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Strategies to eliminate HBV infection

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Abstract

Chronic HBV infection is a major public health concern affecting over 240 million people worldwide. Although suppression of HBV replication is achieved in the majority of patients with currently available newer antivirals, discontinuation of therapy prior to hepatitis B surface antigen loss or seroconversion is associated with relapse of HBV in the majority of cases. Thus, new therapeutic modalities are needed to achieve eradication of the virus from chronically infected patients in the absence of therapy. The basis of HBV persistence includes viral and host factors. Here, we review novel strategies to achieve sustained cure or elimination of HBV. The novel approaches include targeting the viral and or host factors required for viral persistence, and novel immune-based therapies, including therapeutic vaccines.

Keywords

covalently closed circular DNA; cure; elimination; eradication; HBV; host; IL-7; IL-21; immunity; PD-1; PD-L1; receptor; RNAi; seroconversion; therapeutic vaccination; Toll-like receptor; treatment; vaccines; viral entry; virus

Approximately 240 million people worldwide have chronic HBV (CHB) [1]. Current therapy for HBV is aimed at achieving suppression of HBV replication at levels below detection. Although this can be accomplished in almost all patients, long-term management of CHB remains a challenge. Failure to achieve sustained response and HBV persistence is related to the viral factors and inadequate induction of immune response that are seen in

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acute HBV patients, which naturally clears the infection. HLA polymorphisms also determine the variability in host immune response by influencing host susceptibility to HBV infection.

Advancement in the understanding of the basis of HBV persistence has guided the development of strategies that could lead to a functional cure for HBV infection. We discuss the potential strategies under development to achieve a functional cure of hepatitis B by targeting the virus, host or both. Some of these interventions are currently experimental and some have attained preclinical validation, whereas few have reached active clinical trials at this time.

Current therapy for chronic HBV

Current therapy for CHB includes interferon (IFN) or PEGylated IFN- α (PEG-IFN- α), and/or nucleoside/nucleotide (NA)-based therapy, which includes lamivudine (3TC), adefovir, telbivudine, entecavir and tenofovir. The patients with CHB are at increased risk of progression to cirrhosis and hepatocellular carcinoma (HCC). CHB is responsible for approximately 600,000 deaths owing to its complications [1]. The main goal for current HBV therapy is to alter the progression of liver fibrosis and reduce the occurrence of HCC.

Long-term follow-up studies have shown that treatment with PEG-IFN with or without 3TC results in a prolonged clinical remission with durable viral suppression in hepatitis B envelope antigen (HBeAg)-positive CHB patients, increased rate of hepatitis B surface antigen (HBsAg) seroconversion and improved liver histology [2-4]. Response rate was also higher in genotypes A or B compared with C or D [5-7]; and with lower levels of HBV DNA [5] at baseline and higher levels of ALT [5,7]. Use of PEG-IFN therapy is limited by intolerance and adverse events.

The oral antiviral drugs, initially approved in USA (lamivudine, adefovir and telbivudine) were well tolerated but efficacy of these agents is limited by emergence of antiviral resistance. However, newer agents (entecavir and tenofovir) are more potent, well tolerated, and have a high barrier to antiviral resistance. Long-term follow-up studies on newer agents has shown sustained viral suppression along with improvement in biochemical and histologic evidence of disease [8,9].

Long-term therapy also led to decrease in fibrosis and regression of cirrhosis [10].

HBsAg loss was achieved in only 8% of HBeAg-positive patients after 3 years of tenofovir therapy [11]. Similarly, in another study, HBsAg loss rates for HBeAg positive patients treated with entecavir or lamivudine for 2 years were 5 and 3%, respectively [12].

Despite the tremendous improvement in CHB therapy, currently available treatment has several limitations.

Pitfalls of current therapy

• Chronic suppression without sustained cure

Current antiviral therapies are aimed towards inhibition of viral replication. Sustained viral suppression is associated with improved outcomes and it has been shown that elevated HBV DNA level is a strong predictor of increased risk of cirrhosis [13] and HCC [14,15] in CHB. None of the current therapies lead to HBsAg loss or seroconversion in the majority of patients. In addition, current antiviral therapies do not target defective immune response and persistence of covalently closed circular DNA (cccDNA) in the infected hepatocytes. Thus, the goal of current treatment is to achieve long-term virologic control since elimination or 'cure' is not possible.

• emergence of drug resistance

Long-term use of current antiviral agents (NAs), which target viral polymerase, is associated with selection of drug-resistant mutations. Changes in the viral polymerase also generate HBV surface protein variants due to the overlapping nature of the viral genome [16-18]. Emergence of drug resistance hinders long-term use of NAs.

Current end points of HBV treatment

There are several markers in use to evaluate treatment end points, which can be divided into biochemical, serological, virological and histological. All responses can be estimated at several time points during and after therapy [19].

Biochemical response is defined as normalization of ALT levels. It can be evaluated at several time points on-therapy, at the end and after the end of therapy [19].

Suppression of hepatitis B viral replication with no measurable serum HBV DNA when tested by a sensitive PCR assay during treatment and after completion of treatment is used as a virological end point. However, it may not be sustained long term and reactivation may occur, so it is not a definite marker of HBV elimination.

Serological response for HBeAg applies only to patients with HBeAg-positive CHB and is defined as HBeAg loss and seroconversion to anti-HBe [19]. Serological response for HBsAg applies to all CHB patients and is defined as HBsAg loss and development of anti-HBs [19]. HBsAg loss with or without development of anti-HBs is by far the most valuable surrogate marker for treatment end point. Loss of HBsAg correlates well with prevention of complications like cirrhosis and HCC [20-22].

Histological response is defined as a decrease in necroinflammatory activity (by 2 points in HAI or Ishak's system) without worsening in fibrosis compared with pretreatment histological findings [19]. For a detailed review on present standards for assessing end points of HBV treatment, please refer to [23].

• what does the elimination or sustained cure mean?

Despite the tremendous improvement in CHB therapy with new antivirals, viral replication typically rebounds after the treatment is stopped. Therefore, complete elimination or cure is still not possible with current treatment. The plausible explanation is the persistence of cccDNA, which plays a vital role in persistence and reactivation. Nucleoside analog therapy prevents further formation of cccDNA, but has no effect on existing cccDNA. Ideally, elimination of HBV can be defined by loss of HBsAg and seroconversion to anti-HBs antibody and sustained suppression of HBV DNA. This may also result in depletion or inactivation of cccDNA. Until we reach the point when elimination/eradication of cccDNA can be achieved, HBsAg can be used as a marker of surrogate for the level of transcriptionally active cccDNA. Several studies have shown a positive correlation between transcriptionally active cccDNA and HBsAg levels in CHB patients [24,25].

Basis of HBV persistence

Understanding the basis for HBV persistence is critical in designing therapeutic strategies to eradicate HBV. Chronic HBV infection is characterized by an evolving interplay between viral replication and host immune responses. Both viral and host factors contribute to persistence of HBV. Highly efficient and unique replication mechanism of the virus uses a transcriptional template, cccDNA that is sequestered inside the nucleus, and escapes detection by innate DNA sensing cellular machinery. Another factor is the production of viral proteins (HBsAg, HBeAg), which function as a tolerogen and leads to T-cell exhaustion [26]. Advancement in the understanding of the mechanisms involved in HBV persistence has helped to develop strategies that overcome these factors and could result in sustained virologic remission.

• viral factors

Error prone replication—HBV is a small, enveloped DNA virus with a very unique genomic organization and replication mechanism. Genome length of HBV is only 3200 bp compared with 10,000 bp for HIV and that multiple overlapping open reading frames (ORFs) may impose more constraints against variation on HBV than HIV. Despite the constraint imposed by ORFs, HBV replication is error prone due to lack of proofreading activity of HBV polymerase (error rate of 10^{-4} to 10^{-5}), which leads to an accumulation of a pool of genomic sequences with heterogeneous viral population, also called quasispecies [17,27]. These viral variants have a robust survival advantage in particular, when exposed to multiple selection pressures such as immunological pressure from hepatitis B immunoglobulin, NAs and/or vaccination.

Precore/core mutants—The viral core mRNA encodes a core protein (major nucleocapsid protein), DNA polymerase (which reverse transcribes RNA pregenome), and serves as pregenomic RNA, which acts as a template for reverse transcription. Precore mRNA encodes the precore protein, which is processed in the endoplasmic reticulum (ER) to produce HBeAg; the basal core promoter (BCP), nucleotide 1744-1804, resides in X ORF, and controls transcription of both precore and core regions [28]. A variety of precore and core mutants have been reported. There are two well-studied precore mutations: stop

codon mutation at nt 1896, which results in cessation of HBeAg expression, and a mutation in BCP at nt 1762 and nt 1764, which results in diminished production of HBeAg and a resulting increased host immune response [28]. These mutations lead to the development of HBeAg-negative CHB. Associations of precore mutants and increased pathogenicity have been described. Earlier studies have demonstrated that precore mutants might be associated with severe chronic liver disease and with acute liver failure. It has been shown that patients with detectable precore and/or BCP mutants have a lower probability of response and are less optimal candidates for PEG-IFN therapy [29]. Double mutations in BCP at nt 1762 and nt 1764 are reported to be associated with severe liver disease [30], fulminant hepatitis [31], cirrhosis and HCC [32,33].

HBV genotypes—HBV genotypes account for the heterogeneity in clinical manifestations and treatment response among patients with chronic hepatitis B in different parts of the world; several studies reported correlation of HBV genotype with clinical outcomes and response to treatment, especially IFN treatment [34]. To date, ten HBV genotypes (A-J) and several subtypes have been identified, defined by divergence in the entire HBV genomic sequences and distinct geographic distribution. Genotype A is found as an independent risk factor for progression to chronic infection and persistence following acute hepatitis B infection [35]. Acute infection with genotypes A and D results in higher rates of chronicity than genotypes B and C. Patients with genotypes C and D have lower rates of spontaneous HBeAg seroconversion as compared with genotype A and B. There is also a clear association between HBV genotypes, and precore and BCP mutations. Genotype C has a higher frequency of double mutation in BCP A1762T/G1764A, pre-S deletion and is associated with higher viral load than genotype B. Similarly, genotype D has a higher prevalence of BCP A1762T/G1764A mutation than genotype A. Genotype C and D are associated with more severe liver disease, including cirrhosis and HCC. Genotype A and B shows better responses to IFN-based therapy than genotypes C and D, but there are few consistent differences for NAs [36].

cccDNA—A major determinant in the slow kinetics of HBV clearance from infected cells and persistence is the presence of cccDNA. The HBV genome assumes a supercoiled configuration of cccDNA and exists in association with histones and DNA chaperone proteins as a minichromosome. This form allows HBV to persist inside the nucleus by avoiding host innate immune responses. Furthermore, infected hepatocytes have a long half-life, which allows the maintenance of cccDNA in the nuclei of infected cells indefinitely [37] and acts as reservoir for reactivation of viral genome replication. Studies have shown that drug-resistant mutations are archived in the cccDNA and can be rapidly selected out with the use of drugs that exhibits crossresistance [38-40]. The estimated 15-50 copies/cell of cccDNA in the nucleus serve as a store of viral escape variants generated by the error-prone viral polymerase [41]. Antiviral therapies with the currently approved antiviral agents suppress viral replication but do not directly target cccDNA. Thus, inactivation or elimination of cccDNA is one of the potential novel strategies for eradication of HBV.

• Host factors

Host genetics—Persistence of HBV and variability in disease outcome also depends on the multiple host factors. HLA type of an individual is an important factor that determines the variability in host immune response to HBV. Evidence from genome-wide association studies has shown that the HLA DRB locus alleles DRB1*1301/2 are consistently associated with spontaneous resolution of infection [42], whereas HLA-DR7 (DRB1*07) and HLA-DR3 (DRB1*0301) were found to be associated with increased susceptibility to chronic HBV [43-47]. Other HLA types associated with risk of chronic infection were HLA-DPA1(*)0202-DPB1(*)0501 and HLA-DPA1(*)0202-DPB1(*)0301 [48]. HLA-DRB1*0701 and DRB1*0301 have not only been associated with increased susceptibility to chronic infection but also with failure to respond to HBsAg-based vaccine [49,50]. Other alleles strongly associated with no/poor response are DRB1*03, DRB1*07, DQB1*02 and DPB1*1101 [51]. HLA-DRB1*0901, DQA1*0301, DQA1*0501 and DQB1*0301 are found to be consistently associated with persistent HBV infection in different ethnic groups [52-56]. Conceivably, this genotype influences host response by allowing more promiscuous binding of peptides to this allele than others, resulting in a broader T-cell response in subjects with the favorable allele and hence self-limiting infection [57].

Another genome-wide linkage study in siblings with CHB found that a region of linkage on chromosome 21 with single nucleotide polymorphisms spanning the IFN- α receptor II and IL-10 receptor II was associated with chronicity [57,58].

Host-viral interactions—Adaptive immune responses to HBV are blunted in CHB subjects when compared with those who have resolved acute infection. Studies have demonstrated that T cells responding to HBV antigens from these subjects have an exhausted phenotype and are less responsive to HBV antigens [59]. Furthermore, HBV antigens have been shown to interfere with innate immune recognition, by specifically hindering signaling of Toll-like receptor (TLR) 2, 7 and 9 molecules. These molecules are vital in generating an effective innate adaptive crosstalk that is the cornerstone of an effective anti-HBV immunity.

Novel strategies to achieve sustained virologic remission

• Targeting the virus

Targeting viral entry—HBV is an enveloped virus with tropism to infect hepatocytes and viral entry is mediated through specific interactions of viral membrane proteins with cellular receptors. Targeting viral entry with receptor antagonists provides us with new opportunities to treat HBV. Recently, *in vitro* studies, using the primary hepatocytes from tupaia, Yan *et al.* reported a functional receptor for HBV, sodium taurocholate cotransporting polypeptide (NTCP) [60]. NTCP is a sodium-dependent transporter for taurocholic acid, which is expressed at the basolateral membrane of hepatocytes and responsible for most Na⁺-dependent bile acid uptake in hepatocytes. In this regard, Myrcludex-B, a synthetic lipopeptide derived from pre-S1 domain of the HBV envelope protein, which specifically targets the NTCP has been shown to efficiently block HBV infection in *in vitro* [61,62] and in uPA/SCID mice reconstituted with human hepatocytes infected with HBV [63,64]. A

Phase IIa clinical study in CHB patients, investigating the safety, tolerability and efficacy of multiple doses of Myrcludex B in comparison with the control group receiving standard therapy with NAs, is recently completed. Results are awaited.

Targeting viral assembly/encapsidation—HBV persistence and transmission require HBV replication, which depends on the assembly of a core particle composed of capsid protein (Cp), polymerase, and pregenomic RNA. Assembly is one of the critical steps in viral replication, which could be an attractive target for therapeutics. There are multiple classes of compounds discovered that could dysregulate or inhibit virion assembly and encapsidation. Heteroaryldihydropyrimidines are compounds that inhibit HBV virion production *in vitro* and *in vivo* by preventing encapsidation [65,66]. One of the most studied heteroaryldihydropyrimidine compounds is Bay 41-4109, which inhibits capsid formation, concomitant with a reduced half-life of the core protein. These drugs inhibit viral replication by inducing assembly inappropriately and, when in excess, by misdirecting assembly, decreasing the stability of normal capsids [67-69]. These compounds are also active against HBV mutants resistant to NAs [70].

Similarly, phenylpropenamides have also been shown to inhibit viral encapsidation, and are found to be active against 3TC-resistant strains [70-72]. Phenylpropenamides are shown to induce tertiary and quaternary structural changes in HBV capsids. AT-130 (phenylpropenamide derivative) has been shown to bind to a promiscuous pocket at the dimer-dimer interface that favors a unique quasiequivalent binding site in the capsid and can serve as an effective antiviral agent. It decreases viral production by initiating virion assembly at the wrong point in time, resulting in morphologically normal capsids that are empty and noninfectious [73,74]. Clinical efficacy of these compounds has not been reported yet and needs to be studied.

Targeting HBsAg secretion—HBV persistence results from an ineffective anti-viral immune response towards the virus. The exact mechanism by which HBV escapes immunity is poorly understood. The initial response to viral infection results in activation of innate immune responses such as the production of type I IFNs (IFN- α and IFN- β).

Studies on HBV-infected chimpanzees demonstrated a complete lack of induction of type-1 IFN and IFN response genes during early stages of infection. It was recently shown that type-1 IFN responses are also lacking in acute HBV patients [75,76]. In this regard, the early stages of acute HBV are characterized by induction of IL-10 rather than type I IFN, accompanied by a temporary attenuation of natural killer (NK) cell and T-cell responses [77]. The suppression of innate immune response can also be mediated by direct interference of HBV antigens with host cells. High levels of HBsAg in the range of 400 $\mu\text{g/ml}$ (0.4% of total serum protein) have been demonstrated in HBV infected patients [78-80] and are thought to play an important role in suppressing the HBV-specific immune response. In this regard, recent reports have suggested that HBsAg acts directly on dendritic cells to limit cytokine production [81,82]. Thus, control of HBsAg secretion could potentially enable its use with the therapeutic vaccine or as a combination therapy with NAs for the treatment of HBV. Several classes of drugs have been studied to reduce HBsAg secretion [83]. *In vitro* data showed that nonspecific antimicrobial nitazoxanide and its active metabolite,

tizoxanide, reduced the levels of extracellular HBsAg, HBeAg, as well as the levels of intracellular HBcAg in a dose-dependent manner *in vitro*. Nitazoxanide was found to exhibit selective inhibition of intracellular HBV replication and extracellular virus production in cell cultures, and synergistic activity in combination with lamivudine or adefovir against HBV [83]. Recently, a series of novel triazolo-pyrimidine inhibitors of HBsAg secretion was identified using the HBV-expressing cell line HepG2.2.15, through high-throughput screening. The parent compound was shown to not be an inhibitor of viral genomic replication but rather a specific inhibitor of HBV envelope secretion. These triazolo-pyrimidine derivatives were also active in inhibiting HBsAg secretion of HBV variants that are resistant to current NAs [78,80]. The exact mechanism of action for these compounds is still under investigation, and it is unclear at the moment if the reduction of HBsAg secretion would be able to enhance HBV specific immunity *in vivo*.

The HBV genome is an extremely compact structure (~3 kb), which encompasses four overlapping ORFs encoding for viral polymerase/reverse transcriptase; the capsid-forming core protein; three envelope proteins called large (LHBs), middle (MHBs) and small hepatitis B surface antigens; and the regulatory X protein.

The HBV virion is a double-shelled sphere with an inner nucleocapsid and an outer lipoprotein envelope. In addition to infectious virion, HBV produces two other types of particles (subviral empty envelope particles and subviral naked capsid particles). The three envelope proteins are present in different proportions in three types of HBV particles. Noninfectious subviral particles (SVPs) share antigenic features of the virus envelope and are thought to presumably act as a decoy for the immune system [84]. In the past, it was believed that budding of infectious virions and SVPs used the same pathway. However, recent reports suggest that budding of infectious virions depends on functions of the multivesicular body pathway [84].

Nucleic acid polymer, amphipathic oligonucleotide is currently undergoing a proof-of-concept trial in patients with CHB. Rep 9AC' blocks the secretion of HBsAg SVPs without affecting the secretion of infectious virion (which is line with recent studies, suggesting that SVPs and infectious virion use different pathway for budding). Patients treated with REP 9AC' (nucleic acid polymer) cleared HBsAg from serum and some achieved seroconversion. Updated interim results were presented at the 49th annual meeting of the European Association for the Study of the Liver held in Amsterdam (The Netherlands). Patients who had cleared HBsAg from their blood with REP 9AC' monotherapy were subjected to combination treatment with REP 9AC' and either PEG-IFN or Thymosin- α (compound with immunomodulatory activity). Further increases in anti-HBV antibodies were observed in all patients with combination treatment. Further updates on this trial are awaited.

Targeting envelopment—All three HBV envelope proteins share the same S domain and contain N-linked glycosylation at amino acid 146 within the S domain. MHBs and LHBs contain the pre-S2 domain, while N-terminal of LHBs protein contains the pre-S1 domain. Envelopment of hepatitis B core particle (capsids) containing the HBV genome depends on the interaction of nonglycosylated pre-S sequences facing to the cytosol with defined regions on the core particle. The transport of the enveloped virus particles and SVPs

particles may indeed depend on glycosylation and processing of the viral MHBs glycoprotein.

Glucosidase inhibitors inhibit viral morphogenesis and infectivity, most likely by inhibition of glycosylation of envelope protein in ER [85-89]. This approach is important to disrupt the development of HBV envelope, thereby generating quasispecies, that is defective in its ability to bind target cells and establish infection. This is another promising approach to target HBV.

Targeting cccDNA—Current NA-based treatment can block the replication and formation of new cccDNA, but existing cccDNA in already infected cells is not affected directly by current therapies and it has a long half-life (33-50 days). The viral cccDNA in the nucleus serve as a store of viral escape variants generated by the error-prone viral polymerase and escape mutants conferring drug-resistance, which can cause drug resistance or viral rebound upon cessation of treatments.

Inactivation/elimination/degradation of cccDNA—A recently developed therapeutic approach that directly targets HBV cccDNA within cells is by use of zinc-finger nucleases (ZFNs). Zinc finger proteins can be used to block the transcription of cccDNA. ZFNs have proved as one of most versatile and effective classes of gene targeting reagents in recent years. ZFNs have separate DNA-binding and DNA-cleavage domains. For more detail on ZFN engineering please read reference [90].

There is evidence *in vitro* that ZFPs can be used to specifically target the cccDNA of duck HBV infection and inhibit viral transcription and replication [91]. Expression of the ZFPs in LMH cells undergoing the DHBV viral lifecycle resulted in decreased expression of viral RNA and protein expression compared with the empty vector control, without any apparent toxicity effects. In addition, the production of viral particles was also decreased in the presence of the expressed ZFPs [91].

ZFNs are able to cleave HBV DNA in hepatoma cells *in vitro*. However, it raises clinical challenge of off target effects and specifically delivering ZFNs to the liver of infected subjects [92]. This challenge could be overcome by the application of vector platform for delivery. Studies using adeno-associated virus as a vector platform is being explored to deliver designer nucleases to target cells. Adeno-associated virus vectors were found to be safe in clinical and preclinical applications [93].

Recently Cai *et al.* identified two structurally related disubstituted sulfonamides (DSS), termed CCC-0975 and CCC-0346, which were confirmed as inhibitors of cccDNA production, with low micromolar EC₅₀ in cell culture. The author demonstrated that DSS was able to synchronously reduce levels of HBV cccDNA and its putative precursor, deproteinized relaxed circular DNA (DP-rcDNA), without directly inhibiting HBV DNA replication in cell culture or reduction in viral polymerase activity. However, DSS compounds did not promote the intracellular decay of HBV DP-rc DNA and cccDNA, suggesting that the compounds interfere primarily with rcDNA conversion into cccDNA. In

addition, CCC-0975 was shown to reduce cccDNA biosynthesis in duck HBV-infected primary duck hepatocytes [94].

Another very recent study demonstrated APOBEC-dependent degradation of HBV cccDNA induced by IFN- α and lymphotoxin- β receptor on HBV-infected, differentiated HepaRG (dHepaRG) cells and primary human hepatocytes. HBV core protein mediated the interaction with nuclear cccDNA, resulting in cytidine deamination, apurinic/apyrimidinic site formation, and finally cccDNA degradation that prevented HBV reactivation [95].

Epigenetic silencing of cccDNA—Another approach of targeting cccDNA is by epigenetically silencing or transcriptional repression. There is evidence of epigenetic silencing of cccDNA by IFN- α in cell culture and in humanized mice. IFN- α was shown to suppress HBV replication by targeting the epigenetic control of cccDNA function and transcription [96].

Targeting viral mRNA—Viral mRNA can be directly targeted using antisense oligonucleotides, ribozymes, or RNAi. *In vitro* data exist that show that HBV transcript levels can be reduced by using anti-sense oligonucleotides [97], hairpin ribozymes [98] or hammerhead ribozymes using lentiviral vector for delivery [99].

RNAi is one of the fastest moving fields in modern biotechnology. RNAi is a process by which small interfering RNA molecules induces gene silence at post-transcriptional level to effectively knock down the expression of genes of interest. In mammalian cells, it can be used specifically to target the degradation of cellular mRNA [100]. Due to extensive use of ORFs with the DNA genome in HBV, multiple HBV RNAs will make the virus susceptible for RNAi [101]. Several studies have shown that viral mRNA and HBV replication can be inhibited by using RNAi in cell cultures and in mice models [102-105]. McCaffrey *et al.* showed that RNAi could be applied to inhibit production of HBV replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with a HBV plasmid. RNAi was able to inhibit all the steps of HBV replication that occur in cell culture and in mice. They found four separate lines of evidence to establish that RNAi substantially inhibited HBV in mice: RNAi expression significantly reduced secreted HBsAg in serum; HBV RNAs were substantially reduced in mouse liver; HBV genomic DNA was reduced to undetectable levels in mouse liver; and the number of cells staining for HBV core antigen (HBcAg) was substantially decreased [103]. RNA-i could be used as a novel therapeutic approach, however, several challenges such as efficient delivery *in vivo*, RNA instability and off target effects exists and needs to be overcome before it can be exploited in treatment of CHB patients.

• Targeting the host

Recovery from acute HBV infection is associated with robust innate and adaptive immune responses. Innate immune response is the first line of defense against viral infections and results in production of type I IFN, which leads to suppression of viral replication, mediation of NK cell-mediated killing of viral infected cells, and supports the efficient maturation and site recruitment of adaptive immunity through production of proinflammatory cytokines and chemokines [59,106]. These IFN enhance the first defense against viral infections and

modulate both innate and adaptive immune cells [77,107]. The principal producers of type I IFN are the plasmacytoid dendritic cells (pDC) that respond to viruses and other pathogens primarily through the recognition of pathogen-associated molecular patterns by two intracellular TLRs: TLR7 and TLR9 [77,108]. Triggering of TLR leads to activation of pDC and production of high levels of type I IFNs, along with the release of other cytokines, including TNF- α , IL-6 and cell surface costimulatory molecules. pDC also activate NK cells and T lymphocytes, allowing further priming and regulation of antiviral immunity [107,109-110]. Efficient priming of the adaptive immune system causes functional maturation and expansion of distinct B- and T-cell clones, which are able to specifically recognize the infectious agents. This process leads to control of infection and generates a memory response that increases the host ability to block subsequent infections with the same pathogens [59].

In CHB patients, HBV is associated with blunting of innate and adaptive immune responses. Hence, strategies that would augment innate immune responses may also enhance adaptive anti-HBV immunity. Several studies have demonstrated that expressions of TLR (TLR 2, TLR 3, TLR4, TLR7 and TLR9) were decreased in CHB patients [111-114]. In this regard, HBV interferes with TLR2, 7 and 9 signaling, which are considered to play an important role in the control of infection and elimination of virally infected cells.

TLR agonists—Studies have shown that when HBV transgenic mice were injected with ligands specific for TLR 2-9, liver of these HBV transgenic mice produce IFN- α , - β and - γ to inhibit HBV replication, suggesting that HBV replication can be controlled by the activation of innate immune response in the liver [115]. The inhibition of HBV replication was accomplished at a post-transcriptional level by suppressing the assembly or stability of HBV RNA-containing capsids [115,116]. These provide evidence that TLR activation directly inhibits HBV replication [117,118]. However, HBV somehow evades innate recognition by TLRs as a strategy to escape innate immune response by its ability to disrupt TLR expression and inhibit TLR signaling cascades [117,118]. It has been reported that the expression of TLRs in hepatocytes and other cells is downregulated in the presence of various HBV viral products [112,119-123]. Although HBV circumvents endogenous type I IFN pathways, it is plausible that the use of exogenous IFN induction using the TLR7 agonist may reinstate the IFN- α response. When combined with a strategy that results in maximal suppression of HBV replication *in vivo* using NAs, exogenous IFN stimulation via TLR agonists may result in development of protective immunity. Several studies have shown that long-term suppression of HBV using NA results in partial reconstitution of adaptive immunity. In this regard, an adjuvant therapy using TLR agonist may be able to accelerate this process of immune reconstitution and HBV clearance. First, TLR agonists are available as oral compounds enabling rapid uptake by the liver. Second, they may allow being combined along with other NAs as a single pill. Finally, similar to injected IFN, TLR agonists induce IFN production, triggering the production of cytokines to facilitate intracellular communication and cellular trafficking. However, through the use of TLR agonists this antiviral state can be induced at the liver, eliminating the adverse events associated with systemic innate immune activation.

Lanford *et al.*, investigated the effects of immune activation with GS-9620, an orally administered agonist of TLR-7, in chimpanzees chronically infected with HBV. GS-9620 was administered to chimpanzees every other day for 4 weeks at 1 mg/kg and, after a 1-week rest, for a second cycle of 4 weeks at 2 mg/kg. TLR-7 agonists induced prolonged suppression of HBV DNA in both the serum and liver [124]. GS-9620 administration induced the production of IFN- α and various cytokines and chemokines. In addition, it activated all lymphocyte subsets to induce interferon stimulated genes (ISGs) [124]. HBV DNA was reduced, in addition to serum levels of HBsAg, HBeAg and HBV antigen positive hepatocytes. However, an increase in liver enzymes and immune cell infiltration was observed during the period of decrease in both intrahepatic and serum viral load.

In early studies to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of oral GS-9620 in healthy volunteers, oral doses (single dose of 0.3, 1, 2, 3, 4, 6, 8 or 12 mg) were well absorbed and well tolerated. Adverse events associated with IFN treatment were seen in subjects who received the 8 or 12 mg dose and serum IFN- α was only detected at these doses although activation or ISGs were seen at doses $\geq$ 2 mg [125]. Two Phase I clinical trials of GS-9620 on CHB patients are completed. One in virologically suppressed subjects with CHB, and other treatment naive subjects with CHB [126,127]. It will be interesting to know the results of these trials.

Cytokines—The use of cytokines as immunomodulatory therapy for the treatment of chronic viral infections has been extensively studied. Most promising candidates that may be beneficial in CHB patients are IL-7 and IL-21 [128-130].

IL-7—IL-7 is absolutely essential for primary T-cell development and probably has an important role in the normal B-cell development process. IL-7 also plays a role in the development of some dendritic cell (DC) subsets. IL-7-mediated signaling in DCs has been shown to regulate peripheral CD4⁺ T-cell homeostasis [131]. IL-7 therapy for CHB patients would be to enhance, rejuvenate and restore immune response to HBV. Preclinical data generated from numerous model systems have shown that IL-7 has potent immunorestorative effects, as well as vaccine adjuvant effects and beneficial effects in the setting of adoptive cell therapy. CYT107 is a second-generation recombinant human IL-7 product made by Cytheris SA via a recombinant mammalian cell culture system [132]. A Phase I/II randomized, open labeled, controlled, dose-escalation study of repeated administration of recombinant human IL-7 (CYT107), in combination with standard antiviral treatment and vaccination in HBeAg-negative CHB patients is ongoing [133].

IL-21—IL-21 mediates an important function in the induction and maintenance of effector CD8⁺ T cells. Several studies done on chronic lymphocytic choriomeningitis virus (LCMV) infection in mice have shown that IL-21 signaling is required to maintain a functional pool of effector CD8⁺ T cells. Mice deficient in IL-21 or IL-21R show a progressive decline in the number of virus-specific polyfunctional effector CD8⁺ T cells, which correlates with poor viral control [134-136]. In CHB infection, IL-21 was shown to be critical in promoting immune responses that can control infection in mice [137].

In a longitudinal study of CHB patients, it was demonstrated that patients treated with antivirals with complete suppression had significantly higher levels of serum IL-21 than those who did not [138]. High serum IL-21 concentrations were also predictive of HBeAg seroconversion, a clinically important outcome associated with control of HBV infection [138]. Recombinant IL-21 is a new immune modulator currently undergoing Phase I and II testing in cancer patients [139]. Use of recombinant IL-21 for CHB might be a promising approach as a combination therapy for CHB patients receiving NAs.

Programmed death-1/programmed death ligand-1—Chronic and persistent HBV infection is associated with weak and functionally impaired immune response. The persistent exposure to viral antigens leads to virus-specific CD4 and CD8 cell dysfunction or deletion, and with prolonged exposure leads to exhaustion of T-cell response.

Recent data suggest these virus specific T cells hyperexpress the PD-1 molecule and interaction between programmed death-1 (PD-1) receptor on lymphocytes and its ligand programmed death ligand-1 (PD-L1)/2 plays an important role in T-cell exhaustion [140-145]. *In vitro* and *in vivo* data suggest that inhibition of PD-1/PD-L1/2 ligand, which blocks the engagement of PD-1 with its ligand (PD-L1/2), has shown improvement in the antiviral functions of these T cells [146-152]. Studies have shown that HBV-specific T cells express PD-1 at a higher level than other T cells and exhibit exhaustive functionality as determined by cytokine secretion [140-141,143-144]. Attempts have been made to reverse the PD-1-PD-L interaction *in vitro* to rejuvenate HBV-specific immunity. In this regard, *in vitro* blockade of PD-1/PDL1 in a woodchuck hepatitis model with chronically infected WHV showed restoration of T-cell function [153]. Moreover, *in vivo* blockade of PD1/PD-L1 along with therapeutic vaccination and antiviral NA treatment in persistently WHV-infected woodchucks showed that this combination resulted in potent antiviral effect and improved function of woodchuck hepatitis core antigen-specific CD8 T cells [154].

Fisicaro *et al.* examined the role of T-cell exhaustion in the pathogenesis of chronic HBV infection in patients with CHB. They compared phenotype and function of intrahepatic and circulating HBV-specific T cells, and effect of PD-1/PD-L1 blockade. Results showed that intrahepatic HBV-specific CD8 cells express higher PD-1 and lower IL-7 receptor, CD127 levels. PD-1/PD-L1 blockade led to T-cell restoration, both in the periphery and in the liver, with better functional improvement among intrahepatic T cells [155].

Intravenous PD-1 and PD-L1 antibodies have been tested on patients with advanced cancer. Antibody-mediated blockade of PD-L1 induced durable tumor regression and prolonged stabilization of disease in patients with advanced cancers [156]. However, use of anti-PD-1 antibody was associated with a relatively high frequency of grade 3 or 4 adverse events (adverse events occurred in 14% of patients including three deaths from pulmonary toxicity) [157]. Utilization of PD-1 blockade as a therapeutic modality for CHB patients is being investigated. This approach is unique and offers an excellent opportunity to revive exhausted T cells in CHB, thereby allowing restoration of adaptive immunity against HBV and offers a fair chance of achieving sustained virologic remission. Utilizing any approach to block central immunoregulatory mechanisms is associated with unsuspected complications. These constitute a risk profile that may not be acceptable to otherwise healthy CHB patients as

compared with terminally ill cancer patients. Use of anti-PD-1 antibodies offers the best hope of blocking PD-1/L12 interaction, as PD-L1 block would still allow PD-1-PDL2 interaction, however, anti-PD-1 antibodies have been associated with most adverse events and may require further studies prior to application as a therapeutic agent for CHB.

Tregs—Tregs consist of different T-cell subpopulations, including naturally occurring CD4⁺ CD25⁺ Tregs, induced Tregs (IL-10 producing CD4⁺ type I Tregs (Tr1) and T helper type 3 (Th3) cells), and CD4⁺ CD25⁺ T cells that develop in the periphery by conversion of CD4⁺ CD25⁻ T cells. Experimental data suggest that circulating CD4⁺ CD25⁺ Tregs may suppress HBV-specific T-cell responses in CHB patients resulting in persistence of HBV [158].

Despite all the above observations, a study on WHV-infected woodchucks, treated with IL-12 in combination with a TGF- β inhibitory peptide or Treg depletion showed that TGF- β inhibition or Treg depletion had no antiviral effect, instead an enhancement of the intrahepatic tolerogenic environment was observed [159]. Since there are no distinct phenotypic characteristics of Tregs available to target, it will be a difficult task to interfere with the function of Tregs *in vivo*.

Therapeutic vaccinations—Therapeutic vaccination presents a promising strategy in approach towards HBV eradication. As discussed before, HBV-specific T-cell exhaustion due to persistent antigen stimulation is considered a major determinant of HBV persistence or chronicity. A therapeutic vaccine, which could induce a potent CD4⁺ T-cell response, counteract immune tolerance, activate humoral immune response and stimulate CD8⁺ T cells directed against one or more HBV antigens, could achieve sustained control of CHB. During the past several years, different therapeutic vaccines have been developed and investigated in CHB patients with different clinical outcomes.

Several categories of therapeutic vaccines are being developed for CHB infection, which includes: vaccines based on recombinant HBV proteins, HBV-envelope subviral particles, naked DNA eventually combined with viral vectors and vaccines based on T-cell peptide epitopes derived from different HBV proteins [160-162].

Early classical therapeutic vaccines were based on the HBsAg protein that proved to be excellent in terms of its prophylactic potential, however, use of these vaccines have failed to reach expectations in terms of therapeutic efficacy. Antiviral effect of conventional prophylactic HBsAg-based vaccines were only transient and did not result in sustained viral suppression [163]. This is probably because most patients with CHB have diminished HBV responses due to exhaustive T-cell response and do not respond to classical immunizations.

Therapeutic vaccination based on recombinant HBV proteins or HBV-envelope sub-viral particles

Immunogenic - complexes: A double-blind placebo-controlled Phase IIb trial of a therapeutic HBV vaccine based on antigen-antibody immune complexes (HBsAg with antiHBs immunoglobulin) has been conducted in 242 patients with CHB presenting some initial evidence of clinical and virological efficacy [164]. These patients received antigen-

antibody immune complexes with alum as adjuvant with the aim of targeting DCs. The rationale of this combination was based on the hypothesis that immune complex-loaded DCs were superior in efficiently priming HBV-specific CD8⁺ cytotoxic T cells responses *in vivo* compared with naturally occurring immune complexes [165].

However, the results of Phase III clinical trial failed to show the therapeutic efficacy of immune complex-based vaccination [166].

HbsAg & HbcAg combination: Another approach involves the nasal HBV vaccine candidate, comprising HBsAg and core (HBcAg) as vaccine antigens. The nasal HBV vaccine candidate is based on the results of preclinical studies that have demonstrated a good immunogenicity and safety profile. Recombinant HBcAg can act as a potent Th1 adjuvant to HBsAg, as well as a strong immunogen [167,168]. This was tested in a Phase I double-blinded, placebo-controlled randomized clinical trial in healthy volunteers that demonstrated the safety and immunologic efficacy of this combination approach [169]. At present, a Phase III clinical trial is ongoing with HBsAg/HBcAg-based combined vaccine through the nasal and subcutaneous route in CHB patients and results are awaited [170].

whole recombinant yeast-based therapeutic vaccine: A yeast-based immunotherapy platform, Tarmogens (targeted molecular immunogen), is currently under development. Tarmogen incorporates multiple viral antigens, expressing HBV X, surface (S), and core Ags (X-S-core) and has been shown to induce both CD4⁺ and CD8⁺ T-cell responses in healthy and CHB patients *ex vivo*. The GS-4774 is a tarmogen that consists of whole, heat-killed, recombinant *Saccharomyces cerevisiae* yeast, which is genetically modified to express HBV antigens. Use of entire yeast results in preferential uptake/processing by dendritic cells rather than B cells to present HBV antigens to T cells, theoretically resulting in a more efficacious cellular immunity compared with a predominantly humoral immunity with the use of subunit vaccines.

Guo *et al.* demonstrated antigen-specific T-cell responses generated in mice immunized with two candidate vaccines mentioned above [171]. In addition, the data showed that GS 4774 (X-S core) significantly protected mice from tumors engineered to express HBV antigens. In a second study by Kemmler *et al.*, both tarmogens elicited HBV-specific T-cell responses *ex vivo* in samples collected from healthy individuals and donors with CHB [172].

Recently, murine and human immunogenicity models were used to evaluate the type and magnitude of HBV-Ag specific T-cell responses elicited by the vaccine. Mice immunized with yeast expressing X-S-core showed T-cell responses to X, S and core. Both CD4⁺ and CD8⁺ T-cell responses were observed. Human T cells transduced with HBc18-27 and HBs183-91 specific T-cell receptors (TCRs) produced IFN- γ following incubation with X-S-core-pulsed DCs. Furthermore, stimulation of peripheral blood mononuclear cells isolated from CHB patients or from HBV vaccine recipients with autologous DCs pulsed with X-S-core or a related product (S-core) resulted in pronounced expansions of HBV Ag-specific T cells possessing a cytolytic phenotype. These data indicate that X-S-core-expressing yeast elicits functional adaptive immune responses. This therapeutic vaccine seems promising in

inducing HBV-specific T-cell responses in patients with CHB [King TH *et al.* UnpublishedData].

Tarmogen, GS-4774 is being tested in combination with direct-acting antivirals in CHB patients to determine if the combination can increase rates of HBsAg seroconversion. A Phase I trial on GS-4774 has been completed and a Phase II, randomized, open-label study to evaluate the safety and efficacy of GS-4774 for the treatment of virally suppressed subjects with CHB is currently ongoing. Ongoing Phase II studies will determine whether yeast-based vaccines would result in unexpected allergic adverse reactions to common yeast infections in these patients.

Adeno-virus-based therapeutic vaccination: TG1050 is a therapeutic vaccination based on a recombinant nonreplicative human adenovirus serotype 5, expressing multiple specific HBV antigens (truncated core, modified polymerase and HBsAg domains). The product has been designed to prime *de novo* and/or stimulate functional T cells expected to control the HBV replication and to clear HBV. In an experiment by Perrine Martin *et al.*, TG1050 was found to induce high levels of T cells targeting core, polymerase and HBsAg domains in naive mice. In the Adeno-associated virus (AAV) tolerant mouse model, a single injection of TG1050 was shown to induce functional and long lasting T cells producing IFN- γ , TNF- α and IL-2, which were detected in spleen and liver without elevation of ALT. Experiments are ongoing to analyze immunological and virological effects of multiple injections of TG1050 as well as longer follow-ups [173].

• Therapeutic vaccination based on DNA & T-cell peptide epitope

DNA vaccine: DNA vaccines carry the potential to induce T-cell responses. Both CD4⁺ and CD8⁺ cells were elicited by the DNA vaccine, with the helper cells being of the Th1 phenotype, secreting IFN- γ [174]. This vaccine was shown to activate not only the T-cell responses specific to HBV but also NK cells [175].

In one study using DNA vaccine comprising of most HBV genes encoding multiple HBV proteins (i.e., envelope, nucleocapsid and polymerase) plus genetically engineered IL-12 DNA (IL-12N222L) was used in 12 CHB carriers who were being treated with antiviral, 3TC. Detectable HBV-specific IFN- γ secreting T-cell responses were observed at the end of treatment and during a follow-up. These type 1 T-cell responses, particularly CD4⁺ memory T-cell responses were maintained for 40 weeks after the therapy was stopped and correlated with virological responses [176].

T-cell peptide epitope vaccine: In a Phase I trial conducted in healthy volunteers, a polyepitope-based vaccine was used. This consisted of a DNA vector coding for a string of 30 HBV-derived cytotoxic T-cell epitopes linked to 16 Th epitopes, which was expected to be presented to T cells by a large number of HLA molecules. This vaccine was found to be safe and well tolerated in all healthy volunteers [177].

• **Combination approach**—Chronic HBV infection is associated with T-cell exhaustion in the presence of HBV replication and antigen load. Reducing the viral load with antiviral therapy prior to vaccination could provide improvement in vaccine efficacy. Studies have

shown that therapeutic vaccinations are more effective when used in patients with low HBV DNA load in serum at the start of treatment [178,179]. Recent vaccination trials are based on this approach. Therapeutic vaccination is used concomitantly with antiviral agents to induce T-cell restoration with suppression of viral replication and antigen load, which is an important factor responsible for induction of tolerance [180,181].

Conclusion & future perspective

Since sustained virologic remission of HBV does not occur as a result of existing treatment, novel strategies will have to be adopted. A learned approach to achieve this goal is to circumvent the various factors by which HBV establishes chronic infection in humans. Targeting a combination of viral and host factor offers the best possible chance for accomplishing this objective. Safety of a variety of these approaches, especially those targeting host immune responses have not yet been established. If these approaches are safe, they offer a unique perspective to target host immunity against HBV proteins and eventually develop protective immunity and control of HBV replication. A key viral target remains cccDNA, which requires unique approaches, which have shown *in vitro* effectiveness. The challenges remain on developing successful delivery system that allows every single infected cell to be subjected to disruption of ccDNA. In reality, a combination approach would be the most appropriate, effective and pragmatic technique to achieve sustained virologic remission of most CHB patients (Figure 1, Table 1 & Table 2).

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- of interest;
- of considerable interest

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EXECUTIVE SUMMARY

Current therapy for chronic hepatitis B

- Current therapy for chronic hepatitis B (CHB) includes IFN, PEG-IFN- α , and or nucleoside/nucleotide analogs (NAs).

Current goal & end points of HBV treatment

- Currently, the goal of HBV treatment is the prevention of long-term complications, such as cirrhosis and hepatocellular carcinoma.
- End points of HBV treatment are:
 - Suppression of HBV DNA from serum after completion of therapy;
 - Clearance of HBeAg in HBeAg positive patients with or without development of anti-HBe-Ab;
 - Normalization of biochemical markers;
 - Histologic decrease in necro-inflammatory score and possibly regression of fibrosis on liver biopsy.

Pitfalls of current therapy

- Sustained viral suppression off therapy or 'cure' of HBV is seldom achieved with current therapy.
- Clinical use of PEG-IFN- α for treatment of CHB is limited owing to low response rates and side effects. Durable response to PEG-IFN- α is higher in genotype A or B compared with C or D.
- HBV replication cycle relies on reverse transcription, which is error prone with mutations estimated to occur at a rate of approximately 10^{-4} substitutions per base per cycle, which could lead to pre-existing mutations in an infected individual along with emergence of resistance under pressure of NAs.
- Use of oral antivirals (NAs) is limited owing to prolonged duration of therapy leading to impaired adherence, and also emergence of antiviral resistance.
- Newer NAs (tenofovir and entecavir) has shown to be more effective in terms of efficacy, with very high barrier to emergence of resistance. However, rate of sustained elimination is still very low.

Basis of HBV persistence

- Both viral and host factors including host virus interactions contribute to persistence of HBV.
- Most subjects with acute HBV infection resolve viral hepatitis spontaneously.
- Most chronic subjects have impaired innate and adaptive immunity.
- Error prone replication, precore mutants and persistence of cccDNA are the major viral determinants of CHB.

- HBV proteins target key immune cells and surface molecules to circumvent mounting of an antiviral immunity.

Novel strategies to achieve sustained virologic remission

- Potential approaches to target virus include inhibition of viral entry, HBsAg production, and elimination or silencing of cccDNA; most of these studies are currently in preclinical stages/experimental (*in vitro* and *in vivo* data).
- Approaches to target the host include nonspecific inhibition of immunoregulatory pathways and boosting of HBV specific immunity (preclinical, Phase I and II data).
- Realistically, a combination approach may be necessary to achieve sustained virologic remission.

Figure 1. HBV lifecycle showing novel approaches for viral targets

(A) Viral entry; (B) cccDNA; (C) viral mRNA; (D) assembly/encapsidation; (E) envelopment in ER; (F) SVP and HBsAg. Mature viral capsid enveloped in ER and is secreted through MVBs. SVPs are processed and secreted through the ER/Golgi pathway. Possible novel targets are represented by question marks. cccDNA: Covalently closed circular DNA; DSS: Disubstituted sulfonamide; ER: Endoplasmic reticulum; HAP: Heteroaryldihydropyrimidines; HBsAg: Hepatitis B surface antigen; MVB: Multivesicular body; NTCP: Sodium taurocholate cotransporting polypeptide; SVP: Subviral particle; ZFN: Zinc finger nuclease.

Table 1
Viral factors

Factors	Targets	Results	Ref.
Viral entry (NTCP receptor at basolateral membrane of hepatocytes)	Mycludex-B	Specifically targets NTCP and efficiently block infection Phase II trial on CHB patients recently completed - awaiting results	Cell culture: [61,62] uPA/SCID mice reconstituted with PHH infected with HBV: [63,64]
Viral assembly/ encapsidation	HAPs; Bay 41-4109 (HAP compound) Phenylpropenamides; AT-61; AT-130	Inhibit HBV virion production by inappropriate assembly of capsids (no clinical data available yet) Induces structural changes in HBV capsids; initiates virion assembly at wrong point in time (no clinical data available yet)	Cell culture: [65,68-69] HBV Cp(Cp149) expressed in <i>Escherichia coli</i> : [66,67] Cell culture: [71] HBV Cp(Cp149) expressed in <i>E. coli</i> : [73,74]
HBsAg secretion/ inhibition	I: Nitazoxanide and its active metabolite, tizoxanide II: Triazolo-pyrimidine inhibitors III: NAP (Rep 9AC)	I and II: selective inhibition of intracellular HBV replication and extracellular virus production; reduce levels of extracellular HBsAg, HBeAg, and levels of intracellular HBcAg in a dose-dependent manner. Synergistic activity in combination with 3TC or adefovir against HBsAg secretion <i>in vitro</i> III: blocks HBsAg SVPs without affecting secretion of virions	I: cell culture: [83] II: cell culture: [78,80] III: Proof of concept Phase I/II clinical trials on going. Interim results presented at the 48th annual meeting of EASL [182]
Envelopment	Glucosidase inhibitors	Inhibition of glycosylation of envelope proteins in ER	Study in animal model (WHV): [87,88] Cell culture (Hep G2 cells): [86,89]
cccDNA	ZFNs AAV vector platform to deliver ZFNs DSS: CCC-0975 and CCC-0346 IFN- α and lymphotoxin- β receptor - APOBEC-dependent degradation of cccDNA IFN- α	Block transcription of cccDNA leading to inhibition of viral replication Block conversion of rcDNA to cccDNA IFN- α and lymphotoxin- β receptor activation upregulated APOBEC3A and APOBEC3B cytidine deaminases resp. resulting in cccDNA degradation Epigenetic silencing of cccDNA; suppress HBV replication by transcriptional repression of cccDNA	Cell culture (LMH cells + DHBV): [91] Cell culture (Huh7/pTHBV2 HBV model system): [92,93] Cell culture (DHBV infected PDH): [94] Cell culture (HBV-infected cells, PHH, and dHepaRG cells): [95] Cell culture and humanized mice: [96]
Viral mRNA	I: antisense oligonucleotide II: ribozymes III: RNAi	<i>In vitro</i> evidence of inhibition of viral replication	I: cell culture: [97] II: cell culture: [98,99] III: cell culture and mice transfected with an HBV plasmid (small animal model): [102-104]

3TC: Lamivudine; cccDNA: Covalently closed circular DNA; CHB: Chronic HBV; Cp: HBV core promoter; DHBV infected PDH: Duck HBV-infected primary duck hepatocytes; dHepaRG: Differentiated HepaRG cells; DSS: Disubstituted sulfonamides; EAST: European Association for the Study of the Liver; HAP: Heteroaryl dihydropyridine; HBcAg: Hepatitis B core antigen; HbeAg: Hepatitis B envelope antigen; HbsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NTCP: Sodium taurocholate cotransporting polypeptide; PHH: Primary Human hepatocytes; ZFN: Zinc finger nuclease.

Table 2
Host factors

Factors	Targets	Results	Ref.
Innate immunity			
PRRs (TLRs)	TLR-7 agonist: GS-9620	Suppression of HBV DNA in both serum and liver of HBV-infected chimpanzees; induction of IFN- α and cytokines Phase I trials in CHB patients	Animal study: [124] Phase I study in healthy volunteers: [125] Phase I trials in CHB (completed) [126,127]
Cytokines	IL-7 Recombinant human IL-7 (rhIL-7)-CYT 107 IL-21 Recombinant IL-21	Preclinical data: Immunorestorative and vaccine adjuvant effect Phase I-II clinical trial on CHB patients ongoing Possible immunorestorative effect when used in combination with antivirals	Small animal study (LCMV mice): [128,129] Small animal study (LCMV mice): [134–135,137]
Adaptive immunity			
Inhibitory T lymphocytes	PD-1/PD-L1	Blockade of PD-1/PD-L1 interaction leading to restoration of T-cell function	Small animal study (LCMV mice): [146] Wood chucks (WHV infected): [153,154]
Humoral & adaptive immunity			
Therapeutic vaccination	Immunogenic complexes: HBsAg with anti-HBs immunoglobulin)	Priming of HBV-specific CD8 ⁺ CTL responses	Clinical trial: [165] Phase II b clinical trial: [164] Phase III clinical trial: [166]
	HBsAg and HBcAg Combination: NASVAC	Recombinant HBcAg act as potent Th1 adjuvant to HBsAg and strong immunogen Phase I clinical trial in HVs demonstrated safety and immunologic efficacy Phase III clinical trial is on going in CHB patients	Phase I clinical trial: [169] Phase III clinical trial: [170]
	Whole recombinant yeast-based therapeutic vaccine: tarmogens: GS-4774 (formerly GI-13020)	Antigen-specific T-cell responses; tarmogens elicited HBV-specific T-cell responses <i>ex vivo</i> in samples collected from HVs and donors with CHB; induce both CD4 ⁺ and CD8 ⁺ T-cell responses in <i>ex vivo</i> model; induce HBV-specific T-cell response	[172,183] Phase I trial in HV completed: [184] Phase II trial in CHB patients: [185]
	Adenovirus-based therapeutic vaccination TG1050	Stimulate polyfunctional, multispecific, robust and long-lasting T cells targeting multiple epitopes from three major viral proteins, expected to control the HBV replication and to elicit viral clearance	[173]
	DNA vaccines	Activate not only the T-cell responses specific to HBV but also natural killer cells	Phase I clinical trial: [175] Proof-of-concept study/CHB carriers: [176]
	T-cell peptide epitope vaccine	String of 30 HBV-derived CTL epitopes linked to 16 Th epitopes presented to T cells by a large number of HLA molecules	Mouse model: [177]

AASLD: American Association for the Study of Liver Diseases; anti-HBs: Hepatitis B surface antibody; CHB: Chronic hepatitis B; CTL: Cytotoxic T lymphocyte; HBcAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen; HV: Healthy volunteer; LCMV: lymphocytic choriomeningitis virus; NASVAC: Nasal HBV vaccine candidate; PD1/PD-L1: Programmed death-1/Programmed death-ligand 1; PRR: Pattern recognition receptor; Th: T helper; TLR: Toll-like receptor.