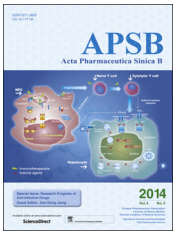
Acta Pharmaceutica Sinica B 2014;4(4):248–257

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



REVIEW

Therapeutic strategies for a functional cure of chronic hepatitis B virus infection

Jinhong Chang, Fang Guo, Xuesen Zhao, Ju-Tao Guo*

Department of Microbiology and Immunology, Drexel University College of Medicine, Doylestown, PA 18902, USA

Received 10 April 2014; revised 20 May 2014; accepted 23 May 2014

KEY WORDS

Hepatitis B virus;
cccDNA;
Antiviral agents

Abstract Treatment of chronic hepatitis B virus (HBV) infection with the viral DNA polymerase inhibitors or pegylated alpha-interferon has led to a significant retardation in HBV-related disease progression and reduction in mortality related to chronic hepatitis B associated liver decompensation and hepatocellular carcinoma. However, chronic HBV infection remains not cured. The reasons for the failure to eradicate HBV infection by long-term antiviral therapy are not completely understood. However, clinical studies suggest that the intrinsic stability of the nuclear form of viral genome, the covalently closed circular (ccc) DNA, sustained low level viral replication under antiviral therapy and homeostatic proliferation of hepatocytes are the critical virological and pathophysiological factors that affect the persistence and therapeutic outcomes of HBV infection. More importantly, despite potent suppression of HBV replication in livers of the treated patients, the dysfunction of HBV-specific antiviral immunity persists. The inability of the immune system to recognize cells harboring HBV infection and to cure or eliminate cells actively producing virus is the biggest challenge to finding a cure. Unraveling the complex virus–host interactions that lead to persistent infection should facilitate the rational design of antivirals and immunotherapeutics to cure chronic HBV infection.

*Corresponding author. Tel.: +1 215 4894929; fax: +1 215 4894920.
E-mail address: Ju-Tao.Guo@drexelmed.edu (Ju-Tao Guo).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2014.05.002

1. Introduction

Hepatitis B virus (HBV) infection is one of the major public health challenges worldwide. While the availability of a vaccine has reduced the number of new HBV infections, it does not benefit the 350 million people already chronically infected by the virus[1]. In fact, approximately one-third of these chronically infected individuals will die from serious liver diseases, such as cirrhosis, hepatocellular carcinoma (HCC) and liver failure, if left untreated[2,3]. The therapeutic goal of chronic HBV infection is thus to decrease the risk of liver disease progression and prevent its detrimental clinical sequelae, which can be achieved by sustained suppression of the virus replication, or ideally by curing the virus infection.

Currently, seven drugs have been approved by the Food and Drug Administration of USA for the treatment of chronic hepatitis B, which include two formulations of alpha-interferon (standard and pegylated) that enhance the host antiviral immune response and five nucleos(t)ide analogs (lamivudine, adefovir, entecavir, telbivudine, and tenofovir) that inhibit HBV DNA polymerase with varying potencies and barriers to resistance[4,5]. At present, the preferred first-line treatment choices are pegylated-interferon alpha-2a (pegIFN-α), entecavir and tenofovir, based on their superior antiviral efficacy and/or high resistance barrier[6]. However, even with the first-line treatment options, pegIFN-α is effective in achieving sustained virological response in only 30% of HBeAg-positive and 40% of HBeAg-negative cases[7–9] and usually associated with severe side-effects[7]. On the other hand, the nucleos(t)ide analogs are well tolerated and potently suppress HBV replication in the vast majority of treated patients. However, even the most potent nucleos(t)ide analogs rarely induce HBV surface antigen (HBsAg) seroconversion, the hallmark of a successful immunologic response to HBV with complete and durable control of infection, or a "functional cure"[10–12]. Hence, long-term, and possibly life-long, nucleos(t)ide analog treatment is required to continuously suppress HBV replication, which may be associated with significant cost burden and limited by drug-associated toxicity. It is, therefore, a pressing need for the introduction of therapeutic regimens that are safer and effective in achieving a functional cure. Apparently, unraveling the complex virus-host interactions that lead to persistent infection and better understanding of the obstacles to a cure are essential for the development of curative therapeutics for chronic HBV infection.

2. Pathobiological features of HBV infection

HBV is the prototype virus of *hepadnaviridae* family and contains a relaxed circular (rc) partially double stranded DNA (3.2 kb in length) genome[13]. A hallmark of HBV genomic DNA replication is protein-primed reverse transcription of a RNA intermediate called pre-genomic (pg) RNA[14,15]. However, unlike that of classic retroviruses, integration of viral genomic DNA into host cellular chromosomes is not an obligatory step in the HBV life cycle. Briefly, as illustrated in Fig. 1, HBV virion binds to its cellular receptor, sodium taurocholate cotransporting polypeptide (NTCP), on the surface of hepatocyte and is subsequently internalized by macropinocytosis[16]. Viral envelope fuses with endosomal membrane to release nucleoacapsid into the cytoplasm, where genomic rcDNA is deproteinized and delivered into the nucleus to be converted into covalently closed circular (ccc) DNA by host cellular DNA repair machinery[17–19]. The cccDNA exists as an episomal minichromosome and serves as the template for the transcription of viral RNAs[20]. The viral pregenomic (pg) RNA is translated to produce both the core protein and DNA polymerase[14]. The DNA polymerase binds to the epsilon sequence within the 5′ portion of pgRNA to prime viral DNA synthesis and initiate nucleocapsid assembly[15,21]. The encapsidated pgRNA is then reverse transcribed into minus strand viral DNA, which serves as a template for the subsequent synthesis of plus strand DNA by viral polymerase. The nucleocapsid matures as rcDNA is formed and can be either enveloped and secreted out of the cell as a virion particle, or delivered into the nucleus to amplify the nuclear cccDNA pool[22–24].

The outcome of HBV infection as well as the severity of HBV-induced liver diseases varies widely among the infected individuals. While over 95% percent of adult-acquired infections are spontaneously cleared within 6 months, more than 90% of exposed neonates and approximately 30% of children aged 1–5 years will develop chronic infection[2,25]. Individuals infected during infancy represent the majority of the global reservoir of chronic carriers. Other than the occasional appearance of ground-glass hepatocytes in chronic HBV carriers, which is presumably due to accumulation of large envelope protein in the endoplasmic reticulum, HBV infection of hepatocytes induces negligible cytopathic effect[26]. It is, therefore, generally believed that the outcome of HBV infection and severity of its associated liver diseases are determined by the nature and magnitude of host immune response against the virus[27,28].

Studies in HBV infected people and chimpanzees show that unlike other viruses that promptly induce an inflammatory cytokine response in the early phase of infection, HBV infection is stealthy and flies under the radar of the host immune sensors for a few weeks before the activation of humoral and T lymphocyte-mediated cellular immune responses[28,29]. While a polyclonal and vigorous HBV-specific T-cell response resolves HBV infection through coordinated kill and cure of infected hepatocytes, a weaker or barely detectable HBV-specific T-cell response fails to eliminate the virus and results in chronic infection[30,31]. Unfortunately, despite extensive research efforts, the precise mechanism by which HBV fails to induce a vigorous immune response in the chronic carriers remains elusive.

3. Virologic goal of chronic hepatitis B therapies

In principle, cure of HBV infection means eradication of the virus from an infected individual, which requires elimination of all extracellular virions and kill or cure of every virally-infected cell in the body. Because either extracellular virions or virally-infected cells dynamically turnover with kinetics regulated by host pathophysiological cues, such as antiviral immune response and proliferation of host cells[32,33], cure of HBV infection can, in theory, be achieved by the inhibition of viral replication for a period of time that allows for complete decay of extracellular infectious virions and the most stable viral replication intermediate, presumably the cccDNA, in infected cells. However, if there are long-living cells infected by HBV, inhibition of viral replication alone may not be possible to eradicate the virus infection. Hence, suppression of HBV gene expression as well as constant immune surveillance of the residual infected cells might be essential for a durable off-therapy control of HBV infection. In fact, it is evident that the cccDNA is not completely eradicated from the liver following resolution of an acute infection, but appears to be controlled at extremely low levels by host antiviral

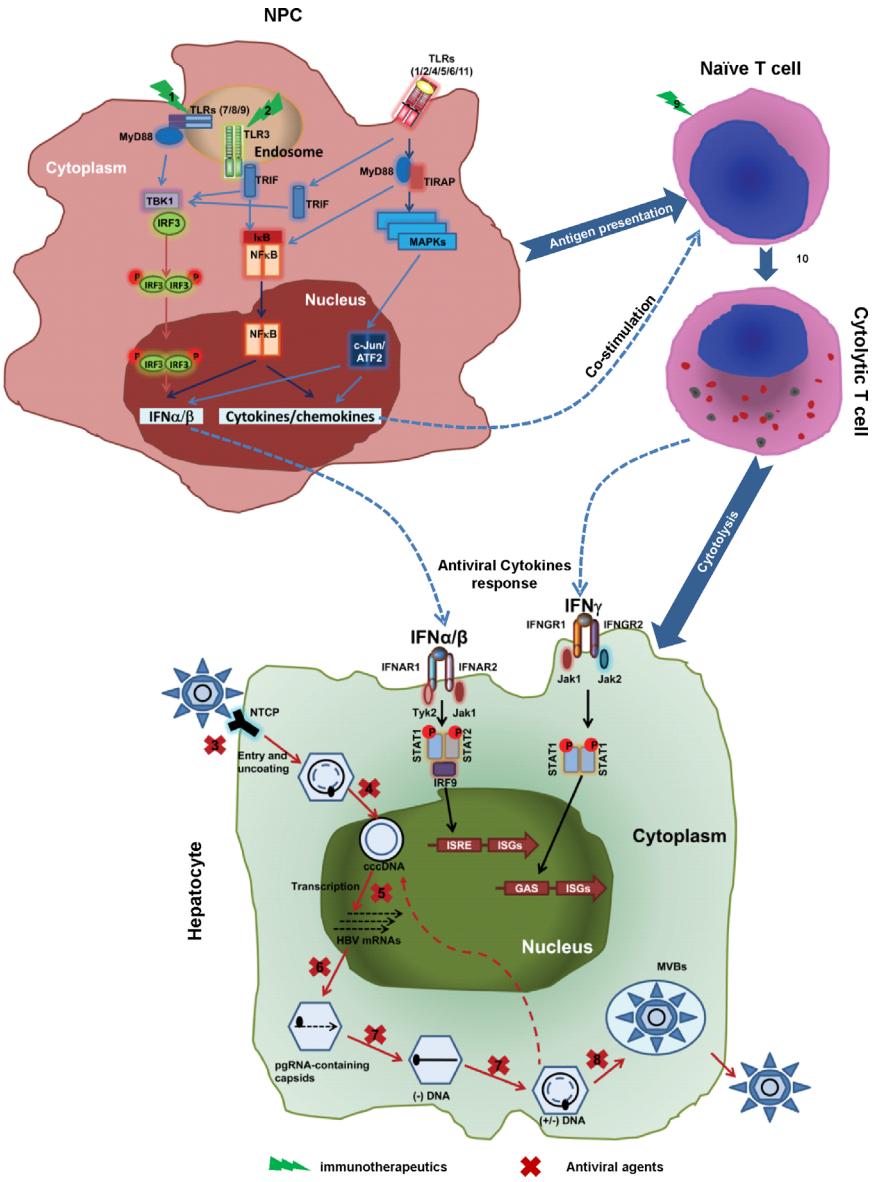


Figure 1 Schematic representation of intrahepatic interplays among hepatocytes, non-parenchymal cells (NPC) and lymphocytes and illustration of antiviral and immunotherapeutic strategies. HBV replication cycle in hepatocytes is illustrated herein and explained in details in text. HBV antigens from infected hepatocytes can be captured and cross-presented by liver-resident antigen-presentation cells, such as dendritic cells and Kupffer cells, to activate HBV-specific T lymphocytes that control HBV infection by either cytolytic kill or cytokine-mediated cure of infected hepatocytes. In addition, activation of TLRs in NPCs by their cognate ligands induces the production of type I IFNs, proinflammatory cytokines and chemokines. The type I IFNs bind to their receptors on hepatocytes to trigger JAK-STAT signaling pathway and induce the expression of ISGs, which limit HBV replication *via* inhibition of cccDNA transcription and encapsidation of HBV pgRNA. The molecular or cellular targets of the ten antiviral (Red cross with number) and immunotherapeutic (Green arrow with number) strategies currently used in clinic or under pre-clinical or clinical development for management of chronic hepatitis B are illustrated herein and explained in details in text and Table 2 in the same numerical fashion.

immune response[10,34,35]. This residual infection becomes clinically relevant only under the condition of systematic immunosuppression[10]. Therefore, a realistic virologic goal of anti-HBV therapy may not necessarily be the eradication of HBV or an absolute cure, but the suppression of viral replication as well as the restoration of host antiviral immune response to sustainably control HBV infection to a condition that is equivalent to that achieved by natural resolution of an acute infection, *i.e.*, a functional cure[11]. However, achievement of the functional cure is currently hampered by the following conditions.

4. Obstacles to a functional cure

4.1. *The intrinsic stability of HBV cccDNA*

Treatment of chronic hepatitis B patients with nucleoside analogs for more than a year reduces HBV load in plasma by more than 4 logs. However, analyses of intrahepatic viral DNA indicate that the antiviral therapies only reduce the cytoplasmic HBV core DNA and nuclear HBV cccDNA by approximately 2 and 1 log, respectively[36–38]. These observations are well corroborated with

the inability of nucleoside analogs to significantly reduce the level of HBsAg antigenemia[39], which is quantitatively correlated with the level of cccDNA. Similarly, extensive analyses of intrahepatic woodchuck hepatitis virus (WHV) and duck hepatitis B virus (DHBV) DNA intermediates under nucleoside analog therapy clearly demonstrated that cccDNA become the predominant form of viral DNA replication intermediates upon long-term suppression of viral DNA replication[40,41]. These findings strongly suggest that cccDNA is the most stable HBV replication intermediate and elimination of cccDNA is the key to cure HBV infection.

As stated above and illustrated in Fig. 1, cccDNA is initially synthesized from the rcDNA from the incoming viral nucleocapsids during HBV infection of a hepatocyte. In the early phase of infection, additional cccDNA are produced from newly synthesized cytoplasmic rcDNA through an intracellular amplification pathway[24,42]. These two pathways culminate in the formation of a regulated steady-state population of 5–50 cccDNA molecules per infected hepatocyte[20,22,43]. Obviously, persistent infection of hepadnaviruses relies on the stable maintenance and proper function of the cccDNA pool in the nuclei of infected hepatocytes as the source of viral RNAs and therefore, cure of HBV infected hepatocytes requires elimination of cccDNA[24,42,44,45].

Because cccDNA cannot amplify itself *via* semiconservative DNA replication, complete inhibition of cytoplasmic rcDNA synthesis by viral polymerase inhibitors should preclude *de novo* cccDNA synthesis. Hence, the cure of HBV infected hepatocytes by nucleoside analogs relies on the decay of pre-existing cccDNA. Accordingly, extensive efforts have been made to determine the half-lives of cccDNA under the variety of condition and obtained apparently contradictory results (Table 1), implying that the rate of cccDNA metabolism varies under different pathobiological conditions. Concerning the potential mechanisms of cccDNA decay, recent studies suggest that inflammatory cytokines, such as IFN-α and lymphotoxin-β, induce intrinsic cellular response to promote the decay of cccDNA through APOBEC3 family enzyme-catalyzed cytidine deamination and subsequent DNA repair process[51,52]. In addition, cccDNA can be diluted during cell division and cccDNA-free cells could arise through multiple rounds of cell division and unequal partitioning of cccDNA molecules into daughter cells[53,54]. Furthermore, studies with integrated WHV DNA as a genetic maker of virally infected hepatocytes during transient and chronic WHV infection of woodchucks unambiguously demonstrated that virus-free hepatocytes can be derived from infected cells[55,56]. In another word, WHV-infected hepatocytes are indeed curable *in vivo*. However, it is not yet clear if the division of infected hepatocytes is required for the host immune response to purge cccDNA *in vivo*.

Based on the mechanistic analyses of cccDNA metabolism, failure of long-term antiviral therapies with viral DNA polymerase inhibitors to eliminate cccDNA is most likely due to either incomplete suppression of HBV rcDNA synthesis, which allows for continuous replenishment of cccDNA pool *via* intracellular amplification pathway, or slow turnover of at least a subpopulation of HBV infected cells that serve as reservoirs of the virus.

4.2. *Incomplete suppression of viral replication*

Although clinical studies on the antiviral efficacy of nucleos(t)ide analogs under the variety of clinical conditions demonstrate striking reduction of viral load in peripheral blood, intrahepatic HBV core DNA and cccDNA are still detectable after long-term antiviral therapy[36–38]. Moreover, sequential accumulation of drug resistance mutations during apparently effective nucleos(t)ide analog therapy provides additional evidence suggesting that residual HBV replication and *de novo* cccDNA synthesis occur under long-term DNA polymerase inhibitor therapy[57]. Interestingly, analyses of viral DNA replication intermediates and core antigen-positive hepatocytes in the livers of WHV-infected woodchucks under the therapy of clevudine demonstrated that after more than 30 weeks of therapy, the predominant WHV DNA species in the liver is cccDNA. However, core-associated viral DNA replication intermediates, such as partial single-stranded DNA, are also clearly detectable. Intriguingly, while the vast majority of hepatocytes become core antigen-negative, a small fraction of hepatocytes expresses core antigen at the level similar to that in the pre-treated hepatocytes[40]. This observation indicates that while majority of infected hepatocytes have been cured after long-term nucleoside analog therapy, the residual viral DNA replication and cccDNA synthesis occur in discrete hepatocytes. In another word, the failure to cure HBV infection is most likely due to a fraction of HBV infected cells that are refractory to nucleoside analog therapies.

Why should this be? Because the nucleoside analogs are prodrugs that require activation by host cellular nucleoside kinases in virally infected cells, it is thus possible that the cells refractory to the therapy are incapable of activating the nucleoside analogs. Alternatively, considering the important role of cell division in elimination of pre-existing cccDNA[41], it is also possible that the refractory cells are long-live cells and have not divided during the therapy. Nevertheless, further understanding the biological feature of the refractory cell population is important for the treatment of chronic HBV infection.

4.3. *Turnover of HBV host cells*

The rate of infected cell turnover is one of the key parameters of HBV infection dynamics *in vivo*. Hepatocyte death, initiated

Table 1 Half-lives of hepadnaviral cccDNA.

Experimental condition	cccDNA half-life (days)	Reference
HepG2 cells transduced by HBV-expressing baculovirus vector	3	46
HBV infected chimpanzees under nucleoside analog therapy	9–14	47
HBV infected chimpanzees during the early phase of clearance	3	48
WHV infected primary woodchuck hepatocytes	>42	45
WHV infected woodchucks under nucleoside analog therapy	33–50	40
Primary hepatocytes from DHBV congenitally infected ducks	3–5	49
DHBV infected woodchucks under nucleoside analog therapy	35–57	50

through attack by antiviral cytotoxic T-lymphocytes (CTL), and compensatory hepatocyte proliferation, are both believed to be major contributing factors in the loss of virus DNA during immune resolution of transient infections. Although non-cytolytic cure of infected hepatocytes have been approved to occur, it is estimated that a minimum of 0.7–1 and approximately 2 complete random turnovers of the hepatocyte population of the liver occurs during the resolution of WHV infection in woodchucks[50] and HBV infection in chimpanzees[48], respectively. Hepatocyte turnover also plays an important role in viral pathogenesis and immune selection of hepatocytes infected with mutant strains of HBV and in the emergence of hepatocytes that appear refractory to HBV infection through clonal expansion. Under the condition of therapeutic inhibition of ongoing HBV DNA replication, the rate of HBV infected cell turnover is a critical determinant of cccDNA decay kinetics[41,58]. Accordingly, hepatocyte turnover has been investigated on the variety of pathobiological conditions by either directly measuring hepatocyte proliferation activity from liver biopsies or mathematic modeling of viral dynamics. These studies from multiple laboratories reveal that while the half-life of hepatocytes in the healthy adult liver is approximately half a year, the median half-lives of infected hepatocytes in patients with chronic hepatitis B are 257 h (=10.7 days) (n=9, range 112–762 h)[59] and 7 days in patients with chronic hepatitis B under lamivudine treatment[60]. The results thus imply the overall rate of hepatocyte turnover is significantly accelerated in patients with chronic hepatitis B, which should favor the eradication of cccDNA with viral replication inhibitor therapies.

4.4. *HBV reservoirs or extrahepatic infection*

While it is possible that a fraction of infected hepatocytes with slower rates of turnover may serve as reservoirs for HBV after immune resolution of transient HBV infection or long-term antiviral therapy, the existence of extrahepatic reservoirs also cannot be ruled out. In fact, although there are reports claiming the existence of HBV DNA or antigens in peripheral lymphocytes and other tissues, productive HBV infection of cell types other than hepatocytes has not yet been convincingly approved[61,62]. However, DHBV and WHV have been demonstrated to have an unanticipated broad cell tropism *in vivo*. For instance, WHV DNA replication intermediates and/or mRNA can be detected in lymphoid cells of spleens, peripheral T and B lymphocytes upon activation[63]. In addition to liver, DHBV antigen expression, DNA replication intermediates and/or mRNA can also be detected in the brain, lung, heart, intestine, kidney, pancreas and spleen[64]. *In situ* hybridization showed evidence of viral replication in the lung epithelium, germinal center of spleen, acinar cell of pancreas and tubular epithelium of kidney[65]. Moreover, DHBV infects both pancreatic α and β endocrine cells and impairs the arginine-stimulated insulin response[66]. Intriguingly, treatment of DHBV congenitally infected ducks with the guanosine analog, ganciclovir, efficiently reduced intrahepatic viral core DNA and reduced core antigen-positive hepatocytes. However, the treatment did not affect viral antigen expression in the bile duct epithelial cells, putative oval cells and DHBV-infected cells in extrahepatic sites such as the pancreas, kidney and spleen[67]. The studies thus showed that cure of HBV infection requires combination therapies targeting all types of infected cells, but not only hepatocytes. However, due to the intrinsic stability of cccDNA in non-dividing cells, control of HBV replication in the long-lived cell types may require elimination of the "reservoir" cells or silence the viral gene expression and replication by host immune response.

4.5. *Dysfunction of antiviral immune response*

Due to the failure of nucleos(t)ide analog therapy to completely eliminate HBV infected cells, viral infection can be restored within a few weeks to several months after the cessation of antiviral therapy. Hence, cure or durable control of a chronic infection by HBV replication inhibitor therapy may not be possible in a host that lacks an antiviral immune response capable of producing antibodies to neutralize residual viruses and cellular immune response that eliminate infected cells and keeps the reservoirs under immune surveillance.

Although failed to resolve HBV infection, specific humoral and cellular immune responses against HBV antigens are readily detectable in chronic HBV carriers, which is distinct from the immune responses observed in patients who resolve transient HBV infection[28,29]. For instance, although antibodies against core proteins, HBeAg, polymerase and X protein can be detected in all or a portion of chronic HBV carriers, antibodies against three envelope proteins are not produced or exist in forms of immuno-complexes. Concerning T cell response, HBV persistence is always associated with defective HBV-specific CD4 and CD8 T cell functions[68]. Despite the phenotypes of the altered immune response in HBV chronic carriers in the different clinical stages have been extensively characterized, the mechanism of HBV infection to induce the dysfunctional immune responses leading to a persistent infection remains elusive[29]. However, it is generally believed that the defective T cell function is maintained primarily by the effect of the prolonged exposure of T cells to high quantities of viral antigens and by the tolerogenic features of hepatocytes and liver resident cells[69,70]. These two combined mechanisms can result in the deletion of HBV-specific T cells or in their functional inactivation (exhaustion), which is characterized by an increased expression of negative co-stimulatory molecules and a dysregulation of co-stimulatory pathways, such as PD-1/PDL1, affect the quality and intensity of the antiviral T cell response[71].

Considering the critical role of the prolonged exposure of T cells to high quantities of viral antigens in the maintenance of HBV-specific T cell dysfunction, it is anticipated that reduction of HBV antigen load through long-term antiviral therapy to significantly reduce cccDNA should help improve T cell function, which might, in turn, lead to sustained suppression or functional cure of HBV infection. However, although as expected that long-term nucleoside analog therapy at least partially restores the function of HBV specific T cells, a durable control of HBV infection could still not be achieved in the majority of the treated patients[72–74]. Hence, although reduction of HBV antigen load is helpful, restoration of functional T cell immune response requires the correction of additional defects in the priming, expansion and differentiation of HBV specific T cells.

5. Strategies toward a functional cure

Obviously, achievement of a functional cure of chronic HBV infection needs to remove one or multiple obstacles discussed above. Strategically, the following four therapeutic approaches, alone or in combination, have the potential to achieve this goal.

5.1. *Combination therapy with antivirals targeting multiple distinct steps of HBV replication*

The key to the success of antiviral therapies against the infection of hepatitis C virus and human immunodeficiency virus is the combination therapy with antivirals targeting multiple distinct viral and/or host functions. Such a therapeutic approach improves antiviral efficacy and prevents emergence of drug-resistant viruses. However, treatment of chronic hepatitis B with combination therapies of two different nucleoside analogs or a nucleoside analog and pegIFN-α does not demonstrate significantly improved clinical benefits[75]. Currently, compounds that inhibit HBV entry into hepatocytes[76], RNase H activity[77] and assembly of nucleocapsids[78–81] are under preclinical and clinical development (Fig. 1 and Table 2). It is anticipated that combination therapies with nucleoside analogs and one or multiple of the novel antivirals should more potently suppress HBV replication and *de novo* cccDNA synthesis in all HBV infected cells, which could potentially improve the rate of functional cure. In absence of a functional cure, it is also expected that the combination therapies may improve the control of HBV replication in the minority of patients for whom nucleoside analog monotherapy is inadequate to suppress viral titers below the clinical detection limit[91]. In addition, the combination therapy may make the antiviral drugs with low genetic barriers to resistance, such as lamivudine, retain effectiveness, which is particularly valuable in developing countries[92].

5.2. *Elimination or functional suppression of HBV cccDNA*

Although it is generally believed that noncytolytic eradication of cccDNA from virally infected cells or permanent silence of its transcription is essential for a cure or durable control of HBV infection, promising approaches and molecular targets for therapeutic elimination and/or silence of cccDNA have not been identified. Despite studies with cccDNA sequence-specific endonucleoases or zinc-finger proteins to cleave cccDNA molecules or inhibit its transcription have been successfully demonstrated in cultured hepatoma cells[93,94], efficient and targeted delivery of the genes that express those antiviral proteins in all HBV infected cells *in vivo* is the biggest challenge toward their clinical application[95]. It is obvious that further understanding the molecular mechanism of cccDNA biosynthesis, maintenance and functional regulation as well as developing convenient cell-based assays for investigation of cccDNA biology and search of cccDNA-targeting compounds are essential for development of antiviral drugs to eliminate or silence cccDNA[51].

For instance, the recent discovery that IFN-α and lymphotoxin-β are capable of inducing non-cytolytic degradation of cccDNA through cytidine deamination invokes possibility to cure HBV infected cells *via* pharmacological activation or argumentation of the host intrinsic antiviral pathway[52]. Moreover, studying the molecular mechanism of IFN-α suppression of hepadnavirus cccDNA transcription reveals that the cytokine induces a distinct silence network to shut off cccDNA transcription. Hence, identification of host and viral proteins that are recruited to cccDNA minichromosomes and suppress its function in response to IFN-α may reveal host and viral targets for rationale development of antiviral drugs to eliminate or transcriptionally silence cccDNA[51,85].

5.3. *Stimulation of intrahepatic innate immune response*

Innate immune response plays an essential role in defending the host from viral infections. Particularly, the cytokine response elicited by activation of host pattern recognition receptors (PRRs) not only contains viral replication and spreading during the early phase of infection, but also orchestrates the activation and development of the adaptive immune response, which ultimately resolves viral infections[96]. Intriguingly, HBV infection has been shown to induce a negligible proinflammatory response during the early phase of infection. Whether this is due to the failure of the virus to activate the PRRs or suppression of the PRR signaling pathways by the virus remains controversial[97]. However, a plethora of evidence suggests that artificial activation of intrahepatic *Toll*-like receptors (TLR)[98,99] and RIG-I-like receptors (RLR)[100,101] induces robust cytokine responses in HBV-replicating mice and efficiently suppresses the virus replication. It is also worth noting that HBV activates other branches of innate immune responses, such as NK cells and NKT cells, although it is compromised by virus-induced immunosuppressive cytokine IL-10 or increase of the inhibitory receptor NKG2A on NK cells[102–104]. Accordingly, as illustrated in

Table 2 Antiviral agents currently in clinical use or under development.

Therapeutic strategies[a]	Representative drugs or compounds	Reference
1. Activation of TLR7	TLR7 agonists	82
2. Activation of TLR3	TLR3 agonists	83
3. Inhibition of virus entry	Myrcludex-B	76
4. Inhibition of cccDNA formation	Disubstituted sulfonamide	84
5. Elimination and/or silence of cccDNA	IFN-α	51,52,85
	Lyphotoxin-β	51
6. Inhibition of nucleocapsid formation	Heteroaryldihydropyrmidines	78
	Phenylpropenamides	80,86
	Sulfamoylbenzamides	81
7. Inhibition of DNA synthesis		
DNA polymerase inhibitors	Nucleos(t)ide analogs	87
RNase H inhibitors		77
8. Inhibition of virion assembly/secretion	Iminosugars	88
	Tetrahydro-tetrazolo-pyrimidine	89
9. Therapeutic vaccinations	Extensively reviewed in	90
10. Promotion of functional CTL differentiation and maturation	Extensively reviewed in	29

[a]The numbers are consistent with that illustrated in Fig. 1.

Fig. 1, pharmacological activation of intrahepatic innate immune response in liver non-parenchymal cells, such as Kupffer cells and dendritic cells, has been considered as a therapeutic approach that not only suppresses HBV replication in hepatocytes *via* induction of antiviral cytokines and activation of NK cells, but also facilitate the priming and development of a successful HBV-specific adaptive immune response[105,106].

Indeed, the great potential of TLR agonists for treatment of chronic hepatitis B has been demonstrated by the promising results obtained with TLR7 small molecular agonists. For instance, four week treatment of woodchucks chronically infected by WHV with GS-9620, a potent and orally available TLR7 agonist, resulted in a greater than 4 log reduction of viral load in all treated animals. Intriguingly, the suppressive effect on the virus was sustained after cessation of treatment and antibody against the surface antigen of WHV became detectable in a subset of woodchucks (Menne et al., 2011, EASL 46th Annual Meeting, Berlin, Germany). Similarly, oral administration of GS-9620 to HBV chronically infected chimpanzees for eight weeks reduced viral load by more than 2 logs and more than ten-fold reduction of viral load persisted for several months after the cessation of treatment. In addition, serum levels of HBsAg, HBeAg and numbers of HBV antigen-positive hepatocytes, were reduced as hepatocyte apoptosis increased. In consistence with the activation of TLR7, GS-9620 administration induced production of interferon-α and other cytokines and chemokines, and activated interferon-stimulated genes[82].

In addition to activate intrahepatic cytokine response, recent studies in a HBV-replicating mice model also showed that blockade of the natural killer cell inhibitory receptor NKG2A increases activity of NK cells and clears HBV infection[107]. This result suggests that interruption of the interaction between NKG2A and its ligand HLA-E might be an ideal therapeutic approach to treat CHB infection in humans.

5.4. *Restoration of HBV specific adaptive immune response*

Current, it is not yet known at what extent the dysfunction of viral specific adaptive immunity in chronic HBV carriers is reversible. Phenotypic analyses of HBV-specific T lymphocytes in chronic carries suggest that the dysfunctional adaptive immune response is, at least in part, due to the exhaustion of cytolytic T cells induced by the prolonged exposure to high quantities of viral antigens. The fact that reduction of HBV antigenemia and viral load in patients receiving long-term effective antiviral therapy partially improves the HBV-specific adaptive immune response seems to support this notion[72–74]. However, it remains to be demonstrated whether more profound and faster reduction of HBsAg antigenemia through inhibition of viral protein expression and replication by hepatocyte-targeting siRNA, such as ARC-520[108] (http://www.arrowheadresearch.com/press-releases/arrowhead-receives-regulatory-approval-begin-phase-2a-trial-chronic-hepatitis-b), or HBsAg secretion by small molecules[89] or nucleic acid-based polymers (REP9AC) (http://replicor.com/antiviral-technologies/hepatitis-b/) could further improve the antiviral immune response and result in a functional cure.

Interestingly, the observation that adoptive transfer of dysfunctional virus-specific CD8 cells from a chronically infected to a naïve uninfected MHC compatible mouse is not sufficient to restore the T cell memory maturation and differentiation process suggests that in addition to reduction of viral antigen load, direct target cellular pathways mediating the immunopathology of HBV infection is essential for the reconstitution of a fully functional immune response against the virus[109]. The differentiation and maturation of functional T-cells are regulated by numerous receptor and ligand interactions in separate cellular compartments at different phases of the immune response[110]. Recent studies suggest that multiple inhibitory receptors, including PD1[111], CTLA-4[112], TIM-3[113] and LAG-3[114], play important roles in T-cell exhaustion during persistent HBV infection. Hence, strategies to directly target the negative co-stimulatory pathways involved in the pathogenesis of T cell exhaustion as well as manipulate the liver microenvironment that regulates T cell function, such as production of immunomodulatory cytokines and regulatory T cells[106,115], have been extensively investigated in HBV replicating mice models, WHV infected woodchucks and humans in clinic. Data obtained from these studies suggest that the function of the different immune regulatory pathways is not redundant in the pathogenesis of T cell exhaustion and combinational interruption of multiple key regulatory pathways might be required to reverse the dysfunctional adaptive immune response[29,116].

Another immunotherapeutic strategy is to induce functional anti-HBV immune responses in chronic HBV carriers by vaccination[117]. During last two decades, vaccination strategies using conventional HBsAg vaccine, immunocomplexes of HBsAg and human anti-HBs, apoptotic cells that express HBV antigens, DNA vaccines or viral vectors expressing HBV proteins have been evaluated in animal models and clinical trials, alone or in combination with antiviral therapy, expression of immune stimulatory cytokines or modulators of T cell function[118–121]. Although functional antiviral immune response could indeed be induced under selected experimental conditions, vaccination strategies for induction of immune response that can resolve or durably control chronic HBV infection remains to be defined[90]. It can be speculated that a successful therapeutic vaccination strategy should, at least, be able to reduce viral load, break the HBV antigen-specific immune tolerance and modulate T cell differentiation and maturation to prevent their exhaustion[122].

6. Concluding remarks

Achievement of a functional cure of chronic HBV infection relies on eradication of cccDNA from the vast majority of virally infected cells as well as sustained immune control of HBV replication in a small number of residual HBV-infected cells. Hence, keys to the success are development of drugs targeting distinct viral and/or host functions to potently suppress HBV replication as well as therapeutics to restore or induce functional antiviral adaptive immune response. However, lack of biologically relevant animal models of chronic HBV infection and efficient HBV infectious cell culture system has hampered our investigation toward understanding of the HBV immunopathogenesis and molecular mechanism of cccDNA metabolism/functional regulation as well as search for immunotherapeutic regimes and cccDNA-targeting antivirals[123,124]. Research to resolve these important issues should ultimately lead to the rational design of antiviral and immunotherapeutic strategies to cure chronic hepatitis B.

References

1. McMahon BJ. Epidemiology and natural history of hepatitis B. *Semin Liver Dis* 2005;**25** suppl 1:S3–8.
2. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997;**337**:1733–45.
3. Lok AS. Prevention of hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2004;**127**:S303–9.

4. Keeffe EB, Dieterich DT, Han SH, Jacobson IM, Martin P, Schiff ER, et al. A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. *Clin Gastroenterol Hepatol* 2008;**6**:1315–41. (quiz 1286).
5. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009;**137**:1593–608.
6. Gish R, Jia JD, Locarnini S, Zoulim F. Selection of chronic hepatitis B therapy with high barrier to resistance. *Lancet Infect Dis* 2012;**12**:341–53.
7. Perrillo R. Benefits and risks of interferon therapy for hepatitis B. *Hepatology* 2009;**49 Suppl 5**:S103–11.
8. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* 2005;**365**:123–9.
9. Lau GK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, et al. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005;**352**:2682–95.
10. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996;**2**:1104–8.
11. Block TM, Gish R, Guo H, Mehta A, Cuconati A, Thomas LW, et al. Chronic hepatitis B: what should be the goal for new therapies? *Antiviral Res* 2013;**98**:27–34.
12. Kim GA, Lim YS, An J, Lee D, Shim JH, Kim KM, et al. HBsAg seroclearance after nucleoside analogue therapy in patients with chronic hepatitis B: clinical outcomes and durability. *Gut* 2013. http://dx.doi.org/10.1136/gutjnl-2013-305517. Published online October 25, 2013.
13. Summers J, O'Connell A, Millman I. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc Natl Acad Sci USA* 1975;**72**:4597–601.
14. Summers J, Mason WS. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982;**29**:403–15.
15. Wang GH, Seeger C. Novel mechanism for reverse transcription in hepatitis B viruses. *J Virol* 1993;**67**:6507–12.
16. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012;**1**:e00049.
17. Guo H, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. *J Virol* 2007;**81**:12472–84.
18. Guo HT, Mao RC, Block TM, Guo JT. Production and function of the cytoplasmic deproteinized relaxed circular DNA of hepadnaviruses. *J Virol* 2010;**84**:387–96.
19. Guo HT, Xu CX, Zhou TL, Block TM, Guo JT. Characterization of the host factors required for hepadnavirus covalently closed circular (ccc) DNA Formation. *PLoS One* 2012;**7**:e43270.
20. Newbold JE, Xin H, Tencza M, Sherman G, Dean J, Bowden S, et al. The covalently closed duplex form of the hepadnavirus genome exists *in situ* as a heterogeneous population of viral minichromosomes. *J Virol* 1995;**69**:3350–7.
21. Wang GH, Seeger C. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* 1992;**71**:663–70.
22. Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000;**64**:51–68.
23. Ganem D, Varmus HE. The molecular biology of the hepatitis B viruses. *Ann Rev Biochem* 1987;**56**:651–93.
24. Wu TT, Coates L, Aldrich CE, Summers J, Mason WS. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* 1990;**175**:255–61.
25. Liang TJ. Hepatitis B: the virus and disease. *Hepatology* 2009;**49**: S13–21.
26. Wang HC, Wu HC, Chen CF, Fausto N, Lei HY, Su IJ. Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress. *Am J Pathol* 2003;**163**:2441–9.
27. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995;**13**:29–60.
28. Guidotti LG, Chisari FV. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 2006;**1**:23–61.
29. Bertoletti A, Ferrari C. Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection. *Gut* 2011;**61**:1754–64.
30. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005;**5**:215–29.
31. Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J Exp Med* 1995;**181**:1047–58.
32. Dandri M, Murray JM, Lutgehetmann M, Volz T, Lohse AW, Petersen J. Virion half-life in chronic hepatitis B infection is strongly correlated with levels of viremia. *Hepatology* 2008;**48**:1079–86.
33. Guo JT, Zhou H, Liu C, Aldrich C, Saputelli J, Whitaker T, et al. Apoptosis and regeneration of hepatocytes during recovery from transient hepadnavirus infections. *J Virol* 2000;**74**:1495–505.
34. Reaiche GY, Le Mire MF, Mason WS, Jilbert AR. The persistence in the liver of residual duck hepatitis B virus covalently closed circular DNA is not dependent upon new viral DNA synthesis. *Virology* 2010;**406**:286–92.
35. Penna A, Artini M, Cavalli A, Levrero M, Bertoletti A, Pilli M, et al. Long-lasting memory T cell responses following self-limited acute hepatitis B. *J Clin Invest* 1996;**98**:1185–94.
36. Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;**126**:1750–8.
37. Wong DK, Seto WK, Fung J, Ip P, Huang FY, Lai CL, et al. Reduction of hepatitis B surface antigen and covalently closed circular DNA by nucleos(t)ide analogues of different potency. *Clin Gastroenterol Hepatol* 2013;**11**:1004–10.
38. Cheng PN, Liu WC, Tsai HW, Wu IC, Chang TT, Young KC. Association of intrahepatic cccDNA reduction with the improvement of liver histology in chronic hepatitis B patients receiving oral antiviral agents. *J Med Virol* 2011;**83**:602–7.
39. Seto WK, Wong DK, Fung J, Huang FY, Lai CL, Yuen MF. Reduction of hepatitis B surface antigen levels and hepatitis B surface antigen seroclearance in chronic hepatitis B patients receiving 10 years of nucleoside analogue therapy. *Hepatology* 2013;**58**:923–31.
40. Zhu Y, Yamamoto T, Cullen J, Saputelli J, Aldrich CE, Miller DS, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. *J Virol* 2001;**75**:311–22.
41. Fourel I, Cullen JM, Saputelli J, Aldrich CE, Schaffer P, Averett DR, et al. Evidence that hepatocyte turnover is required for rapid clearance of duck hepatitis B virus during antiviral therapy of chronically infected ducks. *J Virol* 1994;**68**:8321–30.
42. Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986;**47**:451–60.
43. Beck J, Nassal M. Hepatitis B virus replication. *World J Gastroenterol* 2007;**13**:48–64.
44. Zhang YY, Zhang BH, Theele D, Litwin S, Toll E, Summers J. Single-cell analysis of covalently closed circular DNA copy numbers in a hepadnavirus-infected liver. *Proc Natl Acad Sci USA* 2003;**100**: 12372–7.
45. Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997;**71**:9392–9.
46. Delaney WE 4th, Miller TG, Isom HC. Use of the hepatitis B virus recombinant baculovirus-HepG2 system to study the effects of

(-)-beta-2′,3′-dideoxy-3′-thiacytidine on replication of hepatitis B virus and accumulation of covalently closed circular DNA. *Antimicrob Agents Chemother* 1999;**43**:2017–26.
47. Wieland SF, Spangenberg HC, Thimme R, Purcell RH, Chisari FV. Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees. *Proc Natl Acad Sci USA* 2004;**101**:2129–34.
48. Murray JM, Wieland SF, Purcell RH, Chisari FV. Dynamics of hepatitis B virus clearance in chimpanzees. *Proc Natl Acad Sci USA* 2005;**102**:17780–5.
49. Civitico GM, Locarnini SA. The half-life of duck hepatitis B virus supercoiled DNA in congenitally infected primary hepatocyte cultures. *Virology* 1994;**203**:81–9.
50. Addison WR, Walters KA, Wong WW, Wilson JS, Madej D, Jewell LD, et al. Half-life of the duck hepatitis B virus covalently closed circular DNA pool *in vivo* following inhibition of viral replication. *J Virol* 2002;**76**:6356–63.
51. Liu F, Campagna M, Qi YH, Zhao XS, Xu CX, Li SC, et al. Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. *PLoS Pathog* 2013;**9**:e1003613.
52. Lucifora J, Xia YC, Reisinger F, Zhang K, Stadler D, Cheng XM, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 2014;**343**:1221–8.
53. Lutgehetmann M, Volz T, Kopke A, Broja T, Tigges E, Lohse AW, et al. *In vivo* proliferation of hepadnavirus-infected hepatocytes induces loss of covalently closed circular DNA in mice. *Hepatology* 2010;**52**:16–24.
54. Reaiche-Miller GY, Thorpe M, Low HC, Qiao Q, Scougall CA, Mason WS, et al. Duck hepatitis B virus covalently closed circular DNA appears to survive hepatocyte mitosis in the growing liver. *Virology* 2013;**446**:357–64.
55. Summers J, Jilbert AR, Yang W, Aldrich CE, Saputelli J, Litwin S, et al. Hepatocyte turnover during resolution of a transient hepadnaviral infection. *Proc Natl Acad Sci USA* 2003;**100**:11652–9.
56. Summers J, Mason WS. Residual integrated viral DNA after hepadnavirus clearance by nucleoside analog therapy. *Proc Natl Acad Sci USA* 2004;**101**:638–40.
57. Zoulim F, Durantel D, Deny P. Management and prevention of drug resistance in chronic hepatitis B. *Liver Int* 2009;**29 Suppl 1**:S108–15.
58. Mason WS, Cullen J, Saputelli J, Wu TT, Liu C, London WT, et al. Characterization of the antiviral effects of 2′-carbodeoxyguanosine in ducks chronically infected with duck hepatitis B virus. *Hepatology* 1994;**19**:398–411.
59. Wolters LM, Hansen BE, Niesters HG, DeHertogh D, de Man RA. Viral dynamics during and after entecavir therapy in patients with chronic hepatitis B. *J Hepatol* 2002;**37**:137–44.
60. Wolters LM, Hansen BE, Niesters HG, Zeuzem S, Schalm SW, de Man RA. Viral dynamics in chronic hepatitis B patients during lamivudine therapy. *Liver* 2002;**22**:121–6.
61. Coffin CS, Mulrooney-Cousins PM, van Marle G, Roberts JP, Michalak TI, Terrault NA. Hepatitis B virus quasispecies in hepatic and extrahepatic viral reservoirs in liver transplant recipients on prophylactic therapy. *Liver Transpl* 2011;**17**:955–62.
62. Coffin CS, Mulrooney-Cousins PM, Peters MG, van MG, Roberts JP, Michalak TI, et al. Molecular characterization of intrahepatic and extrahepatic hepatitis B virus (HBV) reservoirs in patients on suppressive antiviral therapy. *J Viral Hepat* 2011;**18**:415–23.
63. Korba BE, Cote PJ, Gerin JL. Mitogen-induced replication of woodchuck hepatitis virus in cultured peripheral blood lymphocytes. *Science* 1988;**241**:1213–6.
64. Hosoda K, Omata M, Uchiumi K, Imazeki F, Yokosuka O, Ito Y, et al. Extrahepatic replication of duck hepatitis B virus: more than expected. *Hepatology* 1990;**11**:44–8.
65. Jilbert AR, Freiman JS, Gowans EJ, Holmes M, Cossart YE, Burrell CJ. Duck hepatitis B virus DNA in liver, spleen, and pancreas: analysis by *in situ* and Southern blot hybridization. *Virology* 1987;**158**:330–8.
66. England JM, Halpern MS. The arginine-stimulated insulin response is impaired in congenital duck hepatitis B virus infection. *Virology* 1988;**165**:299–302.
67. Luscombe C, Pedersen J, Uren E, Locarnini S. Long-term ganciclovir chemotherapy for congenital duck hepatitis B virus infection *in vivo*: effect on intrahepatic-viral DNA, RNA, and protein expression. *Hepatology* 1996;**24**:766–73.
68. Park SH, Rehermann B. Immune responses to HCV and other hepatitis viruses. *Immunity* 2014;**40**:13–24.
69. Bowen DG, Zen M, Holz L, Davis T, McCaughan GW, Bertolino P. The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest* 2004;**114**:701–12.
70. Shin H, Wherry EJ. CD8 T cell dysfunction during chronic viral infection. *Curr Opin Immunol* 2007;**19**:408–15.
71. Wherry EJ. T cell exhaustion. *Nat Immunol* 2011;**12**:492–9.
72. Boni C, Laccabue D, Lampertico P, Giuberti T, Viganò M, Schivazappa S, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. *Gastroenterology* 2012;**143**:963–73.
73. Reijnders JG, Perquin MJ, Zhang NP, Hansen BE, Janssen HLA. Nucleos(t)ide analogues only induce temporary hepatitis B e antigen seroconversion in most patients with chronic hepatitis B. *Gastroenterology* 2010;**139**:491–8.
74. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998;**102**:968–75.
75. Terrault NA. Benefits and risks of combination therapy for hepatitis B. *Hepatology* 2009;**49 Suppl 5**:S122–8.
76. Petersen J, Dandri M, Mier W, Marc Lügehetmann, Volz T, von Weizsäker F, et al. Prevention of hepatitis B virus infection *in vivo* by entry inhibitors derived from the large envelope protein. *Nat Biotechnol* 2008;**26**:335–41.
77. Tavis JE, Cheng X, Hu Y, Totten M, Cao F, Michailidis E, et al. The hepatitis B virus ribonuclease H is sensitive to inhibitors of the human immunodeficiency virus ribonuclease H and integrase enzymes. *PLoS Pathog* 2013;**9**:e1003125.
78. Deres K, Schroder CH, Paessens A, Totten M, Cao F, Michailidis E, et al. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. *Science* 2003;**299**:893–6.
79. Zlotnick A, Mukhopadhyay S. Virus assembly, allostery and antivirals. *Trends Microbiol* 2011;**19**:14–23.
80. King RW, Ladner SK, Miller TJ, Zaifert K, Perni RB, Conway SC, et al. Inhibition of human hepatitis B virus replication by AT-61, a phenylpropenamide derivative, alone and in combination with (-) beta-L-2′,3′-dideoxy-3′-thiacytidine. *Antimicrob Agents Chemother* 1998;**42**:3179–86.
81. Campagna MR, Liu F, Mao R, Mills C, Cai DW, Guo F, et al. Sulfamoylbenzamide derivatives inhibit the assembly of hepatitis B virus nucleocapsids. *J Virol* 2013;**87**:6931–42.
82. Lanford RE, Guerra B, Chavez D, Giavedoni L, Hodara VL, Brasky KM, et al. GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. *Gastroenterology* 2013;**144**:1508–17.
83. Ijichi K, Mitamura K, Ida S, Machida H, Shimada K. *In vivo* antiviral effects of mismatched double-stranded RNA on duck hepatitis B virus. *J Med Virol* 1994;**43**:161–5.
84. Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, et al. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. *Antimicrob Agents Chemother* 2012;**56**:4277–88.
85. Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012;**122**:529–37.
86. Katen SP, Chirapu SR, Finn MG, Zlotnick A. Trapping of hepatitis B virus capsid assembly intermediates by phenylpropenamide assembly accelerators. *ACS Chem Biol* 2010;**5**:1125–36.

87. Dienstag JL. Benefits and risks of nucleoside analog therapy for hepatitis B. *Hepatology* 2009;**49**:S112–21.
88. Block TM, Lu X, Mehta AS, Blumberg BS, Tennant B, Ebling M, et al. Treatment of chronic hepadnavirus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. *Nat Med* 1998;**4**:610–4.
89. Dougherty AM, Guo H, Westby G, Liu Y, Simsek E, Guo JT, et al. A substituted tetrahydro-tetrazolo-pyrimidine is a specific and novel inhibitor of hepatitis B virus surface antigen secretion. *Antimicrob Agents Chemother* 2007;**51**:4427–37.
90. Liu J, Kosinska A, Lu MJ, Roggendorf M. New therapeutic vaccination strategies for the treatment of chronic hepatitis B. *Virol Sin* 2014;**29**:10–6.
91. Yang YJ, Shim JH, Kim KM, Lim YS, Lee HC. Assessment of current criteria for primary non-response in chronic hepatitis B patients receiving entecavir therapy. *Hepatology* 2013;**54**:1603–10.
92. Soriano V, McMahon B. Strategic use of lamivudine in the management of chronic hepatitis B. *Antiviral Res* 2013;**100**:435–8.
93. Hoeksema KA, Tyrrell DL. Inhibition of viral transcription using designed zinc finger proteins. *Methods Mol Biol* 2010;**649**:97–116.
94. Chen JL, Zhang W, Lin JY, Wang F, Wu M, Chen CC, et al. An efficient antiviral strategy for targeting hepatitis B virus genome using transcription activator-like effector nucleases. *Mol Ther* 2014;**22**:303–11.
95. Bloom K, Ely A, Mussolino C, Cathomen T, Arbuthnot P. Inactivation of hepatitis B virus replication in cultured cells and *in vivo* with engineered transcription activator-like effector nucleases. *Mol Ther* 2013;**21**:1889–97.
96. Watts C, West MA, Zaru R. TLR signalling regulated antigen presentation in dendritic cells. *Curr Opin Immunol* 2010;**22**:124–30.
97. Chang J, Block TM, Guo JT. The innate immune response to hepatitis B virus infection: implications for pathogenesis and therapy. *Antiviral Res* 2012;**96**:405–13.
98. Isogawa M, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication *in vivo*. *J Virol* 2005;**79**:7269–72.
99. Wu J, Lu MJ, Meng ZJ, Trippler M, Broering R, Szczeponek A, et al. Toll-like receptor-mediated control of HBV replication by nonparenchymal liver cells in mice. *Hepatology* 2007;**46**:1769–78.
100. Han Q, Zhang C, Zhang J, Tian ZG. Reversal of hepatitis B virus-induced immune tolerance by an immunostimulatory 3p-HBx-siRNAs in a retinoic acid inducible gene I-dependent manner. *Hepatology* 2011;**54**:1179–89.
101. Ebert G, Poeck H, Lucifora J, Baschuk N, Esser K, Esposito I, et al. 5′ Triphosphorylated small interfering RNAs control replication of hepatitis B virus and induce an interferon response in human liver cells and mice. *Gastroenterology* 2011;**141**(696–706)706.
102. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* 2012;**18**:1060–8.
103. Dunn C, Peppa D, Khanna P, Nebbia G, Jones M, Brendish N, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology* 2009;**137**:1289–300.
104. Zhang Z, Zhang SY, Zou ZS, Shi JF, Zhao JJ, Fan R, et al. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. *Hepatology* 2011;**53**:73–85.
105. Publicover J, Gaggar A, Nishimura S, van Horn CM, Goodsell A, Muench MO, et al. Age-dependent hepatic lymphoid organization directs successful immunity to hepatitis B. *J Clin Invest* 2013;**123**:3728–39.
106. Publicover J, Goodsell A, Nishimura S, Vilarinho S, Wang ZE, Avanesyan L, et al. IL-21 is pivotal in determining age-dependent effectiveness of immune responses in a mouse model of human hepatitis B. *J Clin Invest* 2011;**121**:1154–62.
107. Li FL, Wei HR, Wei HM, Gao YF, Xu L, Yin WW, et al. Blocking the natural killer cell inhibitory receptor NKG2A increases activity of human natural killer cells and clears hepatitis B virus infection in mice. *Gastroenterology* 2013;**144**:392–401.
108. Wooddell CI, Rozema DB, Hossbach M, John M, Hamilton HL, Chu QL, et al. Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. *Mol Ther* 2013;**21**:973–85.
109. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci USA* 2004;**101**:16004–9.
110. Odorizzi PM, Wherry EJ. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* 2012;**188**:2957–65.
111. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, et al. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007;**81**:4215–25.
112. Schurich A, Khanna P, Lopes AR, Han KJ, Peppa D, Micco L, et al. Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8 T cells in persistent hepatitis B virus infection. *Hepatology* 2011;**53**:1494–503.
113. Wu W, Shi Y, Li SP, Zhang Y, Liu YN, Wu YH, et al. Blockade of Tim-3 signaling restores the virus-specific $CD8^+$ T-cell response in patients with chronic hepatitis B. *Eur J Immunol* 2012;**42**:1180–91.
114. Li FJ, Zhang Y, Jin GX, Yao L, Wu DQ. Expression of LAG-3 is coincident with the impaired effector function of HBV-specific $CD8^+$ T cell in HCC patients. *Immunol Lett* 2013;**150**:116–22.
115. Ejrnaes M, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, et al. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 2006;**203**:2461–72.
116. Shimizu Y. T cell immunopathogenesis and immunotherapeutic strategies for chronic hepatitis B virus infection. *World J Gastroenterol* 2012;**18**:2443–51.
117. Bertoletti A, Gehring A. Therapeutic vaccination and novel strategies to treat chronic HBV infection. *Expert Rev Gastroenterol Hepatol* 2009;**3**:561–9.
118. Godon O, Fontaine H, Kahi S, Meritet J, Scott-Algara D, Pol S, et al. Immunological and antiviral responses after therapeutic DNA immunization in chronic hepatitis B patients efficiently treated by analogues. *Mol Ther* 2014;**22**:675–84.
119. Bertoletti A, Gehring AJ. Immune therapeutic strategies in chronic hepatitis B virus infection: virus or inflammation control? *PLoS Pathog* 2013;**9**:e1003784.
120. Xu DZ, Wang XY, Shen XL, Gong GZ, Ren H, Guo LM, et al. Results of a phase III clinical trial with an HBsAg–HBIG immunogenic complex therapeutic vaccine for chronic hepatitis B patients: experiences and findings. *J Hepatol* 2013;**59**:450–6.
121. Liu J, Zhang EJ, Ma ZY, Wu WE, Kosinska A, Zhang XY, et al. Enhancing virus-specific immunity *in vivo* by combining therapeutic vaccination and PD-L1 blockade in chronic hepadnaviral infection. *PLoS Pathog* 2014;**10**:e1003856.
122. Xu L, Yin W, Sun R, Wei H, Tian Z. Liver type I regulatory T cells suppress germinal center formation in HBV-tolerant mice. *Proc Natl Acad Sci USA* 2013;**110**:16993–8.
123. Bertoletti A, Maini MK, Ferrari C. The host-pathogen interaction during HBV infection: immunological controversies. *Antivir Ther* 2010;**15 Suppl 3**:S15–24.
124. Zhou TL, Guo HT, Guo JT, Cuconati A, Mehta A, Block TM. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. *Antiviral Res* 2006;**72**:116–24.