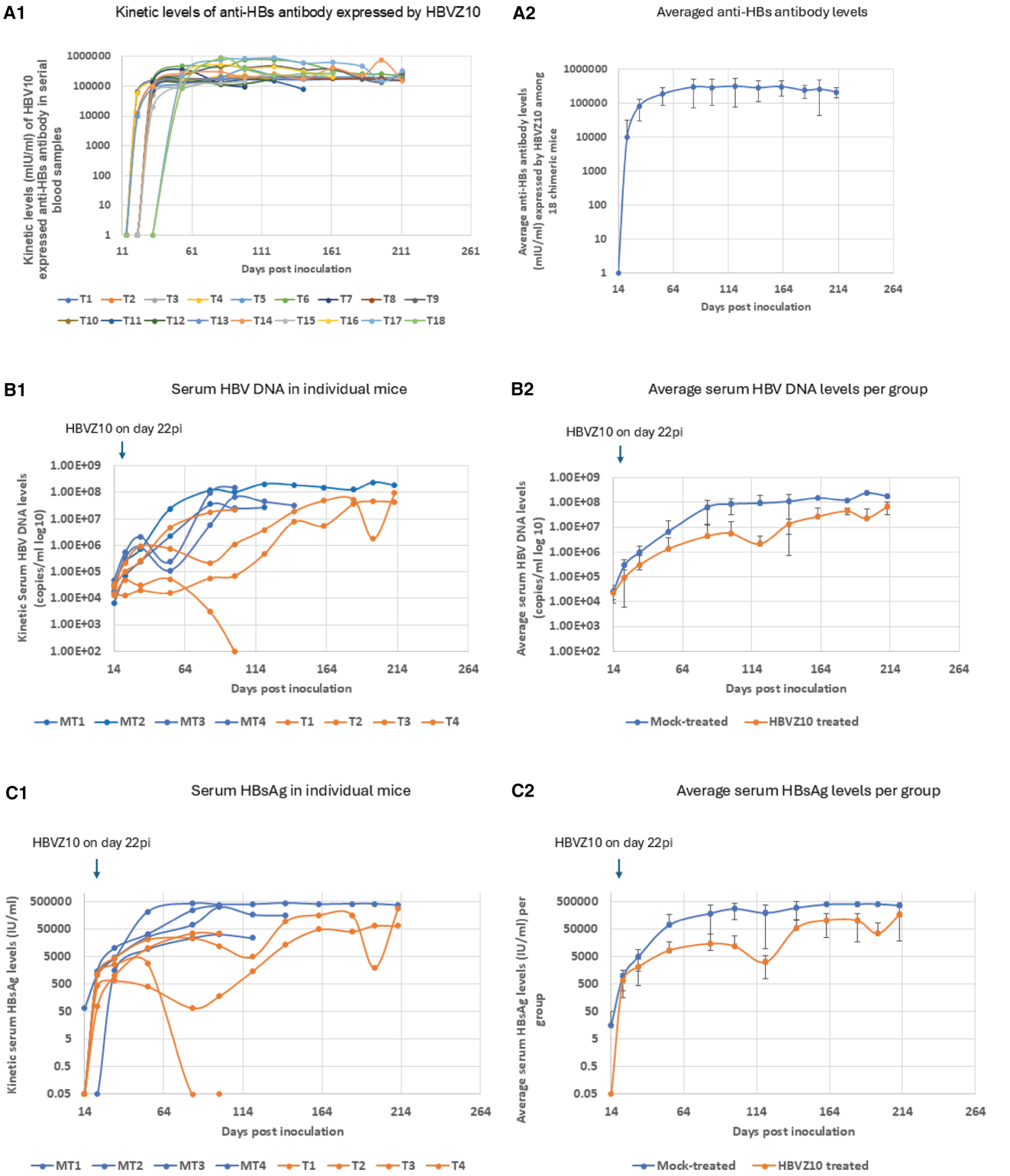
In this study, we demonstrate that a single dose of HBVZ10 achieves sustained anti-HBs antibody levels exceeding 100,000 mIU/mL for over 200 days in chimeric mice. When combined with entecavir (ETV), the treatment reproducibly reduces cccDNA levels by more than 100-fold within a few months, accompanied by a progressive decline in serum HBsAg and HBeAg to undetectable levels. These findings underscore the therapeutic potential of HBVZ10 as a promising candidate and validate the concept of this novel cccDNA elimination strategy in the uPA/SCID chimeric mouse model.

RESULTS

HBVZ10 construct and the specificity of expressed anti-HBs antibody

The late Dr. David Baltimore's laboratory optimized an AAV8 vector to enhance antibody expression,[38–41] achieving up to a 10-fold increase in antibody production compared with unoptimized AAV vectors.[42] This optimized vector already contains sequences encoding the constant regions of human IgG1 γ heavy and λ light chains (Figure S1A). The chick β action promotor in the vector is aimed to express antibodies in skeletal muscle cells (Figure S1B). HBVtech has generated nine AAV vector constructs by cloning different sets of sequences encoding the variable regions of the γ heavy and λ light chains of human anti-HBs antibody genes. Eight of these constructs demonstrated the ability to sustain high levels (up to 500 μg/mL) of anti-HBs antibodies *in vivo* (data not shown). The lead candidate currently in development is HBVZ10.

HBsAg contains a major antigenic determinant known as the "a" determinant and can be further divided into two mutually exclusive subtypes, ad and ay. These subtypes of the "a" antigenic determinant in HBsAg form four distinct serotypes, ADR, ADW, AYR, and AYW.[43,44] These four serotypes not only determine the specificity of elicited anti-HBs antibodies but also represent epidemiological patterns with characteristic geographic distributions. For example, ADR and ADW are most prevalent in East and Southeast Asia,[45–48] regions that together account for nearly half of the world's chronically HBV-infected population.[1] The *in vivo* expression capacity of HBVZ10 was first evaluated in a group of 5 CD1 mice, each of them was intramuscularly administered with 1E10 copies of HBVZ10. A single dose (1E10) of HBVZ10 transduction resulted in sustained high levels (up to 400 μg/mL) of antibody expression for at least 26 weeks and it also shows dose-dependent expression levels (Figure S2A). The HBVZ10 expressed anti-HBs antibody targets the "a" determinant of HBsAg.[43] To evaluate the *in vitro* HBsAg-binding capacity of the expressed anti-HBs antibody by HBVZ10, serum samples were serially diluted and added to 96-well plates coated with each of the four HBsAg serotypes. The expressed antibody exhibited preferential binding to the ADR and ADW serotypes. For



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example, anti-HBs antibodies in day 63 serum samples (corresponding to the expression plateau) from two randomly selected mice (IDs 493 and 452) showed approximately 50% higher OD (optical density) values for binding to HBsAg ADR and ADW compared with AYR and AYW (Figure S2B). Consistently, the antibody demonstrated IC_{50} values of 5.7 ng/mL and 14 ng/mL for neutralization of *in vitro* infection with ADR/ADW and AYR/AYW serotypes, respectively. The broad binding reactivity across all four HBsAg serotypes, together with the favorable IC_{50} values, supports the selection of HBVZ10 as the lead therapeutic candidate for further development.

In vivo expression level, duration, and function of HBVZ10

In addition to the evaluation of HBVZ10 expression in CD1 immunocompetent mice (Figure S2A), the expression capacity of HBVZ10 was also evaluated in immunodeficient mice, i.e., uPA/SCID mice. Following a single injection of HBVZ10 at a dose of 2.5E10 copies or higher, the expressed anti-HBs antibody could reach levels exceeding 100,000 mIU/mL (Figures 2A and 2B). This level is approximately 10,000-fold higher than the level of 12 mIU/mL, which is considered protective after HBV vaccination,[49] more than 100-fold higher than the average level of 306 mIU/mL detected among patients with CHB who have achieved a functional cure[50] and more than 20- to 100-fold higher than the targeted levels for hepatitis B immunoglobulin (HBIG) prophylaxis in the 1st week, 2–12 weeks, and beyond 12 weeks after liver transplantation.[51] Additionally, the expressed anti-HBs antibody was sustained for at least 200 days (Figures 2A and 2B). Such a long-term anti-HBs antibody expression from HBVZ10 is structurally facilitated by forming highly stable AAV genome concatemers in the nuclei of transduced muscle cells[52,53] (Figure 1D4).

The *in vivo* function of the anti-HBs antibody expressed by HBVZ10 was demonstrated in a group of mice ($n = 5$), which received a single dose of 1.8E11 vector copies of HBVZ10 on day 22 post infection (PI) as monotherapy. One mouse was removed from analysis due to death on day 60 PI, likely caused by continuous weight loss. In the remaining animals, HBVZ10-expressed anti-HBs antibody levels ranged from 170,000 to 340,000 mIU/mL and were sustained at steady levels thereafter. HBVZ10 monotherapy slowed HBV spreading, delayed the infection peak, and reduced both serum HBV DNA and HBsAg levels by up to 1–3 logs at most time points in mice T1 and T3 compared to the untreated 4 mice (Figures 2B and 2C). Notably, in mouse T2, both HBV DNA and HBsAg levels declined to undetectable levels (Figures 2B and 2C). However, such a complete response to anti-HBs antibody monotherapy, as observed in mouse T2, appears to be infrequent.

Evaluation of HBVZ10 therapeutic function in blocking cccDNA replenishment *after* infection

Partially blocking cccDNA replenishment

We first evaluated the therapeutic effect of blocking cccDNA replenishment with HBVZ10 as monotherapy. A total of 13 uPA/SCID chimeric mice with humanized livers were infected with HBV for 7 weeks, and then a single dose (1E10 copies) of HBVZ10 was intramuscularly administered on day 49 PI (Figure 3A). As mock-treated control, 4 mice were given 1E10 copies of vector expressing malaria antibody. Additional 4 mice were treated with exogenous mouse anti-HBs antibody (AM31509 PU-N OriGene) as a therapy control. Given that it takes an average of 3 weeks for the expressed anti-HBs antibody to reach plateau levels, the triweekly injections of mouse anti-HBs antibody were started on day 74 PI for up to 9 consecutive times (Figure 3A).

We first observed that the minimal anti-HBs level likely required for serologically completely blocking *de novo* infection was ≥100,000 mIU/mL in mice with serum HBsAg levels around 5,000 IU/mL in 4 mice treated with mouse anti-HBs antibody. As shown in Figures 3B–3E, once serum anti-HBs antibody levels reached or exceeded 100,000 mIU/mL, serum HBsAg became undetectable. Conversely, serum HBsAg reappeared when anti-HBs levels fell below 100,000 mIU/mL (Figures 3B and 3C). An exception was observed in mouse 970: although its anti-HBs antibody level declined below 100,000 mIU/mL at the final time point, serum HBsAg remained undetectable. A possible explanation is that HBsAg had already been suppressed to undetectable levels for over 5 months in this mouse, and the reduced anti-HBs antibody level remained sufficient to maintain control of the HBV infection. This observation suggests that the required anti-HBs antibody threshold may depend on the existing serum HBsAg burden.

Kinetic serum HBsAg levels peaked around day 99 (PI) and remained stable (≥50,000 IU/mL) in mice treated with 1E10 copies of the vector expressing a malaria antibody (Figure 3F). The expressed anti-HBs antibody levels in the 13 mice treated with 1E10 copies of HBVZ10 were below 100,000 mIU/mL, with substantial variability across individuals (Figures 3G and 3H) due to relatively low antibody levels. This variability resulted in different impacts

Figure 2. *In vivo* expression levels and duration of a single dose of HBVZ10 and therapeutic effects of HBVZ10 monotherapy in uPA/SCID chimeric mice with humanized livers

(A1) Kinetic serum anti-HBs antibody levels (mIU/mL) after a single injection of HBVZ10 at a dose of 2.5E10, 1.8E11 or 7E11 genomic copies on day 11, 22 or 44 post inoculation (PI) among 18 individual chimeric mice or replicates. Each of 18 replicates is labeled as T1, T2 … T18. (A2). Average anti-HBs antibody levels (mIU/mL) per group. Note: The chimeric mice lack functional T and B cell immunity and therefore do not produce anti-HBs antibodies in response to HBV infection. A pretreatment serum sample from each replicate was used as a negative control, and all pretreatment anti-HBs antibody levels were plotted as 1 mIU/mL for consistency. (B1) Kinetic serum HBV DNA levels (copies/mL log10) among 8 individual mice. Four of them were mock treated with 1.8E11 copies of AAV vector expressing malaria antibody (blue, labeled as MT1-MT4) and the remaining 4 mice with HBVZ10 monotherapy at a dose of 1.8E11 copies on day 22 PI (orange, labeled as T1-T4). (B2) Average serum HBV DNA levels (copies/mL log10) per group. (C1) Kinetic serum HBsAg levels (IU/mL) among the same 8 individual mice as described in B1. (C2) Average serum HBsAg levels (IU/mL) per group. The lower limit of quantification for serum HBV DNA and HBsAg is 100 copies/mL and 0.05IU/mL, respectively. Error bars: standard deviation (SD).

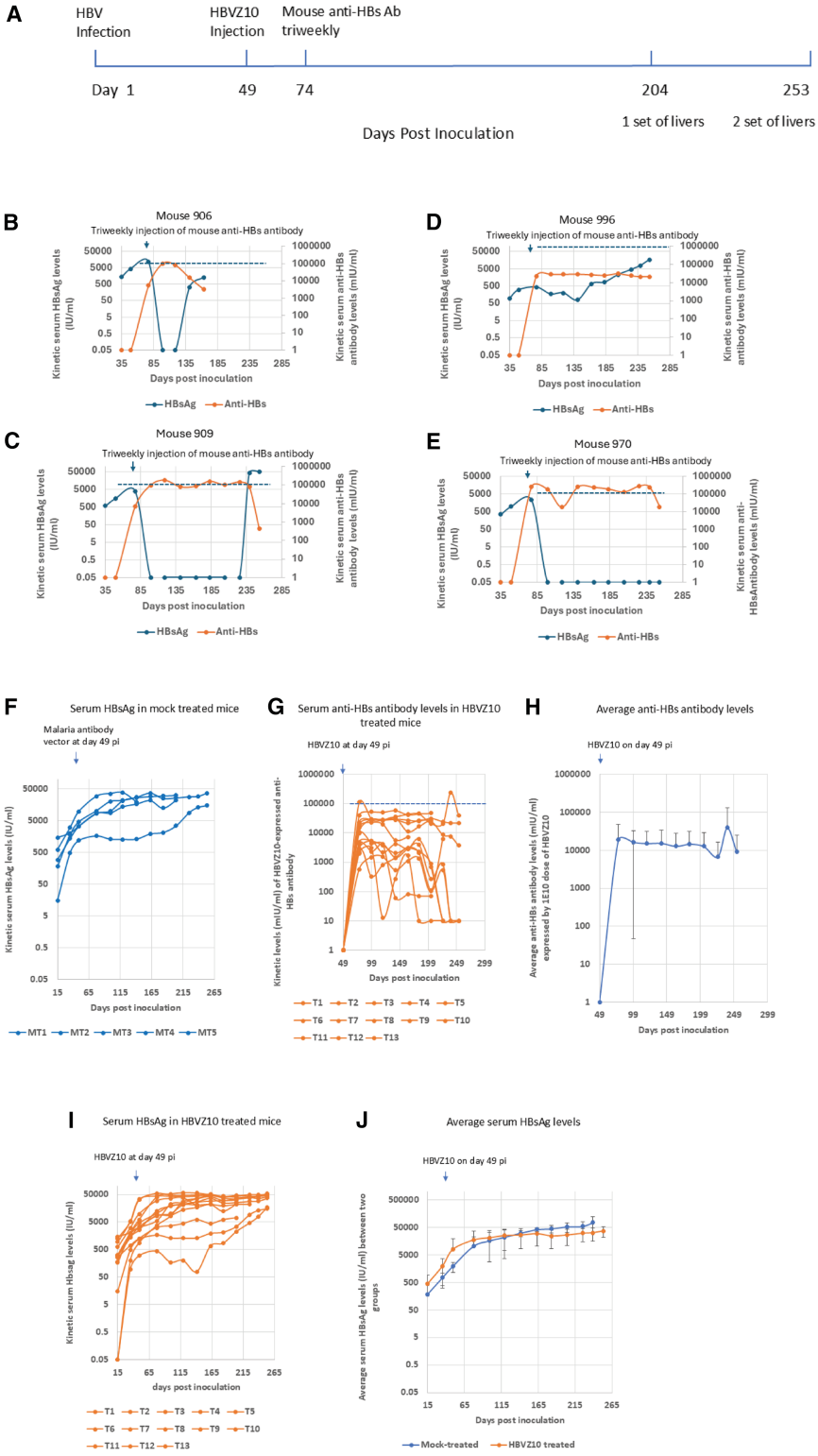

Figure 3. Different serum HBsAg responses to different levels of anti-HBs antibody monotherapy

(A) Experimental procedures and time course of HBV infection and treatment evaluation using uPA/SCID chimeric mice model. (B–E) Kinetic serum HBsAg levels (IU/mL) and anti-HBs antibody levels (mIU/mL) in four infected mice (ID: 906, 909, 996, and 970) treated with triweekly infusions of 250 μg mouse anti-HBs antibody starting on day 74 PI. Note: A dynamic relationship appears to exist between serum HBsAg and anti-HBs antibody levels. For example, in both mice 906 and 909 (2A and B), serum HBsAg became undetectable when anti-HBs antibody levels reached and remained at ≥100,000 mIU/mL, but reappeared when anti-HBs antibody levels declined below 100,000 mIU/mL. (F) Kinetic serum HBsAg levels (IU/mL) in 5 mice (MT1-MT5) mock treated with 1E10 copies of vector expressing malaria antibody on day 49 PI. (G) Kinetic serum anti-HBs antibody levels (mIU/mL) expressed by a single dose (1E10 copies) of HBVZ10 administration on day 49 PI among 13 individual chimeric mice (T1-T13). (H) Average anti-HBs antibody levels (mIU/mL) per group from F. (I) Kinetic serum HBsAg levels (IU/mL) among the 13 mice (T1-T13) treated with a single 1E10 dose of HBVZ10 on day 49 PI, as described in G and H. Note. Mice with relatively lower serum HBsAg levels at baseline (day 49 PI) appeared to respond more effectively to lower levels of anti-HBs antibody treatment than those with higher baseline HBsAg levels. (J) Comparison of average serum HBsAg levels between mock-treated and low-dose HBVZ10-treated groups. A pretreatment serum sample from each mouse was used as a negative control, and all pretreatment anti-HBs antibody levels were plotted as 1 mIU/mL for consistency in (A–D) and (F). Dashed line: anti-HBs antibody level at 100,000 mIU/mL threshold. Error bars: standard deviation (SD).

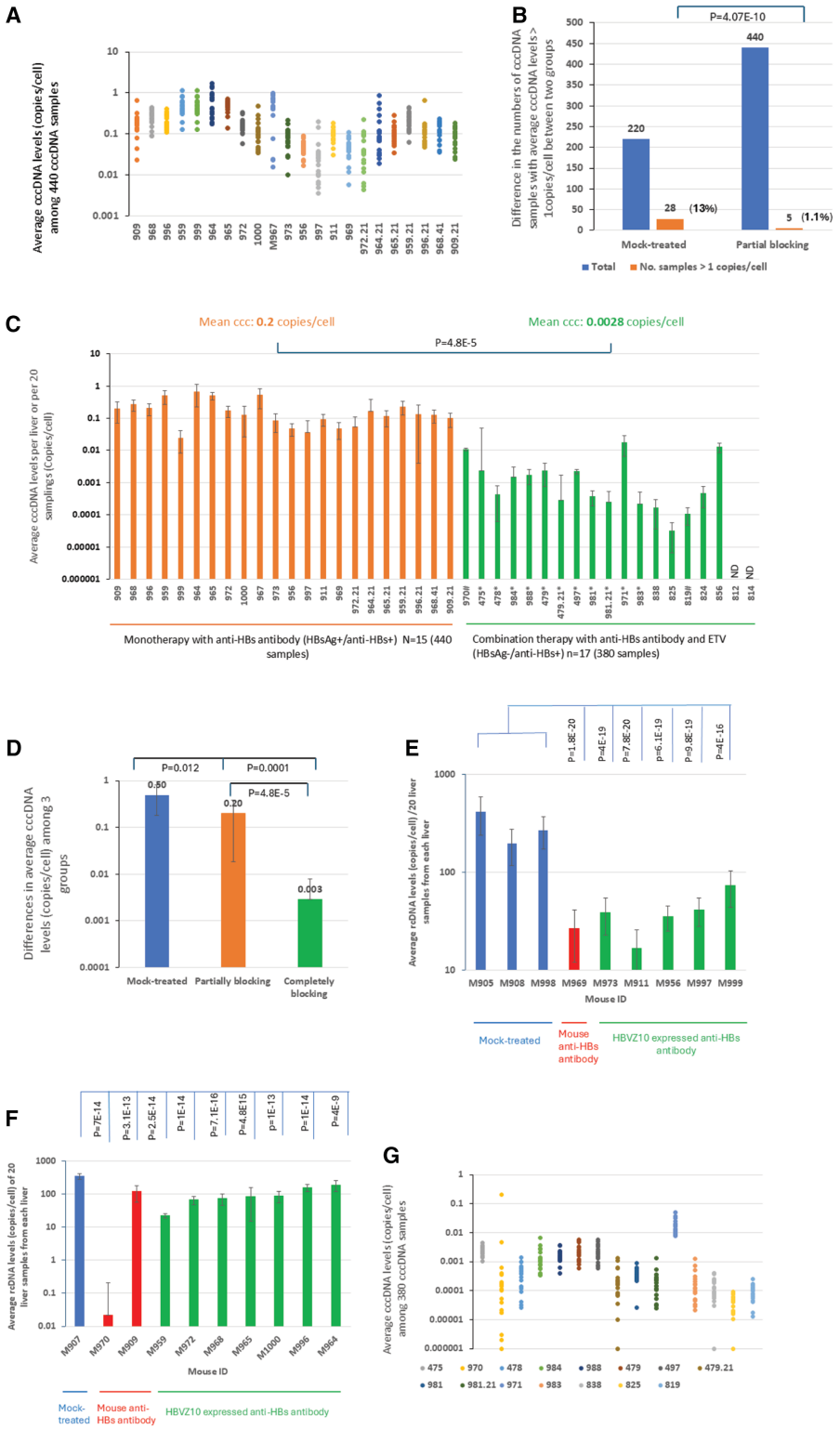

Figure 4. Significant reduction of cccDNA levels in infected chimeric mice upon blocking cccDNA replenishment

(A) Average cccDNA levels (copies/cell) among 440 cccDNA samples isolated from 15 mice, 13 of which received a single administration of HBVZ10 at a dose of 1E10 copies on day 49 PI and the remaining 2 (mice 909 and 996) received mouse anti-HBs antibody infusions at a dose of 250 μg/injection triweekly started on day 74 PI. Each of the

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on serum HBsAg levels, ranging from a 1 to 2 log reduction to no noticeable difference compared to untreated mice (Figure 3I). At the group level, the effect of insufficient anti-HBs antibody monotherapy on serum HBsAg is noticeable but modest (Figure 3J) compared to the impact of sufficient anti-HBs antibody expressed by high dose of HBVZ10 on serum HBsAg (Figure 2C2). Consequently, dual positivity for serum anti-HBs antibody and HBsAg (anti-HBs+/HBsAg+) was observed, indicating that HBV *de novo* infection was only partially blocked in HBVZ10-treated mice.

The presence of HBV cccDNA in infected livers is the root cause of HBV infection. Therefore, the therapeutic impact of HBV curative strategies should be evaluated based on their effects on cccDNA levels. In addition to direct quantification of intrahepatic cccDNA, surrogate markers, such as the kinetics of serum HBV DNA, HBeAg, and HBsAg levels, can be also used to assess cccDNA dynamics and therapeutic efficacy.

A total of 15 livers including the 13 treated with HBVZ10 and the 2 with the mouse anti-HBs antibody monotherapy (mice 909 and 996), whose *de novo* infection was also partially blocked, were harvested at two time points. The first set of six livers and the second set 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, which were analyzed by qPCR using the standards calibrated with Absolute Q (ABQ) digital PCR (dPCR). Average cccDNA levels (copies/cell) among the 440 cccDNA samples varied considerably from 0.04 to 1.7 copies/cell (Figure 4A).

However, average cccDNA levels >1 copies/cell were only detected in five (1.1%) of the 440 samples (Figure 4A), which was significantly lower than the 13% of cccDNA samples in untreated mice (Figure 4B, p = 4.07E−10). The mean cccDNA level in the 15 mice was 0.2 copies/cell, which was significantly lower (p = 0.012) than 0.5 copies/cell in -mock-treated mice (Figures 4C, orange, and 4D).

Intrahepatic rcDNA levels reflect the extent of HBV replication and overall infection burden. Quantification and comparison of rcDNA levels provide additional evidence supporting intrahepatic cccDNA elimination, as the clearance of cccDNA leads to a subsequent reduction in rcDNA levels. Lower cccDNA levels in this group were supported by proportionally lower rcDNA levels (Figures 4E and 4F). These results suggest that cccDNA levels are sensitive to partial blocking of *de novo* infection.

Completely blocking cccDNA replenishment

We then evaluated the impact of serologically blocking *de novo* infection completely or blocking both intracellular and extracellular cccDNA replenishment pathways.

Entecavir (ETV) is an US Food and Drug Administration-approved NA drug that mainly inhibits rcDNA synthesis in infected cells. Since NA-based therapies remarkedly reduces the rcDNA synthesis, they can also reduce cccDNA level by 1–2.9 log[8,54–58] depending on the duration of treatment. This NA-mediated reduction in cccDNA is considered indirect, primarily resulting from inhibition of intracellular cccDNA replenishment through suppression of rcDNA synthesis (Figure 1B). ETV is currently recommended as a first-line therapy for CHB.[59] However, ETV alone cannot fully suppress HBV replication, as residual-infected cells continue to release low levels of virions, driving new rounds of infection that keep replenishing cccDNA pool and prolonging chronic infection course, pointing to a need to target extracellular virions for HBV cure therapy.

Blocking both cccDNA replenishment pathways was accomplished using HBVZ10/anti-HB antibodies to block new rounds of infection, together with 9–12 weeks of ETV therapy to decrease rcDNA synthesis and intracellular recycling pathways (Figure 1C).

ETV monotherapy was used as control. Resembling NA treatment in clinical patients, HBV relapsed following ETV withdrawal in mice treated with 9 weeks of ETV monotherapy. Their cccDNA levels were comparable to mock-treated mice upon termination (see details in progressive cccDNA elimination in the livers was indicated by progressive serum HBeAg reduction upon blocking cccDNA replenishment).

A cccDNA analysis was conducted on 17 mice that achieved HBsAg negative/anti-HBs positive status (see details in next section). The

15 livers underwent an initial round of 20 samplings, and a second round of 20 samplings was conducted for 7 of the 15 livers. The second-round samples were labeled as 972.21, 964.21, and so on. (B) Comparison of the numbers of cccDNA samples with average cccDNA levels >1 copies/cell between mock-treated and anti-HBs antibody-treated groups. The difference in percentages between the two groups were significant (1% vs. 13% p = 4.07E−10 by chi-square test). (C) Average cccDNA levels per liver or per 20 samplings in two groups, the 1st group (15 mice, orange) treated with anti-HBs antibody only (anti-HBs antibody levels <100,000 mIU/mL, resulting in HBsAg+/anti-HBs antibody+ profile, reflecting partial blocking of *de novo* infection), and 2nd group (green) with complete blocking of *de novo* infection, resulting in anti-HBs antibody+/HBsAg-) in 17 mice either under a combination of HBVZ10 with ETV in 15 mice or anti-HBs antibody monotherapy in 2 mice (mouse 819 with a single injection of HBVZ10 at a dose of 1.8E11 and mouse 970 with mouse anti-HBs antibody at a dose of 250 μg/injection triweekly for 9 consecutive times). Anti-HBs antibody levels in group 2 were maintained at ≥100,000 mIU/mL. # anti-HBs antibody monotherapy. * HBVZ10/anti-HBs antibody and 9 weeks of ETV, and remaining mice with HBVZ10 + 12 weeks of ETV. (D) Comparison of average cccDNA levels among three groups: mock-treated (averaged from 220 cccDNA samples, blue), partial blockade of *de novo* infection (averaged from 440 samples, orange), and complete blockade of *de novo* infection (averaged from 380 samples, green). The differences in average cccDNA levels among the three groups were statistically significant (Student's t test). (E and F) Average rcDNA levels (copies/cell) among 15 mice with partial blocking of *de novo* infection, harvested either on day 204 PI (E) or day 253 PI (F). Blue: mock treated with malaria antibody. Green: HBVZ10. Red: mouse anti-HBs antibody in both E and F. (G) Average cccDNA levels among 380 cccDNA samples isolated from 17 mice with complete blocking of de novo infection. p value >0.05 is considered significant. Error bars: standard deviation (SD). 909, 968 … or M905, M908 … etc. are mice ID.

two mice (mouse 970, which received nine injections of mouse anti-HBs antibody at triweekly interval described in Figure 2E and mouse 819 (the same mouse referred as T2 in Figures 1B1 and C1), which received a single dose of 1.8E11 copies of HBVZ10) underwent anti-HBs antibody monotherapy, while the remaining 15 mice were treated with a combination of HBVZ10/anti-HBs antibody and ETV.

Each of the 17 livers that were collected after the peak infection (around day 82 PI) between days 123 and 253 PI was 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 (green in Figures 4C and 4G). Among them, 140 cccDNA samples from 7 mice were analyzed using both qPCR and ABQ dPCR that can directly detect a single copy of cccDNA, showing comparable results (Figure S3). All cccDNA levels were <1 copies/cell, and most of them were <0.01 copies/cell (Figure 4G). The mean cccDNA level in 17 mice was 0.0028 copies/cell, which is >100-fold lower ($p = 0.0001$) than the 0.5 copies/cell in untreated mice and significantly lower ($p = 4.8E{-}5$) than the 0.2 copies/cell in mice with partially blocked *de novo* infection (Figures 4C and 4D). 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 efficient cccDNA elimination and further supports the observation that cccDNA replenishment is required to maintain cccDNA levels, underlying the spontaneous clearance of cccDNA from infected livers.[27]

Kinetic human albumin levels were similarly steady between untreated and treated mice (Figure S4), suggesting that cccDNA elimination mainly resulted from the blocking of cccDNA replenishment and not from the net loss of infected human hepatocytes in treated mice. The steady human albumin levels were consistent with normal ALT (Alanine Aminotransferase) levels during the treatment and follow-up period between days 35 and 207 PI (Figure S5).

Progressive cccDNA elimination in the livers was indicated by progressive serum HBeAg reduction upon blocking cccDNA replenishment

Serum HBeAg is synthesized using pre-core mRNA that must be transcribed from cccDNA[60] and can be used as a surrogate for cccDNA level. Upon blocking of cccDNA replenishment with 12 weeks of ETV therapy (Figure 5A), HBVZ110 expressed anti-HBs antibody levels ≥100,000 mIU/mL (Figure 5B), and serum HBeAg was progressively reduced to undetectable levels from baseline (Figure 5C) among the 8 mice that also achieved progressive reduction of serum HBsAg to undetectable levels (Figure 5D). The baseline levels of HBeAg or HBsAg represent the relative amount of already infected cells before responding to the therapy. A progressive reduction of both the serum HBeAg and HBsAg from the baseline reflects spontaneous cccDNA elimination from the already infected cells since neither ETV nor HBVZ10 directly acts on cccDNA; both primarily affect cccDNA replenishment (Figures 1B and 1C), highlighting the dynamics of cccDNA and the importance of replenishment in maintaining cccDNA levels in infected livers. In addition, both intrahepatic cccDNA and rcDNA levels in these mice with progressive reduction of serum HBeAg and HBsAg were significantly lower than that in mock-treated mice (Figures 5D and 5E).

More than 100-fold reduction in cccDNA level observed in 80 days of treatment of blocking cccDNA replenishment

uPA/SCID chimeric mice 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 6A shows that baseline HBsAg levels on days 54 and 82 PI reached approximately 5,000 IU/mL before a progressive decline in mouse 838, which was treated with HBVZ10 (7E11 copies on day 44 PI) and ETV combination (started on day 54 PI). This was comparable to that in mouse 833 that received HBVZ10 monotherapy on day 44 PI (7E11 copies). Mouse 833 displayed dual positivity for serum HBsAg (Figure 5A) and anti-HBs antibody (Figure 2A) after treatment; thus, *de novo* infection in mouse 833 was considered only partially blocked. Serum HBsAg levels remained steady at approximately 5,000 IU/mL from days 82 and 162 (termination day) in mouse 833 but more than 10-fold lower than that in mock-treated mouse 838. We used the cccDNA levels in mouse 833 as a reference for baseline cccDNA levels before HBsAg clearance in mouse 838. The intracellular HBsAg, rcDNA, and cccDNA levels in mouse 838 were lowered by >100-fold during 80 days from day 82 to day 162 PI compared with those in mouse 833 (Figure 6B). Intracellular HBsAg, rcDNA, and cccDNA levels in mouse 833 were 2- to 3-fold lower though significantly and 100- to 1,000-fold lower in mouse 838 compared to the mock-treated mouse 838 (Figure 6B). The cccDNA results further support that the observed spontaneous cccDNA clearance was efficient and could be transformed into progressive cccDNA elimination upon blocking both cccDNA replenishment pathways.

Add-on of HBVZ10 and mouse anti-HBs antibody to 9-week ETV treatment prevented HBV relapse, progressively reducing both serum HBeAg and HBsAg levels

We conducted another animal experiment to evaluate if HBV relapse can be prevented after ETV withdrawal. HBV infected uPA/SCID chimeric mice were divided into 3 groups: mock-treated, ETV 9-week monotherapy, and 9-week ETV with add-on of HBVZ10. ETV treatment started day 35 PI for 9 weeks. In add-on group, HBVZ10 was added at a dose of 1E10 copies day 70 PI or 5 weeks after starting ETV. HBV infection was monitored for 16 weeks after ETV withdrawal (Figure 7A).

HBV infection relapsed after ETV stopped in the ETV monotherapy group

ETV treatment reduced viremia by 2–3 log. As expected, HBV DNA levels gradually bounced back to mock-treated level (Figure 7B orange vs. blue) after ETV withdrawal.

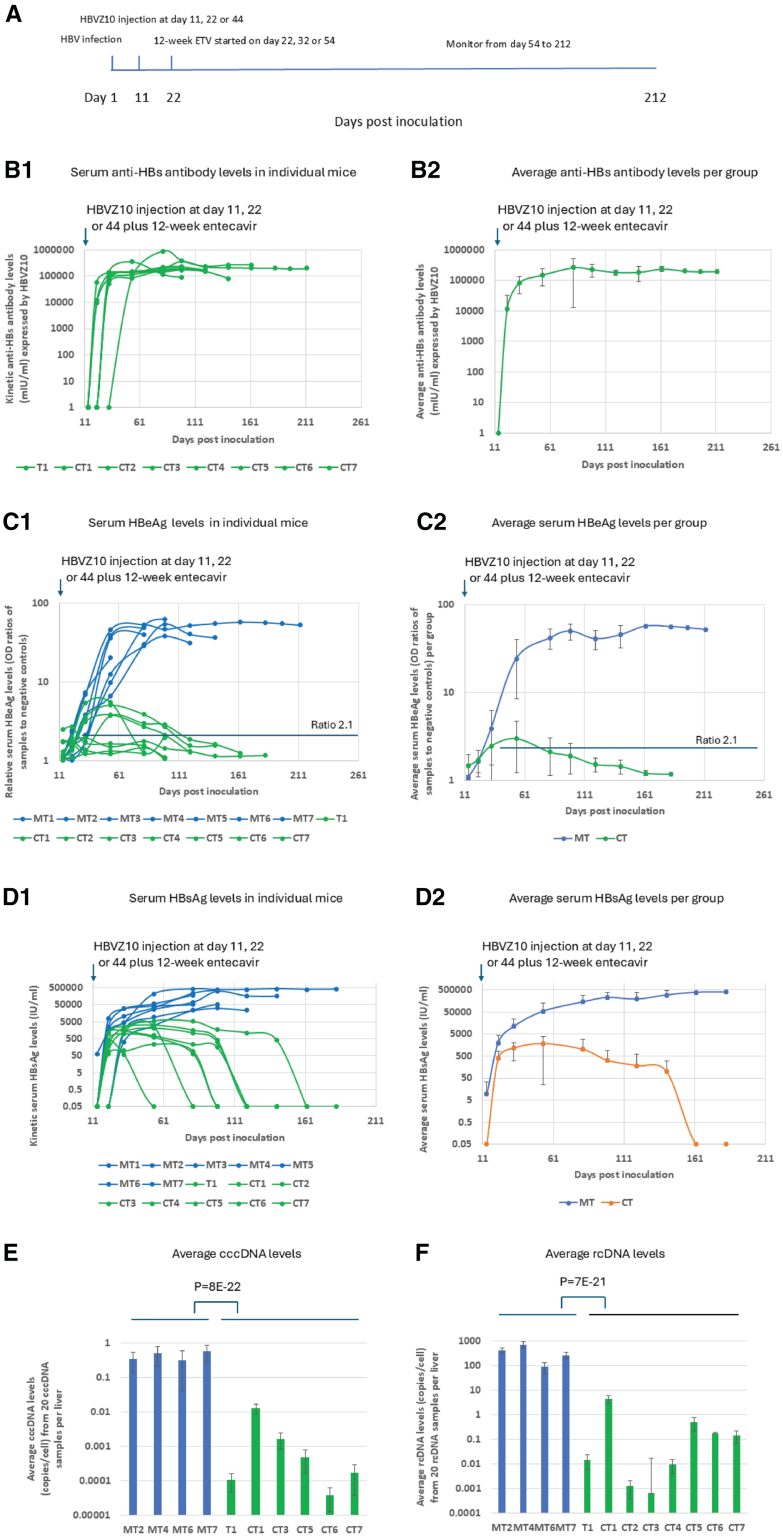


Figure 5. Progressive reduction of both serum HBeAg and HBsAg upon blocking of cccDNA replenishment

(A) Experimental procedures and time course of HBV infection and treatment evaluation using uPA/SCID chimeric mice model. (B1) The expressed anti-HBs antibody levels were >100,000 mIU/mL and sustained at steady levels for 200 days after a single injection of HBVZ10 in 8 chimeric mice (T1 and CT1-CT7) infected with HBV. A pretreatment serum sample from each replicate was used as a negative control, and all pretreatment anti-HBs antibody levels were plotted as 1 mIU/mL for consistency. (B2) Average anti-HBs antibody levels per group. HBVZ10 was administered on day 11 with a dose of 2.5E10 copies for mice CT1-CT4 on day 22 with 1.8E11 copies for mice T1 and CT5-CT6, or on day 44pi with 7E11 copies of HBV10 for mouse CT7. All 7 mice (CT1-CT7) also received 12-week ETV 10 days after HBVZ10 injection. T. HBVZ10 monotherapy. CT: Combination of HBVZ10 with ETV. (C1) Serum HBeAg kinetics among 7 mock-treated mice (MT1-MT7 blue) and 8 treated mice (T1 and CT1-CT7 green). Serum HBeAg levels are expressed as the OD ratio of the tested sample to the mean OD of three negative controls. A ratio greater than 2.1 is considered positive. (C2) Average serum HBeAg levels per group between mock-treated (blue) and treated (green) from B1. (D1) Kinetic serum HBsAg levels (IU/mL) among mock-treated 7 mice (blue) and 8 treated mice (green) from B1. (D2) Average serum HBsAg levels per group between mock-treated (blue) and treated group (green) from C1. (E) Comparison of average cccDNA levels between mock-treated and combination treated groups (cccDNA was undetectable in mice CT2 and CT4 and not plotted). (F) Comparison of average rcDNA levels between mock-treated and combination-treated groups. Statistical significance was determined using Student's *t* test; $p < 0.05$ was considered significant. Error bars: standard deviation (SD).

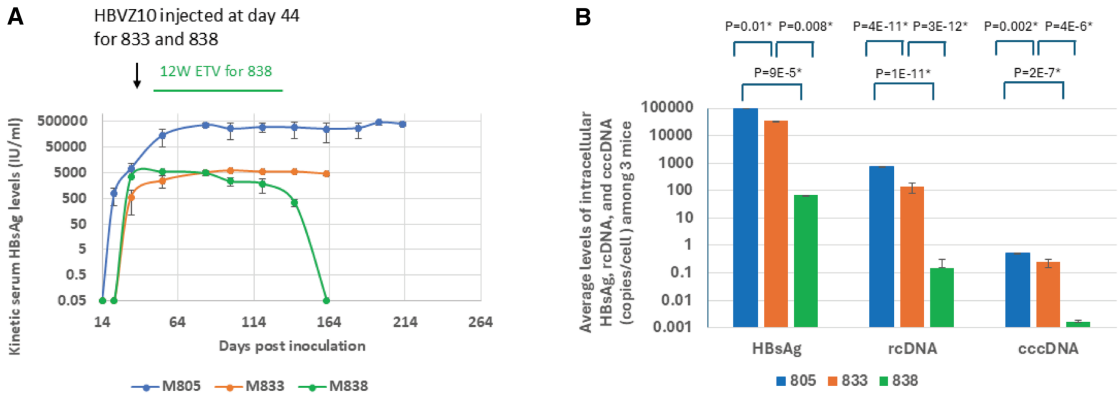


Figure 6. Leveraging spontaneous cccDNA loss into efficient cccDNA elimination by blocking cccDNA replenishment

(A) Kinetic serum HBsAg levels among 3 mice (805. mock-treated; 833. HBVZ10 monotherapy (7E11 copies) on day 44 PI, and 838. Combination therapy: HBVZ10 (7E11 copies administered on day 44 PI, followed 10 days later by the initiation of a 12-week ETV treatment). (B) Comparison of average intracellular HBsAg, rcDNA and cccDNA levels among 3 mice as described in A. The lower limit of serum HBsAg detection is 0.05 IU/mL. Statistical significance was determined using Student's *t* test; $p < 0.05$ was considered significant. Error bars: standard deviation (SD).

HBV relapse was prevented, and serum HBeAg and HBsAg levels were progressively reduced in animals with add-on of HBVZ10/anti-HBs antibodies to 9-week ETV treatment

Since the expressed anti-HBs antibody levels by the low dose of 1E10 copies of HBVZ10 were well below 100,000 mIU/mL, a minimal level required to completely block *de novo* infection with serum HBsAg levels around 5,000 IU/mL, and to test if increasing anti-HBs antibody level would achieve comparable serum HBeAg and HBsAg clearance described in Figures 5B and 5C, we decided to boost anti-HBs antibody levels by injecting mouse anti-HBs antibody (AM31509PU-N, OriGene) at 250 μg/injection triweekly in 8 mice started on day 99 PI or later.

Serum HBV DNA level was also 2–3 log reduced during ETV treatment in this group. No HBV relapse was observed in all 8 animals with add-on therapy during up to 112 days of observation after ETV withdrawal (Figures 7C and 7D).

Anti-HBs antibody levels reached >100,000 mIU/mL (Figure 7E) following the boost. We observed progressive reduction of serum HBsAg to undetectable levels in 8 of mice (Figure 7F), accompanied by progressive reduction of serum HBeAg levels, and 7 of them became undetectable (Figure 7G). An exception was mouse CT5, whose baseline HBeAg level was much higher and remained detectable on the termination day.

Consistent with progressive clearing of serum HBeAg, average cccDNA and rcDNA levels for 20 samplings of each liver were 2–3 log lower in the 5 mice with undetectable serum HBV DNA, HBeAg, and HBsAg than the control mice (Figures 7H and 7I, green).

This add-on experiment and our findings suggest that long-term NA therapy could be safely shortened or discontinued once serum HBV DNA has been reduced to very low or undetectable levels and sustained high levels of circulating anti-HBs antibodies sufficient to prevent *de novo* infection after NA cessation are established.

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

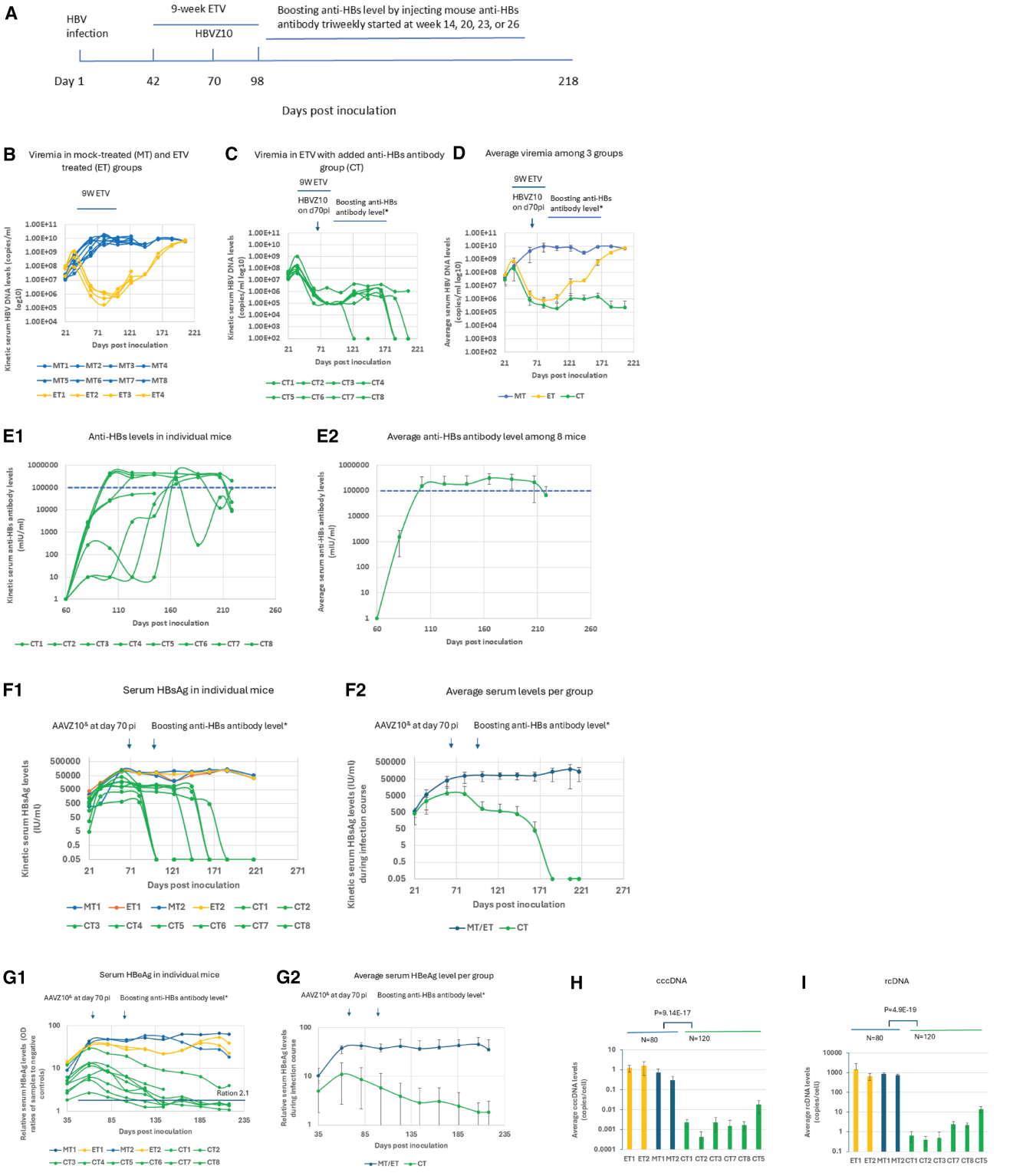
Two series of serum samples from untreated mice and two from treated mice, whose serum HBsAg underwent progressive reduction and became undetectable (Figures 8A1 and 8B1), were subjected to western blot analysis. This showed similar kinetics as detected by ELISA, confirming the progressive reduction in serum HBsAg in two serial serum samples with anti-HBs treatment (Figures 8A2, 8B2, and 8B3).

We also analyzed intrahepatic HBsAg levels in seven mice with progressive serum HBsAg reduction, respectively. Intrahepatic HBsAg was either not detectable or was detectable at low levels by ELISA (Figure 8C1), which was confirmed by western blot analysis (Figure 8C2). In addition, intrahepatic HBc protein 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 HBc protein was detected in none of the 20 liver lysates (Figures 8D1 and 8D2), supporting the notion that the progressive serum HBsAg reduction reflects intracellular HBV clearance upon blocking cccDNA replenishment.

DISCUSSION

This study evaluated HBVZ10 as a novel gene therapy candidate in HBV-infected uPA/SCID chimeric mice with humanized livers and tested a new cccDNA elimination strategy aimed at blocking cccDNA replenishment without directly targeting cccDNA.

Steady cccDNA level in persistent HBV infection is primarily maintained by cccDNA replenishment via two major cccDNA replenishing pathways (Figure 1A), as indicated by this study. Intracellular



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cccDNA replenishment can be blocked by reducing rcDNA synthesis and supply under NA treatment such as ETV (Figure 1B), while both pathways can be blocked by ETV combination with HBVZ10 (Figure 1C).

A major challenge in CHB is continuous new rounds of infection, which replenish the cccDNA pool, prolong the course of chronic infection, and drive HBV relapse following cessation of HBV treatment or reactivate HBV infection after CHB is clinically resolved. A primary factor that allows new rounds of infection is insufficient levels of endogenous anti-HBs antibody that is depleted by a huge pool of serum HBsAg in CHB patients.

Blocking *de novo* infection could theoretically be achieved through intracellular inhibition of HBV replication, provided the inhibition is sufficiently potent to completely halt virion production, thus preventing new rounds of infection. However, currently approved NA drugs, either alone or in combination with emerging therapies such as siRNA or capsid inhibitors, fail to completely suppress virion production. This is evidenced by HBV relapse observed in patients after treatment cessation.[35,61–63] For instance, low-level residual viremia persists in patients even after multi-year of tenofovir disoproxil fumarate (another 1st line NA drug[59]) therapy, as demonstrated by the successful establishment of HBV infection in uPA/SCID mice using sera from these patients as inocula.[64] A typical HBV relapse features a progressively rising viremia from nadir[26,35] or comprises progressively spreading infection from the remaining residual infected cells with cccDNA presence, suggesting that most infected cells may have cleared cccDNA by the time of treatment withdrawal.

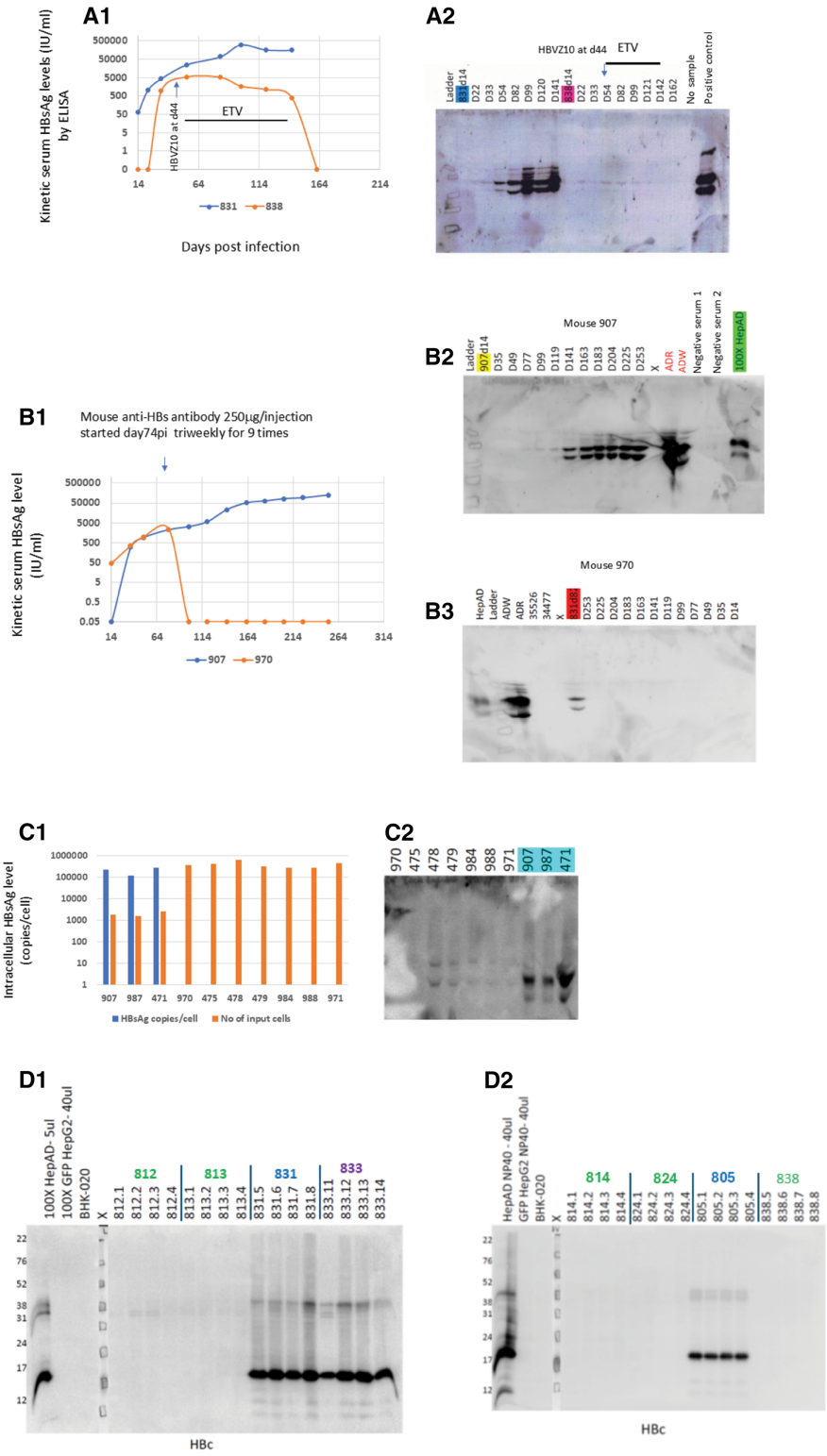
These findings highlight two limitations of current HBV drugs and combination therapies.

(1) Intracellular inhibitors need extracellular partners. Once virions are released from infected cells, an extracellular blocker is required to prevent the released virions from initiating new infections.
(2) There is a need for potent and long-lasting extracellular blockers capable of continuously neutralizing virions in the extracellular space.

The use of anti-HBs antibodies has gained a renewed interest recently with several antibodies being evaluated in clinical trials for CHB and chronic hepatitis D in combination with NA, siRNA or peg-interferon (IFN).[32,34,65] However, exogenous anti-HBs antibodies do not address the fundamental deficiency in endogenous anti-HBs antibody production in CHB. Maintaining effective antibody levels requires repeated high-dose infusions, which pose challenges for patient adherence and can result in subtherapeutic or declining levels if doses are missed or discontinued. In this study, suboptimal anti-HBs levels failed to fully block new rounds of HBV infection. In contrast, increasing anti-HBs levels above 100,000 mIU/mL converted partial blocking to complete blockade of *de novo* infection, leading to progressive elimination of cccDNA. This anti-HBs antibody level-dependent effect may explain the limited or transient efficacy observed in clinical trials using exogenous anti-HBs antibodies at weekly doses of 18 or 75 mg per injection or 300 mg per 4 weeks as monotherapy or combined with siRNA or peg-IFN.[66] For comparison, in HIV clinical trials, each of the two broadly neutralizing antibodies was administered at 30 mg/kg every 3 weeks (a total of 4.2 g of two antibodies for a 70 kg adult)[37] and achieved sustained viral suppression following interruption of antiretroviral therapy, highlighting the possibility to shorten current lifetime medication to a finite course if the antibody is sustained at effective levels. However, both HBV and HIV infections tend to rebound once antibody treatment is discontinued,[37,67] implying that the transient nature of exogenous antibodies may have the difficulty shortening the infinite HBV NA therapy and underscore the need for sustained endogenous antibody expression to achieve this goal.

Figure 7. Add-on of HBVZ10/anti-HBs antibodies to 9-week ETV treatment prevented HBV relapse, leading to progressive reduction of both serum HBeAg and HBsAg levels

(A) Experimental procedures and time course of HBV infection and treatment evaluation using uPA/SCID chimeric mice model. (B) Serum HBV DNA (copies/mL log10) kinetics in mock-treated (MT1-MT8 blue) and ETV monotherapy (ET1-ET4 orange) group. (C) Serum HBV DNA kinetics in 8 ETV-treated mice (CT1-CT8) with add-on with a single dose of 1E10 copies of HBVZ10 on day 70pi, then anti-HBs levels were further boosted with administration of mouse anti-HBs at a dose of 250 μg/injection triweekly started on day 99 or later. (D) Average serum HBV DNA levels (copies/mL log10) among 3 groups. (E1) Serum kinetic anti-HBs antibody levels (mIU/mL) expressed by HBVZ10 and boosted by infusions of mouse anti-HBs antibody among the 8 mice (CT1-CT8). Dashed line: 100,000 mIU/mL. Note: Anti-HBs antibody levels exhibited significant fluctuations in some mice receiving triweekly infusions of mouse anti-HBs antibody, contrasting the steady and consistent antibody levels expressed by sufficient HBVZ10 doses (Figure 4A). A pretreatment serum sample from each replicate was used as a negative control, and all pretreatment anti-HBs antibody levels were plotted as 1 mIU/mL for consistency. (E2) Average anti-HBs antibody levels (mIU/mL) per group. (F1) Kinetic serum HBsAg levels (IU/mL) among 2 mice with mock-treated (MT1 and MT2 blue), 2 mice with 9-week of ETV therapy (ET1 and ET2 yellow), and 8 mice with 9-week of ETV and anti-HBs antibodies add-on (CT1-CT8 green). (F2) Average serum HBsAg levels (IU/mL) per group. (G1) Kinetic serum HBeAg levels among 2 mice with mock-treated (MT1 and MT2 blue), 2 mice with 9-week of ETV therapy (ET1 and ET2 yellow), and 8 mice with 9-week of ETV and anti-HBs antibodies add-on (CT1-CT8 green). Note. Mouse CT5 had relatively higher baseline HBeAg level and HBeAg remained detectable on termination day (day 218 PI) despite progressive reduction. Serum HBeAg levels are expressed as the OD ratio of the tested sample to the mean OD of three negative controls. A ratio greater than 2.1 is considered positive. (G2) Average serum HBeAg levels per group. (H and I) Comparison of intracellular cccDNA and rcDNA levels among 2 livers with 9-week of ETV therapy (ET1 and ET2 yellow), 2 livers with mock-treated (MT1 and MT2 blue), and 6 livers with 9-week of ETV and anti-HBs antibodies add-on, 5 (CT1-CT3, CT7 and CT8) of them achieved progressive reduction of both serum HBeAg and HBsAg to undetectable levels and the remaining one with detectable serum HBeAg on the termination day (CT5). The differences in cccDNA and rcDNA levels were analyzed by Student *t* test, $p < 0.05$ considered significant. Error bars: standard deviations (SD). The limit of detection for HBV DNA is 100 copies/mL and 0.05 IU/mL for serum HBsAg.MT: mock-treated. ET: ETV Monotherapy. CT: 9-week of ETV and anti-HBs antibodies add-on.



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HBVZ10 converts the transduced cells (mainly skeletal muscle cells) into anti-HBs antibody-producing cells. Skeletal muscle cells in adults are nondividing cells and multiple copies of HBVZ10 genomes in the same nuclei form concatemers to allow long-term expression (Figures 1D2–1D4). Thus, HBVZ10 expands endogenous production capacity beyond specific B cells, aiming to address insufficient anti-HBs antibody production in CHB.[31] It offers two advantages over other modalities.

1. *It delivers a durable endogenous expression*. Unlike exogenous anti-HBs antibodies[33,34] and entry inhibitors[68] requiring repeated infusions, HBVZ10 demonstrated its ability to express high levels of anti-HBs antibodies (≥100,000 mIU/mL) sustained for at least 200 days following a single dose of 2.5E10 or more vector copies. A durable endogenous expression is required to shorten the current infinite HBV antiviral therapy without risking HBV relapses and to prevent HBV reactivation after CHB is clinically resolved. Our results indicate that HBVZ10 may possess such potential.
2. *Its antiviral effect is independent of the host immunity*. Unlike therapeutic vaccines[69,70] that deliver antigens but not effector molecules and must depend on the host's adaptive immunity for vaccine functions, HBVZ10 directly expresses anti-HBs antibody independent of the host's immune system. The defective HBV-specific T and B cell immune responses observed in CHB and their heterogeneity across patients[69–72] are major challenges for therapeutic vaccine development. HBVZ10 delivers its therapeutic efficacy regardless of the patient's immune status.

Current HBV combination therapies, often limited to intracellular inhibitors, fail to completely halt HBV replication and new rounds of infection.[61–63] When HBVZ10 combined with ETV, sustained high-level anti-HBs antibody expression complements ETV by durably blocking new rounds of infection with released virions. Meanwhile, ETV inhibits virion production and cccDNA replenishment, thus enhancing the efficiency of HBVZ10-mediated neutralization. This synergistic mechanism improves efficiency for both cccDNA elimination and HBsAg seroclearance. Thus, combining an intracellular replication inhibitor (ETV) with a durable extracellular virion blocker (HBVZ10) may offer a superior regimen compared to all intracellular inhibitors-based combinations.

In this study, we explored a new cccDNA elimination strategy that aims to block two cccDNA replenishment pathways while it leverages the spontaneous clearance of cccDNA in infected cells. Our evaluations showed that this strategy can reproducibly deliver a progressive cccDNA elimination. This was evidenced by >100-fold reductions in cccDNA within months, accompanied by progressive reduction of serum HBeAg and HBsAg and intrahepatic HBsAg and HBcAg to undetectable levels in chimeric mice with humanized livers.

AAV is known to have favorable safety profile and several AAV vector-based gene therapies have been approved.[73] There are two potential issues in HBVZ10 clinical applications. (1) *Possible anti-HBs immunocomplex:* however, clinical trial showed that infusions of high dose of HBIG for treating chronic hepatitis B (560 mg/kg, a total of 39.2 g HBIG required for a 70 kg adult patient) only resulted in minor transient immunocomplex reaction in 2 of 6 patients,[74] which is confirmed by a new clinical trial with infusions of high doses (up to 240,000 IU weekly) of anti-HBs monoclonal antibody.[75] Unlike the infusion of exogenous antibodies, which leads to a rapid spike in antibody levels and may immediately form a large amount of immunocomplexes in the bloodstream, HBVZ10-mediated anti-HBs antibody expression takes 2–3 weeks to reach therapeutic levels. This gradual increase may help prevent the sudden formation of large quantities of immunocomplexes that cannot be promptly cleared by the mononuclear macrophage system. As a result, HBVZ10-driven antibody expression is less likely to overwhelm the host's immunocomplex clearance systems and may reduce the risk of immunocomplex deposition in blood vessels and other tissues. (2) *Possible anti-drug antibodies (ADA)*: the expressed human anti-HBs antibody can be immunogenic to a unique recipient.[76] A treatment with a mild immunosuppressant methotrexate reduced Humira-related ADA from 12% to ∼2%–3%.[77] Such treatment can be explored for HBVZ10-related ADA if it occurred.

Furthermore, the same AAV8 vector has been used to express HIV broadly neutralizing antibodies and is currently under evaluation in phase 1 clinical trial, where it has shown a favorable safety profile with doses up to 2.5×10^{12} vg per kg.[78] These precedents support the feasibility and safety of HBVZ10 in a clinical setting.

There are several limitations associated with the use of this chimeric mouse model. While it supports robust HBV replication and

Figure 8. Progressive reduction in serum HBsAg detected with ELISA was corroborated with western blot results and supported by intracellular HBsAg reduction

(A) Progressive reduction of serum HBsAg detected by ELISA in mouse 838 (A1 orange) who received a single dose of HBVZ10 combined with 12-week ETV and was corroborated by western blot (A2). Mouse 831 was a mock-treated control. 100 × Hep: Positive control with concentrated viral particles from the HepAD38 cell medium. (B) Progressive reduction of serum HBsAg detected by ELISA in mouse 970 (B1 orange) who received mouse anti-HBs antibody monotherapy at a dose of 250 μg/injection triweekly for 9 consecutive times and was corroborated by western blot (B2). Mouse 907 was a mock-treated control. ADR and ADW: HBV positive human sera. 100xHepAD, Positive control with concentrated viral particles from HepAD38 cell medium. 35526 and 34477, negative human sera. X, no sample lane. Intrahepatic HBsAg was undetectable by ELISA (C1) in lysates (30 μL each) from seven mice that achieved an $HBsAg^{-}$/anti-HBs^{+} serum status. Consistently, western blot analysis (C2) of 40 μL liver lysates showed either undetectable or markedly reduced HBsAg levels in these seven mice compared with three untreated controls (mice 907, 987, and 471). (D) Intrahepatic HBc protein analyzed using western blot. 100xHepAD, Positive control with concentrated viral particles from HepAD38 cell medium. 100× GFP-HepG2 cell lysate, negative control. BHK-020, Uninfected liver lysate as negative control. X, no sample lane. Mice 812, 813, 814, 824, and 838 in green were the five mice with progressive reduction of serum HBsAg level upon blocking cccDNA replenishment. Mice 831 and 805 in blue were mock treated. Mouse 833 in purple received a single dose of HBVZ10 monotherapy on day 44pi. Four liver lysates from each liver were analyzed, and numbered as 1, 2, 3, and 4 or 5, 6, 7, and 8 or 11, 12, 13, and 14, respectively after mouse ID number.

recapitulates key features of persistent HBV infection in humans, the humanized liver may not fully reflect the complexity of a native human liver. The absence of functional adaptive immunity in this model enabled us to evaluate the effect of anti-HBs antibody on cccDNA elimination in isolation; however, this may not accurately predict outcomes in humans, where both T and B cell immune responses might influence the results differently. Due to the animals' fragile health, liver biopsies could not be performed at early time points to assess baseline cccDNA levels, and we instead relied on serum HBeAg and HBsAg as surrogate markers. Furthermore, the model supports HBV infection for only 6–7 months, as aging contributes to increased animal attrition. Consequently, we were unable to extend follow-up beyond 16 weeks after ETV withdrawal. The durable efficacy of HBVZ10 needs to be established in human trials.

Our findings show the potential of HBVZ10 as a novel extracellular blocking modality, capable of continuously expressing high levels of anti-HBs antibodies with a single dose. This study also provides preclinical evidence for this new cccDNA elimination strategy and HBVZ10 combination therapy with ETV. HBVZ10-based therapy offers potential for achieving more efficient and durable cccDNA elimination and shortening NA course for treating CHB in humans, which needs to be demonstrated through clinical trials.

MATERIALS AND METHODS

HBVZ10 construct

AAV8 is one of the several AAV gene therapy vectors with high tropism for skeletal muscle cells and has been utilized to deliver therapeutic genes for the treatment of genetic neuromuscular disorder, such as X-linked myotubular myopathy (XLMTM).[79] In phase 1/2 XLMTM clinical trial, muscle biopsies from nine enrolled subjects who received an AAV8 vector-based gene therapy encoding myotubularin 1, demonstrated robust, dose-dependent tissue transduction and myotubularin protein expression,[80] supporting the therapeutic potential of AAV8 in targeting muscle tissue.

To expand endogenous capacity to express anti-HBs antibody in patients with CHB, HBVtech decided to develop a new therapy, candidate based on AAV gene therapy platform. The late Dr. Baltimore's laboratory optimized an AAV8 vector to enhance human antibody expression,[38–41] achieving levels 10 times higher than those produced by unoptimized AAV vector.[42] The size of HBVZ10 expression construct (from 5′ITR to 3′ITR) is 4.4 kb. The specific promoter elements within the AAV8 vector, which drive antibody expression in muscle cells, are illustrated in Figure S1B.

The AAV8-based HBVZ10 is designed to primarily target skeletal muscle cells, as adult muscle tissue consists largely of nondividing cells, a key requirement for achieving long-term anti-HBs antibody expression.

Animals and HBV infection

All animal experiments followed the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and were performed at Noble Life Sciences (NLS) Inc. (Sykesville, MD), a preclinical research contract service provider. The selection of Noble Life Sciences Inc. was approved by the NIAID (National Institute of Allergy and Infectious Diseases) contract office at NIH (NIH-approved Animal Welfare Assurance no. A4633-01). All animal studies were approved by the Institutional Animal Care and Use Committee of Noble Life Sciences protocol NLS-614. All animals received humane care.

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 (Bbiosafety Level 2) room with controlled temperature at 23°C and 12 h-light/dark cycle. All procedures were performed during the light cycle. All animals were fed with gamma-radiated CRF1 (Charles River Formula-1) food and autoclaved water *ad libitum*. An HBV (ADR serotype) inoculum, 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 (Phosphate-buffered saline) in 100 μL volume, was administered intravenously (tail vein) to each chimeric mouse.

Mock treatment controls

All untreated controls in each of three experiments were mock treated with a single dose of administration of a control AAV vector expressing malaria antibody. The dose matched the HBVZ10 dose in the same experiment.

Definition of complete blocking of *de novo* infection

The serologically complete blocking of *de novo* infection was defined as serum anti-HBs antibody +/HBsAg−.

Monitoring HBV infection and human albumin level in blood

Blood was collected triweekly for quantification of serum HBV DNA (qPCR), 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.

Analysis of intrahepatic HBV DNA

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% Triton X-100) with 10 strokes. The homogenized tissue suspension was spun for 2 min at 14,000 rpm and 100 μL of lysate was saved for western blot or ELISA of intracellular HBsAg and the remaining 400 μL transferred to a new microtube for isolation of replicative intermediates while nucleic pellet remained in the tube for cccDNA isolation. Isolated rcDNA and cccDNA samples were quantified using qPCR, as described in Zhang et al.[27]

Quantification of Anti-HBs antibody levels in serum

Serum anti-HBs antibody levels were quantified using the anti-HBs antibody ELISA kit (MONOLISA Anti-HBs EIA, cat. no. 25220, Bio-Rad) with corresponding calibrators (cat. no. 25219, Bio-Rad), following the manufacturer's instructions. Pretreatment serum samples from chimeric mice served as negative controls, and a value of 1 mIU/mL was assigned to all pretreatment samples for consistency in data presentation.

Statistical analysis

HBsAg (IU/mL), HBeAg and HBV DNA (copies/mL), and anti-HBs antibody levels (mIU/mL) are expressed as mean ± standard deviation (SD) per group. Average intracellular rcDNA and cccDNA levels are expressed as copies/cell. Differences in variables are analyzed using Student *t* test (for normally distributed data), or the Kruskal-Wallis and Mann-Whitney *U* test (for non-normally distributed data) (SPSS basic version, SPSS Inc., Chicago, IL), and $p < 0.05$ is considered statistically significant. The number of cells per sampling was calculated using sample weight (mg) multiplied by 1.39E5 cells per mg liver tissue,[81] then normalized by factor 0.7 by considering 70% of human liver cells are hepatocytes.[82] The formula to calculate copies/cell is listed below:

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

DATA AND CODE AVAILABILITY

The data, analytic methods, and study materials related to this study will be made available upon reasonable request to the lead contact, Yong-Yuan Zhang (yongyuanzhang@hbvtech.com).

ACKNOWLEDGMENTS

We would like to thank the late Dr. David Baltimore at the California Institute of Technology for his generous support in developing this AAV vector-based HBV therapy; Dr. Rajen Koshy and Dr. Mindy Davis at National Institute of Allergy and Infectious Diseases (NIAID), for participation, advice, and support of this project; Dr. Alex Balazs at Broad Institute for technical support; and Ms. Amanda Ulloa at NIAID for reading the manuscript. This study (PI Y.-Y.Z.) was supported by NIH research contracts 75N93020C00045 and 75N930220C00042, SBIR grant R43 AI155066-01A, and DoD Discovery Award W81XWH-21-1-0065. F.Z. received public grants overseen by the French National Research Agency (ANR) as part of the second "Investissements d'Avenir" program (reference: ANR-17-RHUS-0003) and by the European Union (grant EU H2020-847939-IP-cure-B). J.H. was funded by a NIH grant, R37AI043453. The funders had no role in study design, data collection, decision to publish, or preparation of this manuscript.

AUTHOR CONTRIBUTIONS

Investigation, project administration, and methodology, B.-H. Z. Investigation, Y. Z. Investigation and project administration, S. H. Investigation, L. L. Methodology, investigation, funding acquisition, and writing – review and editing, J. H. Investigation, writing – review and editing, methodology, and funding acquisition, F. Z. Conceptualization, methodology, investigation, funding acquisition, writing – original draft, and writing – review and editing, Y.-Y. Z.

DECLARATION OF INTERESTS

B.-H.Z. and Y.-Y.Z. are employees of HBVTECH. F.Z. received consulting fees from Aligos, Assembly, Gilead, and GSK, and research funding was provided to INSERM and Lyon University by Assembly and Beam. Y.-Y.Z. is the inventor of wo2023122555a2.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2025.101646.

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OMTM, Volume *33*

Supplemental information

HBVZ10, an AAV8 vector-based new HBV therapy candidate for cccDNA elimination

Bai-Hua Zhang, Yuanping Zhou, Stephen Horrigan, Laura Luckenbaugh, Jianming Hu, Fabien Zoulim, and Yong-Yuan Zhang

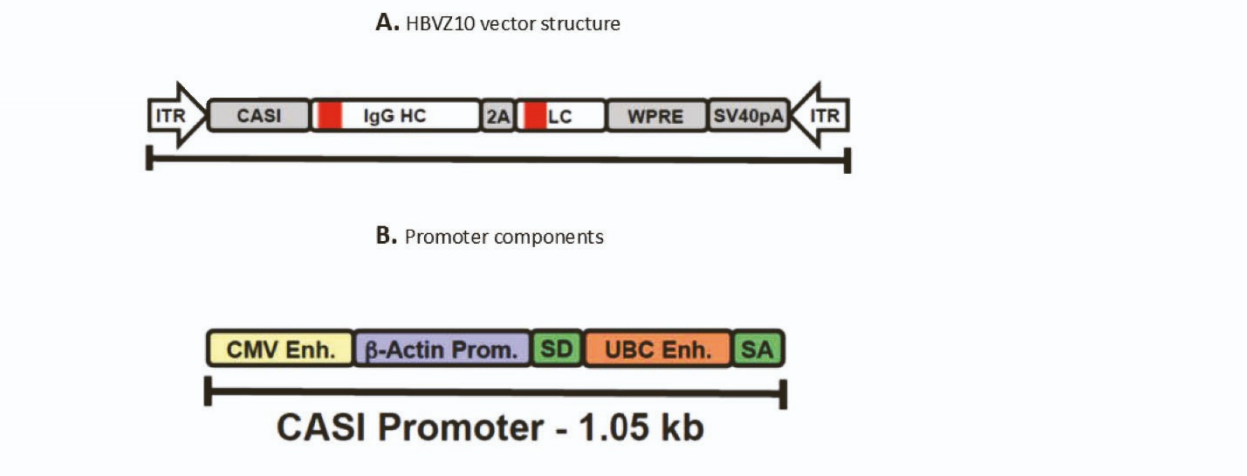

Figure S1 (A)**.** Schematic representation of the AAV8 vector for antibody expression. ITR: inverted terminal repeats. CASI: specific promoter (see 1B). IgG HC: gamma heavy chain constant region. LC: kappa light chain constant region. 2A: a self-cleavage sequence. WPRE: woodchuck hepatitis virus posttranscription regulatory element. SV40pA: SV40 late-polyadenylation signal. (B) Components of the assembled specific promoter for muscle cell expression. Enh, enhancer; Prom, promoter; SD: splicing donor; UBC: Ubiquitin gene C; SA: splicing acceptor**.**

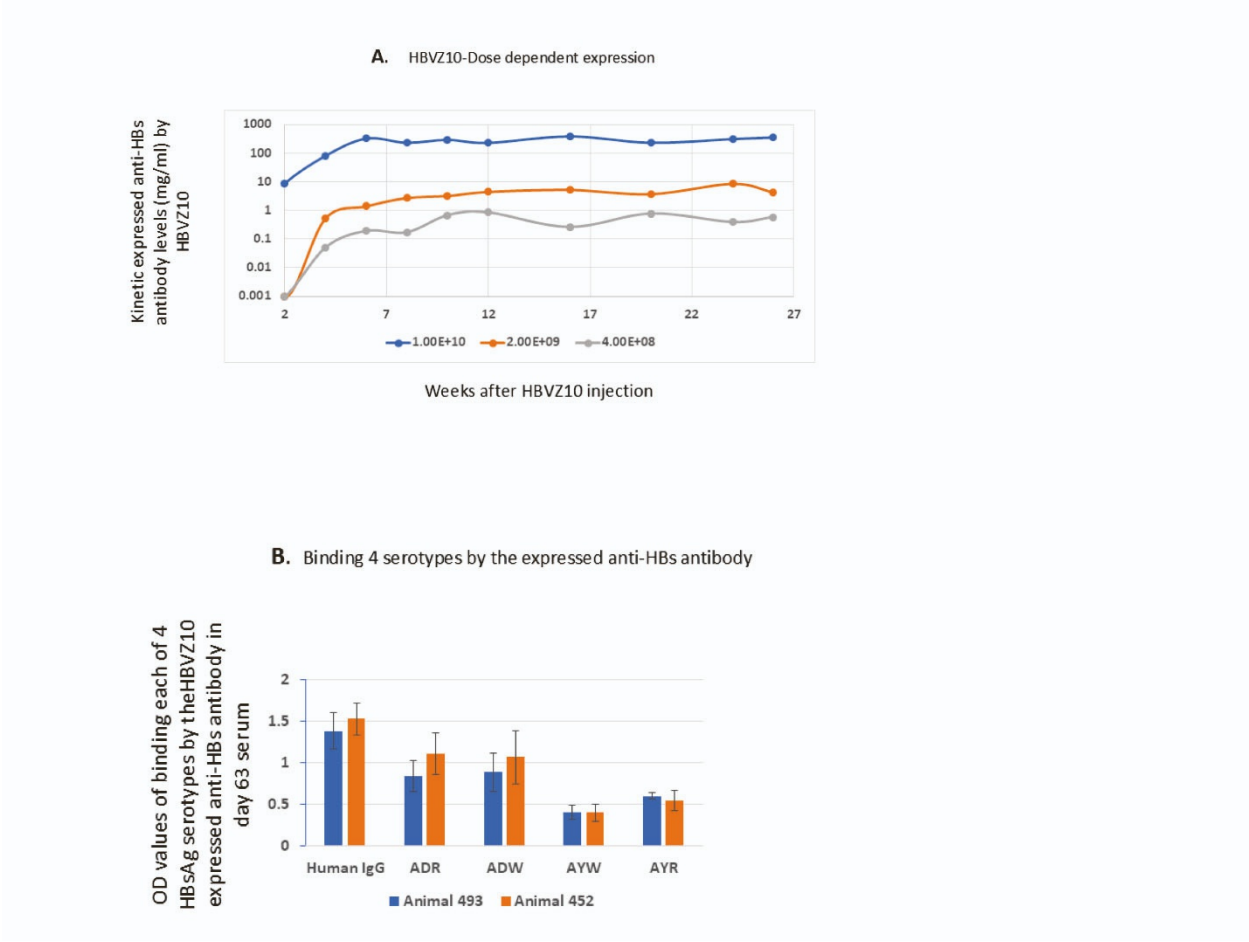

Figure S2. Expressing anti-HBs antibody levels with different doses of HBVZ10 using immunocompetent CD1 mice and its in vitro affinity. (A). Three doses of 1E11, 2E10, and 4E9 copies of HBVZ10 were intramuscularly injected into right thigh muscle of 3 groups of mice, respectively, and biweekly blood samples were collected for measuring anti-HBs antibody concentrations (µg/ml) during 26 weeks of duration. (B). The expressed anti-HBs antibody in vivo by HBVZ10 shows 50% higher affinity to HBsAg ADR and ADW serotype compared to AYW and AYR serotype. ELISA 96-well plate was coated with 100ng of each of human IgG, HBsAg ADR, ADW, AYW, and AYR antigens and 100µl of 1/10,000 diluted serum collected at day 63 pi from two chimeric mice (No: 493 and 452) who were intramuscularly injected with

1E10 copies of HBVZ10 on day 14, were added to corresponding wells for evaluating the ability to bind 4 HBsAg serotypes. Average OD values and standard deviations from triplicate are plotted.

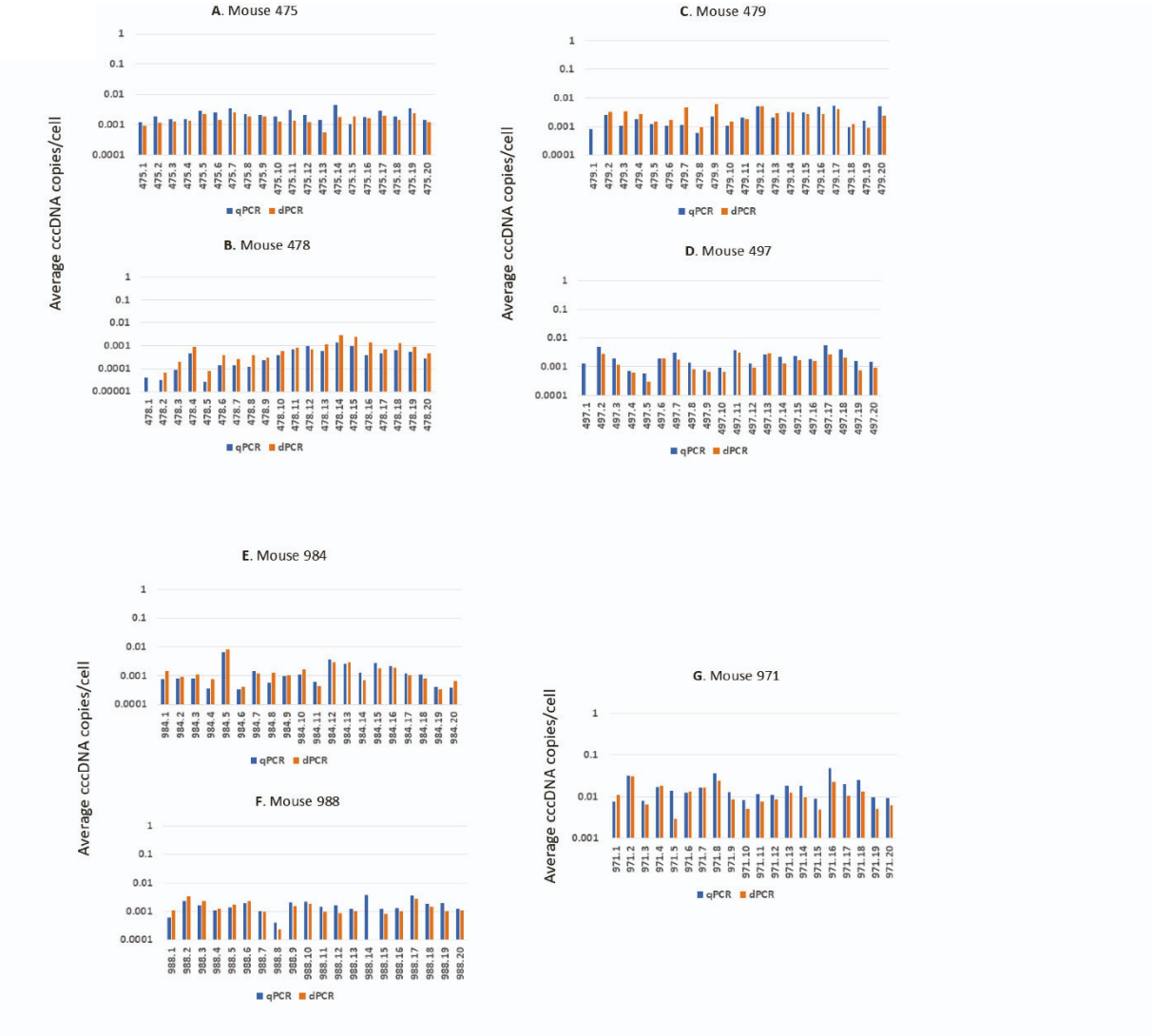

Figure S3. Comparable results of cccDNA quantification between qPCR with ABQ dPCR calibrated standards and ABQ dPCR among 140 cccDNA samples. **A-G** display cccDNA copies/cell among 20 cccDNA samples from mice 475, 478, 479, 497, 984, 988, and 971, respectively.

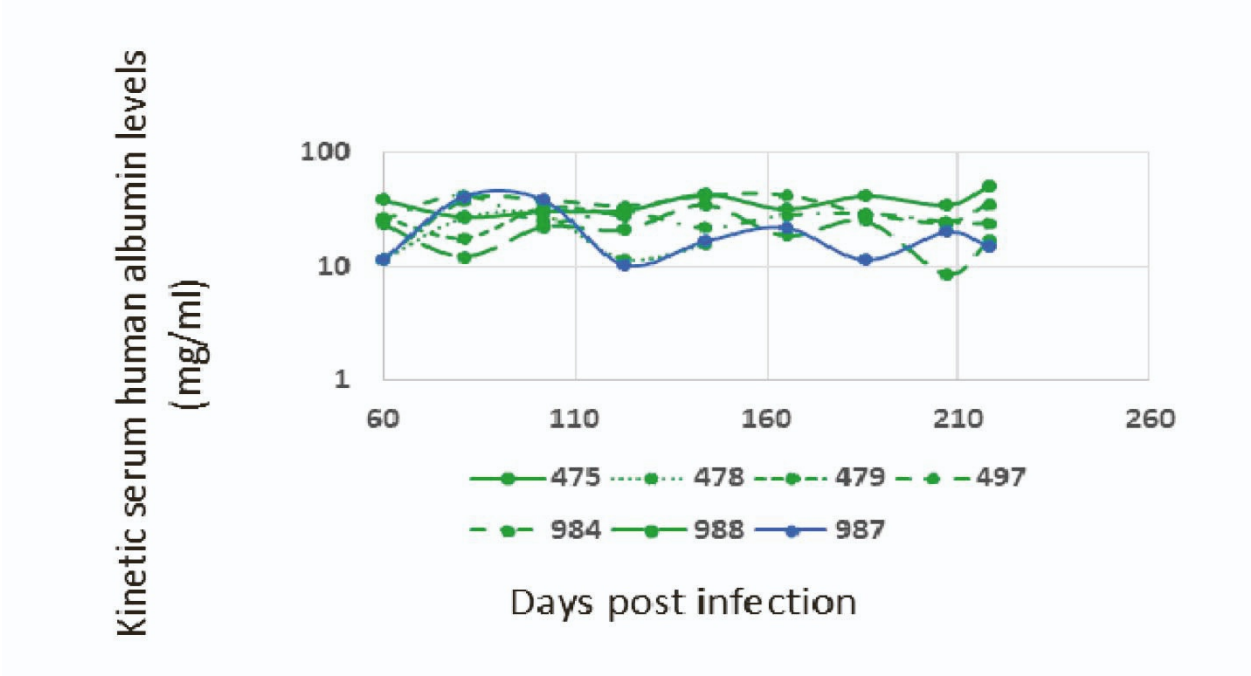

Figure S4. Kinetic serum human albumin levels. Kinetic human albumin levels were quantified in serial samples from 6 treated mice that eliminated cccDNA by >100-fold, accompanied by progressive reduction of serum HBsAg and HBeAg to undetectable levels upon complete blocking of de novo infection (green), and one untreated mouse (blue) as control.

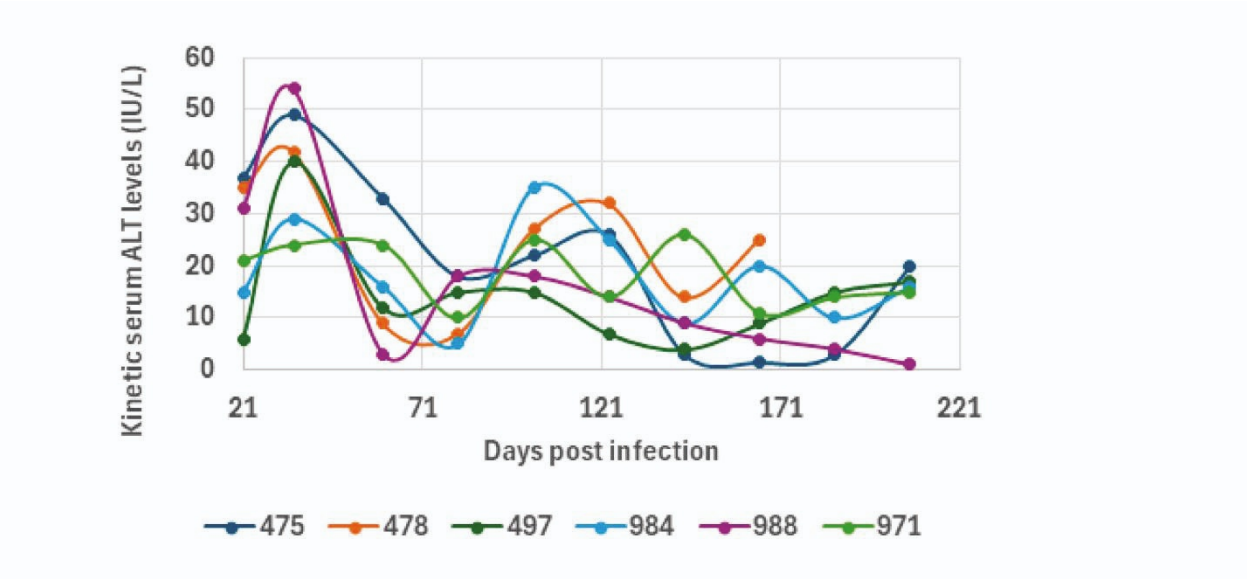

Figure S5. Kinetic ALT levels in serial blood samples of 6 mice treated with HBVZ10/anti-HBs antibody and 9 weeks of entecavir. Average cccDNA levels were reduced by 1-2 log accompanied by progressive reduction of HBeAg and HBsAg.