Chapter 3

# Immunotherapy and Vaccine Development in Viral Hepatitis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55525

## 1. Introduction

More than 300 million people and 170 million people are chronically infected with hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively. To eradicate those viral infections, both prophylactic and therapeutic approaches are required. In HBV infection, there are global programs for prophylactic vaccination. However, some subjects, especially under immunocompromized state, are unable to acquire ant-HBs antibody (Ab) with conventional vaccination, and several attempts to improve the immunogenicity of HB vaccine have been made. On the other hand, development of effective prophylactic HCV vaccine has not been achieved mainly because of high rates of escape mutations within HCV envelope genes. We first focus on the recent development of prophylactic vaccine for HBV and HCV infections.

In the second half of the review, we summarized immunotherapeutic approach for both viral infections. Neither HBV nor HCV is cytopathic, and hepatitis is caused by the host immune response against virus-related peptides expressed on hepatocytes in conjunction with human leukocyte antigens (HLA). In acute self-limiting hepatitis, a broad immune response occurs that is strong enough to eradicate the virus or suppress viral replication [1], indicating that efficient induction of anti-viral immune response could have a potential to control viral infections. However, in chronic hepatitis, there are many mechanisms that hamper the antiviral immune response leading to persistent viral infection.

In chronic HBV infection, strong long-term viral suppression can now be achieved with various nucleoside and nucleotide analogs. However, there are some problems that must be solved in the near future. One of the problems in the treatment of nucleos(t)ide analogs is a low rate of HBe seroconversion even after long-term administration in $HBeAg^+$ patients. Moreover, reactivation rate of HBV replication is high in both $HBeAg^+$ and $HBeAg^-$ patients after cessation of the treatment, although drug-free viral controls would be better than long-term adminis-

tration of the drugs in terms of control of medical costs and avoidance of adverse effects of these agents. Therefore, it would be beneficial to achieve long-term viral eradication even after cessation of nucleos(t)ide analogs in combination with efficient immunotherapy.

On the other hand, antiviral oral drugs, such as protease inhibitor or polymerase inhibitor showing potent antiviral ability [2], have been developed for chronic HCV infection. However, not all patients treated with these drugs could achieve sustained virological response and high medical cost for each patient is a global serious problem. Effective immunotherapy combined with these drugs may improve their antiviral effects and control medical costs also in hepatitis C.

## 2. Problems and recent advances in HBV vaccination

### 2.1. Non-responder for HBV vaccine (Table 1)

In HBV infection, HBV is transmitted at a high incidence rate by parenteral, percutaneous or sexual contact. Therefore, primary protection is very important and universal vaccination regardless of maternal HBsAg status is recommended. Most of countries have introduced HBV vaccination into their national immunization programs and more than 80% of infants have received HBV vaccine three times. In Taiwan, universal vaccination program of all newborns was introduced in 1986. After twenty years of the program, the rate of chronic HBV infection decreased from 9.8% to 1.2% and the risk of childhood HCC has been decreased by 70% [3, 4].

| |
|---|
| Host factors |
| Age > 40 years |
| Obesity |
| Smoking |
| Genetics, certain HLA types |
| Other medical conditions |
| Diabetes |
| Cirrhosis |
| Renal failure |
| Conditions requiring immunosuppressive therapy |
| Unrecognized chronic HBV infection |
| Technical |
| Subcutaneous administration |
| Freezing of vaccine |

**Table 1.** Causes of non-responder to HBV vaccine

Non-responder for HBV vaccination and vaccine escape mutants are important problems to be focused in the future. The general recommendation for non-responders is to repeat a three-dose schedule; 50-75% is expected to respond to the second dose. Non-responders to the second dose should be evaluated for underlying chronic HBV infection. For hemodialysis patients, response may be improved by using double-dose vaccine [5]. Intradermal administration have been tried to improve the effectiveness of vaccination but is technically difficult [6] and long-term efficacy has not been demonstrated. Several trials to improve the effectiveness of HBV vaccination particularly in hemodialysis patients have been investigated (Table 2). Combined use of granulocyte macrophage-colony stimulating factor or levamisole as an adjuvant of HBV vaccination is a promising strategy [7-9]. New chemical adjuvant has also shown an excellent potential [10-12]. On the other hand, Interleukin (IL)-2 is not shown to be effective when used as an adjuvant [13,14]. Recently, a new HBV vaccine including preS lesion, which is known as an essential site for HBV-entry to hepatocyte, has been tried [15,16].

| Materials | Mechanism | Effectiveness | ref |
|---|---|---|---|
| *Adjuvant* | | | |
| GM-CSF | dendritic cell activation | Meta-analysis: OR 4.63 | [7] |
| Levamisole | interferon inducer | Meta-analysis: OR 2.43 | [8] |
| | upregulation of defective immune function | Meta-analysis: OR 2.77 | [9] |
| Interleukin-2 | enhanced cellular immunity | RCT: no significant effect | [13, 14] |
| HB-AS04 | upregulation of CD86, increased cytokine | RCT: Significant at month 3 | [10] |
| (aluminium salt, monophosphoryl lipid (MPL)) | | RCT: Significant | [11] |
| HB-AS02 | TLR4 agonist, improve antigen presentation | RCT: Significant | [12] |
| (MPL, QS21: extract from *Quillaja saponaria*) | | | |
| *Pre-S* | | | |
| Sci-B-Vac/BioHepB | block HBV-entry to hepatocyte? | preS1 antibody 50~60% positive | [15, 16] |
| (preS1/PreS2/preS) | | | |

GM-CSF;granulocyte macrophage-colony stimulating factor, OR; Odds ratio, RCT; randomized controlled trial, TLR; toll-like receptor

**Table 2.** Human trials to improve the effectiveness of HBV vaccination

### 2.2. Escape mutant

Mutations in the small-S protein, commonly glycine to arginine substitution at codon 145 (G145R), have been found in some children born from mothers infected with hepatitis B [17]. Although these mutants have been found in many parts of the world, the prevalence appears

to be low and decline in the efficacy of HBV vaccine has not been reported. However, a recent report showed that the mutant HBV was transmitted by sexual contact with a subject who had received universal HBV vaccination [18]. Application of HBV vaccine including preS protein to block infection of HBs mutant could be an effective strategy and should be investigated in the future.

## 3. Recent advances in HCV vaccine

In the analysis of secondary HCV infection after spontaneously clear primary infection, increased rate of spontaneous viral clearance and broader T-cell responses was found [19]. Actually, an animal study using chimpanzee showed that T-cell vaccine elicits effective immunity against HCV challenge thorough early $CD4^+$ and $CD8^+$ T-cell response [20]. These indicate the induction of protective memory responses against HCV during natural infection and also suggest a possibility for the development of effective prophylactic HCV vaccines.

B-cell response against HCV through the production of neutralizing antibodies has been analyzed for the development of HCV vaccine. In acute HCV infection, the detection of neutralizing antibodies and consequent rapid clearance of HCV had been reported [24]. On the other hand, delayed induction of these neutralizing antibodies has been shown in patients developing chronic HCV infection, [21]. These observations represent that the neutralizing antibodies could not control HCV infection and there are escape mechanisms of HCV from those antibodies. Actually, rapid evolution in the envelope glycoprotein sequences has been demonstrated as the mechanism of HCV evasion [22]. The HCV envelope glycoproteins, E1 and E2, have proven to be the essential region not only for viral attachment but also for viral endocytosis into hepatocytes [23]. Hypervariable region (HVR) is known to be located at the N-terminus of E2 region and is highly immunogenic and the region is necessary for binding to scavenger receptor class B type I (SR-BI), a lipoprotein receptor molecule involved in HCV entry [24,25]. Furthermore, conformational epitope within E2 is known to be conserved among various genotypes of HCV and is necessary for binding to CD81 [26,27]. While, E1 displays a relatively high degree of conservation within subtypes, also suggesting a high degree of intergenotypic cross-neutralization potential [28].

$CD4^+$ and $CD8^+$ T-cell are also important in viral clearance [29-31]. $CD4^+$ T-cells against conserved protein epitopes, such as HCV core, nonstructural (NS)3, NS4 and NS5, have been associated with self-limited infection of HCV. Trials for HCV prophylactic vaccines can be divided into two strategies to induce T-cell response or neutralizing antibodies, but both should be addressed together for an effective prophylaxis strategy. Since acute HCV infection is usually asymptomatic and is not associated with liver failure, prevention from acute to chronic HCV infection is another key goal of vaccine development.

Preclinical evaluation of prophylactic vaccine has been performed in chimpanzee, the only established model for the study of HCV infection in an immunocompetent host. These preclinical trials and the results are summarized in Table 3. Not only humoral responses but also cellular responses were elicited by vaccination, leading to viral clearance after HCV

challenge [20,32-37]. Because many chimpanzees spontaneously resolve acute hepatitis C in unvaccinated control groups, definite conclusions for the efficacy of HCV vaccination should be analyzed in human studies. Most vaccine candidates were successful in inducing immune response and reducing viral load. However, protection of infection following challenge with heterologous strains was limited. Although these limited protection shows the difficulty in developing a vaccine against different isolates, these preclinical trials certainly provides information on potential and design of vaccine candidates. As phase I human clinical trial, E1/E2 vaccine adjuvanted with MF59 (an oil-in-water emulsion) was administered to healthy adults, and neutralizing antibodies could be induced without adverse events [38]. Furthermore, these antibodies showed the neutralizing capacities against heterologous virus strains [39]. Further trials should be made for the development of effective HCV vaccines.

| Materials | Outocome | ref |
|---|---|---|
| E1/E2 protein | 21 vaccinated/24 controls | [32] |
| | 2/12 chronic infection after homologous challenge | |
| | 1/9 chronic infection after heterologous challenge | |
| | Strong humoral immune response | |
| | 15/24 chronic infection in controls | |
| DNA plasmid: E2 | 2 vaccinated/1 controls | [33] |
| | 2/2: viral clearance | |
| | High anti-E2 antibodies in one vaccine | |
| | E2-specific CD4 T-cell response in the second | |
| DNA plasmid: Core-E1-E2, NS3-5 | 6 vaccinated/2 controls | [34] |
| | 2/6: viral clearance | |
| | HCV-specific antibody and T-cell response | |
| | Reduced peak viral load in all animals<br>1 sterilizing immunity: high anti-E2 and strong cellular response | |
| | 1/2 controls: viral clearance | |
| Adenovirus NS3-NS5B | 5 vaccinated/5 controls | [20] |
| | 4/5: viral clearance after 18 months | |
| | Peripheral and intrahepatic CD8 T-cell response | |
| | 3/5 controls: viral clearance | |
| HCV like particle: Core E1-E2 | 4 vaccinated/4 controls | [35] |
| | 4/4: viral clearance after 12 months | |
| | HCV specific CD4 and CD8 T-cell response | |

| Materials | Outocome | ref |
|---|---|---|
| | 1/4 controls: viral clearance | |
| DNA plasmid: Core E1-E2 NS3 | 4 vaccinated/2 controls | [36] |
| | 1/4: viral clearance | |
| | Reduction of HCV load in serum and liver | |
| | Strong HCV CD4 response | |
| | Anti-E1 and anti-E2 specific antibodies | |
| Vaccinia virus Core E1-E2-P7 -NS3-NS3 | 4 vaccinated/2 controls | [37] |
| | 1/4: viral clearance after homologous | |
| | T-cell response: vigorous IFNγ production and moderate proliferation | |

**Table 3.** Preclinical trials of HCV vaccines in chimpanzees

## 4. Immnunotherapy for viral hepatitis and vaccine development

To develop efficient immunotherapy, understanding of immune response for eradication or suppression of hepatitis virus during acute hepatitis is important. Moreover, the immunosuppressive mechanisms leading to persistent viral infection need to be analyzed.

### 4.1. Immune response in acute viral hepatitis

Immunological analysis has been extensively performed in transgenic and chimpanzee models of acute HBV infection. In one model, transgenic mice, in which infectious HBV virions replicate in the liver with expression of all HBV-related antigens, were injected with HBsAg-specific cytotoxic T lymphocytes (CTLs) that had been induced in nontransgenic mice. The transgenic mice produced interferon (IFN)-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$, which purged viral RNA and DNA without destroying infected hepatocytes [40,41]. Importantly, this noncytolytic clearance of intracellular HBV is more efficient at controlling HBV replication than the killing of infected hepatocytes. This was confirmed in a chimpanzee infection model [42] and incubation phase of acute hepatitis B in humans [43].

The same is essentially true in acute HCV infection. Multispecific and vigorous CTL responses against HCV antigens are important for successful eradication of the virus. Moreover, a $CD4^+$ T cell response at an early stage of acute infection and persistence of the response are apparent in acute infection [44]. In contrast to acute HBV infection, the majority of patients with acute HCV infection progress to persistent infection, and the mechanisms underlying failure to eradicate the virus have been analyzed. The failure of $CD4^+$ T cell function is a key factor in HCV persistence and $CD4^+$ T cells from persistent infection do not produce Th1 cytokines, such as IFN-$\gamma$ and IL-2, but produce IL-4 and IL-10, clearly distinct from those seen

http://dx.doi.org/10.5772/55525

in patients with recovery [45]. Moreover, an early and strong Th1 response has been shown to play an important role in disease resolution.

The contributions of $CD4^+$ and $CD8^+$ T cells to the control of viral infection were analyzed in a chimpanzee model of acute hepatitis B and C by depleting either T cell population with monoclonal antibodies (Abs). The data showed that both $CD4^+$ and $CD8^+$ T cells are required for virus elimination [46,47].

### 4.2. Hierarchy of T cell response in viral hepatitis

The antigen-specificity of the T cell response to HBV in acute hepatitis has been analyzed, and it is clear that acute viral hepatitis involves a vigorous CTL response to multiple epitopes in the viral nucleocapsid, envelope, and polymerase proteins, while these are not seen in patients with chronic hepatitis [1]. Although multi-specificity of the CTL response is characteristic in acute hepatitis, there is known to be a hierarchy of epitope-specific $CD8^+$ T cell responses determined by cytokine production after peptide stimulation. In acute hepatitis B, $CD8^+$ T cell response to HBc18-27 (HLA-A2 restricted epitope) is dominant followed by the response to polymerase epitope (455 – 463), whereas envelope epitopes are always subdominant [48]. The hierarchy is clearly distinct from that observed in chronic hepatitis, in which the $CD8^+$ T cell response to envelope epitope (183 – 191) is always dominant. Interestingly, chronic hepatitis patients with lower HBV DNA levels in the serum show greater responses to HBc18-27 than those with high HBV DNA. These findings imply that the T cell response to HBcAg is important for viral control, which is important for designing peptide vaccines for the treatment of chronic HBV infection.

In acute HCV infection, the CTL responses were directed against multiple viral epitopes, in particular within the structural (core) and nonstructural (NS) regions of the virus (NS3, NS4, and NS5), and the CTL frequencies were higher in patients with acute infection [30,49]than in those who develop persistent infection. The hierarchy of HCV epitopes has not been analyzed extensively, but resolution of primary infection in the chimpanzee was shown to be associated with a dominant $CD4^+$ T cells response against epitopes including NS3 (GYKVLVLNPSV) [50].

### 4.3. Immune response in chronic viral hepatitis

In contrast to acute hepatitis, the T cell response to HBV is weak and is narrowly focused in chronically infected patients [51], suggesting that it may be a cause of persistent infection.

HBV-specific helper and CTLs are barely detectable in peripheral blood of patients with chronic hepatitis B (CHB) [52], possibly due to exhaustion by high viral load or tolerance to HBV.

In contrast to chronic HBV infection, CTL response against various HCV epitopes including core and envelope and NS regions can be detected in chronic HCV infection, especially in liver-infiltrating lymphocytes [53]. Although intrahepatic CTL response was shown associated with low viral load [54], the CTL response is not enough to terminate HCV infection possibly due to the presence of immunosuppressive mechanisms similar to chronic HBV infection.

## 4.4. Immunosuppressive mechanisms responsible for persistent hepatitis virus infection

### *4.4.1. Regulatory T cells (Tregs)*

In HBV infection, significant accumulation of $CD4^+CD25^+FoxP3^+$ Treg cells in the liver was found in patients with chronic HBV infection. Moreover, patients with high viral load have a higher proportion of Tregs in the liver [55], suggesting that intrahepatic Tregs suppress antiviral immune responses in the liver in chronic hepatitis B virus infection. In HCV infection, several groups have also shown a higher frequency of $CD4^+CD25^+$ regulatory T cells in the blood of chronically HCV-infected patients versus recovered or healthy individuals [56,57] and the presence of $CD4^+FoxP3^+$ T cells in the liver of chronically HCV-infected patients [58].

### *4.4.2. Programmed Death-1 (PD-1)*

PD-1 is a surface receptor critical for the regulation of T cell function [59,60]. Binding to PD-1 by its ligands PD-L1 and PD-L2 results in the antigen-specific inhibition of T cell proliferation, cytokine production, and cytolytic function, leading to exhaustion of T cells.

Intrahepatic HBV-specific $CD8^+$ T cells express higher levels of PD-1, and upregulation of intrahepatic PD-1/PD-L1 is associated with liver inflammation and ALT elevation [64]. PD-1/PD-L1 blockade increased $CD8^+$ T cell proliferation and enhanced IFN-$\gamma$ and IL-2 production by intrahepatic lymphocytes [61].

In chronic HCV infection, circulating and intrahepatic HCV-specific $CD8^+$ T cells were found to express high levels of PD-1 [62], and PD-1 expression level in the liver is higher than that in peripheral blood. Increased expression of PD-1 is associated with $CD8^+$ T cell dysfunction, and functional restoration is achieved by blocking the signal from PD-1 [63]. Interestingly, HCV core protein induces PD-1 and PD-L1 on T cells from healthy donors [64], indicating that immunosuppressive ability of HCV core protein is mediated by the upregulation of inhibitory molecules on T cells. Increased PD-1 expression on HCV-specific CTLs was reported to be significantly associated with poor response to antiviral therapy [65].

### *4.4.3. IL-10*

IL-10 is an important cytokine with anti-inflammatory properties, and is produced by activated monocytes/macrophages and T cell subsets, including Treg and Th1 cells [66]. In chronic HBV infection, HBcAg stimulates the production of IL-10, which negatively regulates HBcAg-specific Th17 cell responses in CHB patients [67].

In HCV infection, HCV proteins have been shown to induce IL-10 from monocytes in patients with chronic HCV infection, leading to suppression of antiviral immune response [68].

### *4.4.4. T-cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3)*

Recently, an inhibitory molecule, Tim-3, has been reported. A high frequency of Tim3-expressing $CD4^+$ and $CD8^+$ T cells are found in chronic HBV infection, and the frequency of

Tim-3$^+$ T cells was positively correlated with the severity of liver inflammation, and negatively correlated with plasma IFN-γ levels [69]. Tim-3 was also highly expressed on CD4$^+$ and CD8$^+$ T cells in HCV infection, with the highest levels seen on HCV-specific CTLs. Tim-3 expression is associated with reduced Th1/Tc1 cytokine production, and blocking the Tim-3 – Tim-3 ligand interaction could enhance CD4$^+$ and CD8$^+$ T cell proliferation in response to HCV-specific antigens [70].

*4.4.5. Dysfunction of Dendritic Cells (DCs)*

In patients with CHB, maturation of DCs from peripheral blood of patients after incubation with cytokines is lower than that of normal subjects with lower expression of HLA-DR and costimulatory molecules in the former population [71], leading to low allostimulatory function of DCs from CHB patients. Interestingly, impaired function of monocyte-derived DCs from patients with CHB could be reversed by inhibiting viral replication with nucleoside analogs such as lamivudine [72]. Type 2 precursor plasmacytoid dendritic cells (pDCs), which are the most important cells in antiviral innate immunity, were also reported to have quantitative and qualitative impairment in patients with chronic HBV infection [73]. Recently, HBV itself was shown to inhibit the functions of pDCs [74].

In chronic hepatitis C, DCs from patients also show impaired immunostimulatory function, which could be induced by HCV [75] or NS4 protein [76]. Monocyte-derived DCs from HCV patients were shown to induce proliferation of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells, which limit proliferation of HCV-specific T lymphocytes [77]. DCs in HCV patients thus inhibit T cell responses via a variety of mechanisms.

## 5. Immunotherapeutic trials for viral hepatitis

Previous basic analyses and human trials in HBV infection are listed in Tables 4 and 5, respectively, and those in HCV infection are summarized in Tables 6 and 7.

| Animal model | Immunotherapy | Results | Ref |
|---|---|---|---|
| ***Peptide vaccination*** | | | |
| HBV transgenic mice | A synthesized fusion peptide, consisting HBcAg18-27 and HIV Tat49-57 adjuvanted with CpG ODN | Decreased in serum HBV DNA levels and the expression levels of HBsAg and HBcAg in the liver | [79] |
| ***Protein vaccination*** | | | |
| HBV transgenic mice | HBsAg vaccine | Most of the mice showed reduction of HBV DNA levels and disappearance of HBeAg and HBsAg | [83] |

| Animal model | Immunotherapy | Results | Ref |
|---|---|---|---|
| Woodchuck hepatitis Virus infection | Combination of vaccine of HBV large surface protein and clevudine | Restored T-cell response to Pre-S and S region. | [85] |
| Mice | Chimeric HBsAg-preS1 protein | Primed both HBcAg-specific T cells and antibodies to preS1. | [86] |
| Balb/c and HBV transgenic mice | Chimeric HBsAg-preS1 protein | Induced strong anti-HBc and moderate anti-preS1 immune response, and reduced HBsAg and HBV DNA in HBV-Tg mice. | [96] |
| Balb/c mice | Chimeric protein with HBcAg and carboxy terminus of the Hsp65 | Induced moderate anti-HBc immune response and strong HBcAg-specific T cells response. | [97] |
| Balb/c and HBV transgenic mice | HBsAg, HBcAg and heat shock protein gp96 | Decreased serum HBsAg and HBcAg expression in hepatocytes by 45% and 90%, respectively. Decreased serum HBV DNA to below or close to the detection limit. | [98] |
| Balb/c and HBV transgenic mice | Fusion protein with protein transduction domains from HIV-1-Tat and HBcAg | Induced HBcAg-specific CTLs and enhanced production of IFN-γ, IL-2, IL-4 and IL-10. Reduced HBV DNA and HBsAg in the serum and HBsAg expression in liver tissue of HBV transgenic mice. | [99] |
| ***DNA immunization*** | | | |
| Woodchuck | DNA vaccine expressing WHsAg was administered by electroporation | Induced dose-dependent antibody and T cell responses to WHsAg more efficiently than conventional hypodermic needle injection. | [104] |
| Acute DHBV infection | DNA vaccine expressing DHBc and Pre-S/S and entecavir<br>Boosted with fowl poxvirus vectors expressing DHBc and Pre-S/S | Cleared DHBV infection at a rate of 100%. | [105] |
| Chronic DHBV infection | DNA vaccine encoding the HBV large envelope and/or core protein with or without lamivudine | Reduced viremia and liver DHBV cccDNA in 33% of ducks.<br>Seroconversion to anti-pre S in 67% of ducks showing cccDNA clearance. | [106] |
| HBV transgenic mice | DNA vaccine expressing HBcAg fused to extracellular | Reduced serum HBV DNA and HBcAg in the liver. Clearance of serum HBsAg was also observed. | [107] |

| Animal model | Immunotherapy | Results | Ref |
|---|---|---|---|
| | domain of CTLA-4. Mice were challenged by pAAVNBV1.2 | | |
| HBV transgenic mice | DNA vaccine expressing HBsAg fused to extracellular domain of CTLA-4. | Serum levels of HBsAg and HBV DNA were decreased by induction of anti-HBs Ab and HBsAg-specific CD8+ T cell response. | [108] |
| ***DC immunization*** | | | |
| HBV transgenic mice | Activated bone marrow-derived DCs | Broke CTL tolerance to HBsAg. | [121] |
| HBV transgenic mice | HBV-specific peptide-pulsed DCs | Reduced in the serum HBsAg and HBV DNA. | [122] |
| HBV transgenic mice | Anti-CD40 agonistic monoclonal Ab | Induced noncytopathic inhibition of HBV replication mediated by antiviral cytokines (IL-12 and TNF-α) produced by activated intrahepatic APCs. | [123] |
| ***Cytokines and adjuvants*** | | | |
| HBV transgenic mice | Recombinant IL-12 | Markedly inhibited HBV replication in the liver. | [132] |
| HBV transgenic mice | α-galactosylceramide that can activate NKT cells | Induced complete inhibition of HBV replication. | [133] |
| HBV transgenic mice | Recombinant IL-18 | Inhibited HBV replication noncytopathically, mediated by activation of resident intrahepatic NK cells and NKT cells. | [134] |
| ***Gene therapy*** | | | |
| HBsAg transgenic mice | Lentivectors expressing HBsAg and IgFc fusion Ag | Induced seroconversion to anti-HBs. | [100] |
| ***Adjuvant*** | | | |
| HBV transgenic mice | Cationic lipid DNA complexes and HBsAg | Suppressed HBV DNA in hepatocytes non-cytopathically. | [101] |
| Woodchuck | Cationic liposomes and non-coding DNA was administered with WHsAg intramuscularly | Induced rapid and high Ab and T cell response to WHsAg. | [102] |

CpG ODN; CpG oligodeoxynucleotide, WHV; woodchuck hepatitis virus, DHBV; duck hepatitis B virus, cccDNA; covalently closed circular DNA, DC; dendritic cells, CTL; cytotoxic T lymphocytes, APC; Antigen-presenting cells, NKT; natural killer T, Ig; immunoglobulin

**Table 4.** Immunotherapeutic approaches for animal models of HBV infection

| Immunotherapy | Results | Ref |
|---|---|---|
| ***Peptide vaccination*** | | |
| A vaccine with HBc18-27 peptide comprised of a T-helper cell epitope and two palmitic acid residues | Low levels of CTL activity were induced but no significant changes in liver biochemistry or viral serology were observed. | [80] |
| ***Protein vaccination*** | | |
| PreS2/S (GenHevac B) or S (Recombivax). | HBe/anti-HBe seroconversion was observed in 13% and HBV DNA negativity was in 16% of the treated patients. | [84] |
| Oral administration of HBV envelope proteins (HBsAg+preS1+preS2) | Induced histological improvement in 30%, HBeAg negativity in 26.3% and HBsAg-specific T cell proliferation in 78% of the treated patients. | [87] |
| Combination of lamivudine and HBsAg vaccine | Induced sustained negativity of HBV DNA in 1/4 of the patients. | [88] |
| The combination with lamivudune and HBsAg vaccine in $HBeAg^+$ cases | No improvement of HBe seroconversion rate was observed in comparison with lamivudine therapy alone. | [89] |
| Combination of lamivudine and HBsAg vaccine | HBV DNA became undetectable in 64% of the patients, and was decreased in the remaining patients. | [90] |
| Intradermal HBsAg vaccine and laimvudine in combination with IL-2 | Induced significant HBV DNA loss in the serum in two of five of the treated patients. | [91] |
| IFN-α-2b monotherapy (9 months) or IFN-α-2b plus pre-S2/S vaccine | Induced greater reduction in HBV DNA in patients with combination HBV therapy than those who received IFN-α-2b monotherapy. | [92] |
| Complexes composed of yeast-derived hepatitis B surface antigen (HBsAg) and antibodies | HBeAg seroconversion rate was 21,6% and was correlated with decrease of HBsAg and HBV DNA. | [93] |
| HB preS/S vaccine (GenHevac B) | Caused no effect on HBV DNA and seroconversion of HBeAg to HBeAg in the immunotolerant phase of children with chronic HBV infection. | [94,95] |
| ***DNA immunization*** | | |
| DNA vaccine encoding HBV envelope protein | Induced an increase in HBV-specific IFN-γ-secreting T cells in nonresponders to conventional therapies, and HBV DNA levels were transiently decreased in 50% of vaccinated patients. | [109] |
| DNA vaccine encoding PreS and S in patients with lamivudine breakthrough | Induced IFN-γ-producing T cells specific for preS or S antigen. Two of 10 patients showed seroconversion to anti-HBe. | [110] |
| DNA vaccine encoding HBsAg followed by recombinant modified vaccinia virus Ankara expressing HBsAg | Failed in decrease in AST or ALT and did not reduce HBV viremias. | [111] |
| ***DC immunization*** | | |

| Immunotherapy | Results | Ref |
|---|---|---|
| Activated DCs from PBL pulsed with HBsAg | Induced anti-HBs and HBsAg-specific cellular immnunity in some patients. | [124] |
| PBL-derived DCs from chronic hepatitis B incubated with a cocktail of cytokines: IL1-β, PGE2, IL-6 and TNF-α, and pulsed with HBsAg or HBcAg | Induced autologous T cell proliferation and Ag-specific IFN-γ production. | [125] |
| Peripheral blood-derived DCs, activated with GM-CSF and IL-4 pulsed with HBsAg. | Both patients with normal and elevated ALT responded equally to DC vaccine and 53% of the patients showed induction of HBeAg negativity. | [126] |
| Activated DCs from PBL with GM-CSF and IL-4, pulsed with two peptides, HBc18-27 and PreS2 44-53. | Undetectable HBV DNA was achieved in 46.3% and 3.13% of $HBeAg^-$ and $HBeAg^+$ patients, respectively. ALT normalization was observed in 69% and 30.5% of $HBeAg^-$ and $HBeAg^+$ patients, respectively. | [127] |
| ***Cytokines*** | | |
| GM-CSF | Safe and tolerable up to 1.0mg/kg body weight, and induced HBV DNA negativity in 4/8 patients. | [135] |
| Combination therapy with GM-CSF and HBsAg vaccine in HBV carrier children | Significantly reduced serum HBV DNA. | [136] |
| High dose of IL-12 (0.5μg/kg) | HBV DNA clearance was observed in 25% of the patients. | [137] |
| Combination of IL-12 and lamivudine | Stimulated T cell response to HBV with IFN-γ production. However, IL-12 was unable to suppress re-elevation of HBV DNA after cessation of lamivudine. | [138] |
| Combination of IL-12 and IL-18 | Stimulated IFN-γ production by CD4+ T cells isolated from peripheral blood in response to HBcAg, and the effect was greater than those observed with either cytokine alone. | [139] |
| ***Thymosin-α 1(Talpha1)*** | | |
| Combination of Talpha1 and IFN-α | No significant differences was observed as compared with IFN-α monotherapy with respect to HBeAg seroconversion, changes in histology, normalization of ALT or loss of HBV DNA. | [140] |
| Talpha1 alone | At 12 months after cessation of therapy, 36.4% of patients treated with 1.6mg of Talpha1 achieved ALT normalization, 15% achieved HBV DNA clearance by transcription-mediated amplification, and 22.8% achieved clearance of HBeAg. | [141] |
| Comparative effect of Talpha1 and IFN-α | Talpha1 treatment was more effective in achieving ALT normalization and HBV DNA negativity at the end of the follow-up period than IFN-α. | [142] |

| Immunotherapy | Results | Ref |
|---|---|---|
| Combination of Talpha1 and lamivudine | No any additional antiviral effect compared with lamivudine monotherapy as assessed by HBe seroconversion and the emergence of viral breakthrough. | [143] |
| Combination therapy with lamivudine and Talpha1 | Induced significantly higher rates of ALT normalization, virological response, and HBeAg seroconversion than lamivudine monotherapy. | [145] |
| CTL; cytotoxic T lymphocytes, GM-CSF; granulocyte macrophage-colony stimulating factor | | |

**Table 5.** Immunotherapeutic trials for chronic HBV infection in humans

| Animal model | Vaccine | Results | Ref. |
|---|---|---|---|
| ***Protein vaccination*** | | | |
| Chimpanzee | Recombinant HCV-like particles containing core, E1 and E2 proteins | Increased in peripheral and intrahepatic T cell proliferative responses against the HCV proteins. | [35] |
| ***DNA immunization*** | | | |
| HCV transgenic mouse model | The combination of DNA vaccination encoding HCV core and mouse IL-2 | Broke tolerance against HCV and activates previously tolerant T cells. | [112] |
| Mice expressing HCV antigens in the liver | HCV NS3/NS4 DNA vaccine | Induced HCV-specific CD8+ T cells expressing IFN-γ and CCR5 and cleared HCV NS3 expressing hepatocytes. | [113] |
| Mice | Murine DCs with CFm40L transfected with adenovirus encoding HCV NS3 | Induced CD4+ and CD8+ T cell response against HCV NS3. | [114] |
| Mice | DCs transfected with adenovirus encoding HCV NS3 | Induced multiepitopic CD4+ (Th1) and D8+ T cell response and down-regulated the expression of HCV RNA in the liver. | [115] |
| Balb/c and HLA-A2.1 trangenic mice | DCs transfected with adenovirus encoding HCV NS3 | Induced NS3-specific cell mediated and humoral immune response. | [116] |
| Chimpanzee with HCV-challenge | Recombinant adenoviral vectors encoding the HCV NS3-5B (genotype 1b) and with NS3-5B–encoding plasmid DNA in a combined modality regimen | HCV NS3-NS5, HCV-specific T cells appeared earlier, maintained better functionality, and persisted at higher frequencies. The T cells controlled HCV-challenge. | [117] |
| **DC immunization** | | | |

| Animal model | Vaccine | Results | Ref. |
|---|---|---|---|
| HCV transgenic mouse model | DCs treated with peptide inhibitors of IL-10 | Induced strong anti-HCV T cell responses | [128] |
| ***Gene therapy*** | | | |
| C57BL/6 and BALB/c mice | Adenovirus-besed HCV vaccine by fusing HCV NS3 to MHC class II chaperone protein invariant chain | Induced CD8+ T cells expressing IFN-γ, TNF-α, IL-2, CD27 and CD127. The CD8+ T cells protected mice from infection with recombinant vaccinia virus expressing HCV NS3 of heterologous 1b strains | [129] |

**Table 6.** Immunotherapeutic approaches for animal models of HCV infection

| Immunotherapy | Results | Ref. |
|---|---|---|
| ***Peptide vaccination*** | | |
| A vaccine, IC41, containing 7 relevant HCV T cell epitopes and the Th1 adjuvant poly-L-arginine | Induced HCV-specific Th1/Tc1 responses in a subset of HCV patients not responding to or relapsing from standard therapy. However, only a minimal decrease in HCV viremia was induced by the vaccination. | [81] |
| Vaccination with a peptide derived from HCV core protein | Induced both cellular and humoral responses in nearly all HCV patients with different HLA class I-A alleles, and reduced serum ALT and AFP levels in 29% and 50% of patients, respectively. | [82] |
| ***DNA vaccination*** | | |
| A new vaccine, CIGB0230, consisting of a mixture of plasmid expressing HCV structural antigens and HCV recombinant core protein | Induced specific T cell proliferation and IFN-γ production in 73%. More than 40% of the vaccines showed improvement of liver histology, despite persistent detection of HCV RNA. | [99] |
| ***DC vaccination*** | | |
| Human DCs from HCV-infected patienst with CFh40L transfected with adenovirus encoding HCV NS3 | Induced CD4+ and CD8+ T cell response against HCV NS3 in HCV-infected patients | [114] |
| Monocyte-derived DCs loaded with lipopeptides consisting of HCV-specific HLA-A2.1-restricted CTL epitopes | Induced HCV-specific CD8+ T cell responses with IFN-γ production in PBL in HCV patients in whom conventional IFN-based therapy has failed. However, ALT levels were not elevated and viral load was not decreased. | [118] |
| Human DCs infected with adenoviral vectors harboring HCV core and NS3 | Induced CD4+ and CD8+ T cell response against HCV core and NS3 in healthy subjects | [130] |
| Human DCs infected with adenoviral vectors harboring HCV NS genes | DCs transfected with adenovirus NS3/NS4 efficiently induced HCV-specific immunity in healthy subjects | [131] |

| Immunotherapy | Results | Ref. |
|---|---|---|
| ***Cytokine*** | | |
| Talpha1 | Patients with chronic HCV infection who had been nonresponders to prior IFN-α and ribavirin were treated with Talpha1, PEG-IFN α-2a, and ribavirin for 48 weeks. Twenty-four percent of the treated patients with genotype 1 achieved a sustained virological response. | [144] |
| ***Blockade of inhibitory signals*** | | |
| Blocking PD-1, CTLA-4 and IL-10 combined with therapeutic vaccination | Synergistically enhanced functional CD8+ T cell response and improve viral control in chronically infected mice. Moreover, addition of stimulatory signals, such as IL-2, could further increase the efficacy of the therapy in chronic viral infection | [146] |
| Combined blockade of CTLA-4 and PD-1 | Combined blockade of CTLA-4 and PD-1, but not blocking of either molecule, can reverse CD8+ T cell exhaustion in HCV infected patients | [147] |
| Blocking Tim-3/Tim-3 ligand | Blocking Tim-3/Tim-3 ligand induced intrahepatic T cell proliferation and IFN-γ production in response to HCV antigens in HCV-infected patients | [70] |
| Blockade of Tim-3 on human HCV-specific CTLs | Blockade of Tim-3 on human HCV-specific CTLs fron HCV-infected patients increased cytotoxicity against an HCVAg-expressing hepatocyte cell line that expresses HCV epitopes | [149] |
| ***Gene transfection*** | | |
| Human T cells transduced with HCV TCR specific for HCV NS3 1071-1081 (HLA A2-restricted epitope) | T cells recognized the peptide and produced IFN-γ, IL-2 and TNF-α in healthy subjects | [119] |
| Two adenoviral vectors expressing NS3, 4 and 5 proteins from HCV genotype 1B | Induced HCV-specific CD4+ and CD8+ T cells subsets secreting IL-2, IFN-γ, and TNF-α and could be sustained for at least a year after boosting. | [120] |
| ***Recombinant virus*** | | |
| Recombinant poxvirus vaccine, TG4040, that expresses the hepatitis C virus (HCV) proteins NS3, NS4, and NS5B. | It was safe and well-tolerated. It induced HCV-specific immune response and a transient decrease in HCV viremia (>1log) in 33% of HCV-infected patients. | [103] |

CTLA-4; cytotoxic T lymphocyte antigen-4, PD-1; programmed death-1, Tim-3; T cell Immunoglobulin and Mucin domain containing-3

**Table 7.** Immunotherapeutic approach for chronic HCV infection in humans

### 5.1. Suppression of viral replication

High viral load has been shown to suppress $CD4^+$ and $CD8^+$ T cells in addition to induction of Tregs, which could be reversed by antiviral therapy [78]. Therefore, immunotherapy followed by restoration of virus-specific T cell response with antiviral therapy could be more efficient.

### 5.2. Induction of immune response to hepatitis virus

#### *5.2.1. Peptide immunization*

A fusion peptide consisting of HBc18-27 and HIV Tat49-57 was synthesized, and the vaccination induced significant anti-viral effect in HBV transgenic mice [79]. However, in humans, peptide vaccine containing highly immunogenic HBc18-27 administered to CHB patients, showed disappointing results [80], because there was no induction of a significant antiviral T cell response.

In HCV infection, a vaccine, IC41, containing 7 HCV T cell epitopes and the Th1 adjuvant induced HCV-specific Th1/Tc1 responses in chronic HCV patients, but anti-viral effects were minimal [81]. Another HCV vaccine with a peptide derived from HCV core protein induced both cellular and humoral responses in HCV patients and reduced serum alanine aminotransferase (ALT) and alpha-fetoprotein (AFP) in some patients [82].

#### *5.2.2. Protein immunization*

In a model of HBV in transgenic mice, HBsAg vaccine in complete Freund's adjuvant once a month for 12 months induced reduction in HBV DNA, and the disappearance of HBeAg and HBsAg in most mice treated [83]. Interestingly, some mice developed anti-HBs in the sera. However, several human trials with HBsAg vaccine showed limited efficacy if used as monotherapy.

Recently, HB vaccine containing not only S protein but also preS has been used with increased immunogenicity [84-88], or has been combined with lamivudine [89,90], IL-2 [91] or IFN-$\alpha$ [92] leading to potential improvement of clinical efficacy [93-95]. Moreover, vaccines containing HBcAg have been developed, and some showed significant anti-viral effect in HBV transgenic mice [96-99]. Because T cell response to HBcAg is important for viral control, these vaccines may have a promising immunothepapeutic potential also in humans. Recently, some trials to enhance the immunogenicity of HBV vaccine in combination with adjuvant or by using viral vectors have been made [100-102].

In HCV infection, a recombinant poxvirus vaccine expressing HCV NS3, NS4 and NS5B, TG4040, has been recently developed [103] and administered to HCV patients. The vaccine was safe and induced HCV-specific cellular immune response and reduction in viremia. These data are encouraging, and further large scale clinical trials need to be done.

#### *5.2.3. DNA immunization*

Injection of plasmid DNA has been shown to strongly elicit both cellular and humoral immune responses. DNA vaccine is now shown to be safe and well-tolerated, and has been tried in humans with some encouraging anti-viral effects both in mice and humans [104].

In a model of duck hepatitis B virus infection, DNA vaccine encoding HBV large envelope and/or core protein was shown to induce reduction in not only viremia [105] but also cccDNA in the liver in one third of ducks receiving DNA monotherapy or combination treatment along with lamivudine [106]. This finding is encouraging because clearance of cccDNA from the liver is the goal of treatment for HBV infection, but is difficult to achieve using IFN-$\alpha$ or nucleoside analogs. More recently, DNA vaccine expressing HBcAg or HBsAg in combination with extracellular domain of CTLA-4 have been developed and showed significant anti-viral effects in HBV transgenic mice [107,108]. In humans, safety and therapeutic potential of DNA vaccines have been already explored in chronic HBV carriers [109-111].

In HCV infection, DNA vaccine encoding HCV core and IL-2 breaks tolerance and activates previously tolerant T cells in HCV transgenic mice [112]. NS3-specific T cells were induced by DNA immunization in mice models [113-116]. In chimpanzee models, NS3-specific T cells were also induced and HCV-challenge could be controlled [117]. In HCV patients, a new DNA vaccine, CIGB0230, consisting of a mixture of plasmid expressing HCV structural antigens induced HCV-specific T cell response and improved liver histology [118]. Furthermore, trials to elicit HCV-specific T cells response by transduction of HCV-specific T cell receptor or by new type vaccines have been made [119,120].

*5.2.4. DC immunization*

DCs are specialized antigen-presenting cells that can induce strong immune responses in T and B cell. We have previously shown that activated bone marrow-derived DCs can break CTL tolerance to HBsAg in HBV transgenic mice [121]. Thereafter, several immunotherapies with activated DCs have been applied in both animals and humans. In a recent study performed in HBV transgenic mice, peptide-pulsed DCs were shown to significantly reduce the concentrations of serum HBsAg and HBV DNA [122], indicating therapeutic potential in chronic HBV infection. Moreover, when intrahepatic antigen-presenting cells, including DCs, were activated by injection of anti-CD40 agonistic Ab, HBV replication was inhibited by a noncytopathic mechanism possibly through production of antiviral cytokines such as TNF-$\alpha$ and IL-12 [123]. Although no CTL response against HBV antigens was reported in this study, the in vivo activation of DCs could be an alternative way for inducing antiviral immune responses including possible activation of CTLs against HBV. In humans, injection of activated DCs loaded with HBV peptide or protein achieved the induction of HBV-specific immunity [124,125] and a reduction in HBV DNA level in some patients [126,127]. HBeAg negativity was achieved in more than half of the treated patients in one study [126].

In HCV infection, DCs treated with peptide inhibitors of IL-10 were shown to induce strong anti-HCV T cells response in HCV transgenic mice [128], suggesting a strategy to augment the immunogenic function of DCs. Recently, murine DCs infected with adenovirus encoding HCV NS3 were used as vaccines, and showed induction of NS3-specific T cell response and antiviral effect [129]. In humans, DCs infected with adenovirus vectors harboring HCV core or NS genes, especially NS3, were administered in healthy subjects [130,131] and HCV patients [114,118], and those DCs induced $CD4^+$ and $CD8^+$ T cell response in both populations. Although preparation of activated and mature DCs incurs financial costs and requires experienced researchers, immunotherapy with DCs is a promising method.

*5.2.5. Cytokines and Thymosin-α1 (Talpha1)*

Cytokines, such as IL-12 [132] and IL-18 [133], and the activation of NKT cells [134] were shown to inhibit HBV replication noncytopathically in HBV transgenic mice. In humans, GM-CSF [135,136] and IL-12 [137-139] have been used for treatment with some antiviral effects. They have been used as monotherapy or in combination with HBsAg vaccine or lamivudine.

Talpha1, a synthetic 28-amino acid peptide, is able to enhance the Thl immune response and also exerts a direct antiviral mechanism of action. It has been used for the treatment of chronic HBV [140-143] and HCV [144] infection in humans, and showed antiviral effect with some efficacy. Although antiviral effect by the addition of Talpha1 to lamivudine or IFN-α therapy was controversial, a meta analysis demonstrated that the combination therapy with lamivudine and Talpha1 showed significantly higher rates of ALT normalization, virological response, and HBeAg seroconversion as compared with lamivudine monotherapy [145]. It is of note that HBeAg seroconversion rate was 45% in the combination group, which was significantly higher than that with lamivudine monotherapy (15%).

*5.2.6. Blockade of inhibitory signals*

There have been several basic attempts to improve the efficacy of immunotherapy. Among these reports, augmentation or restoration of T cell response by blocking the inhibitory signals have been extensively analyzed in vitro. It has been demonstrated that exhausted T cells

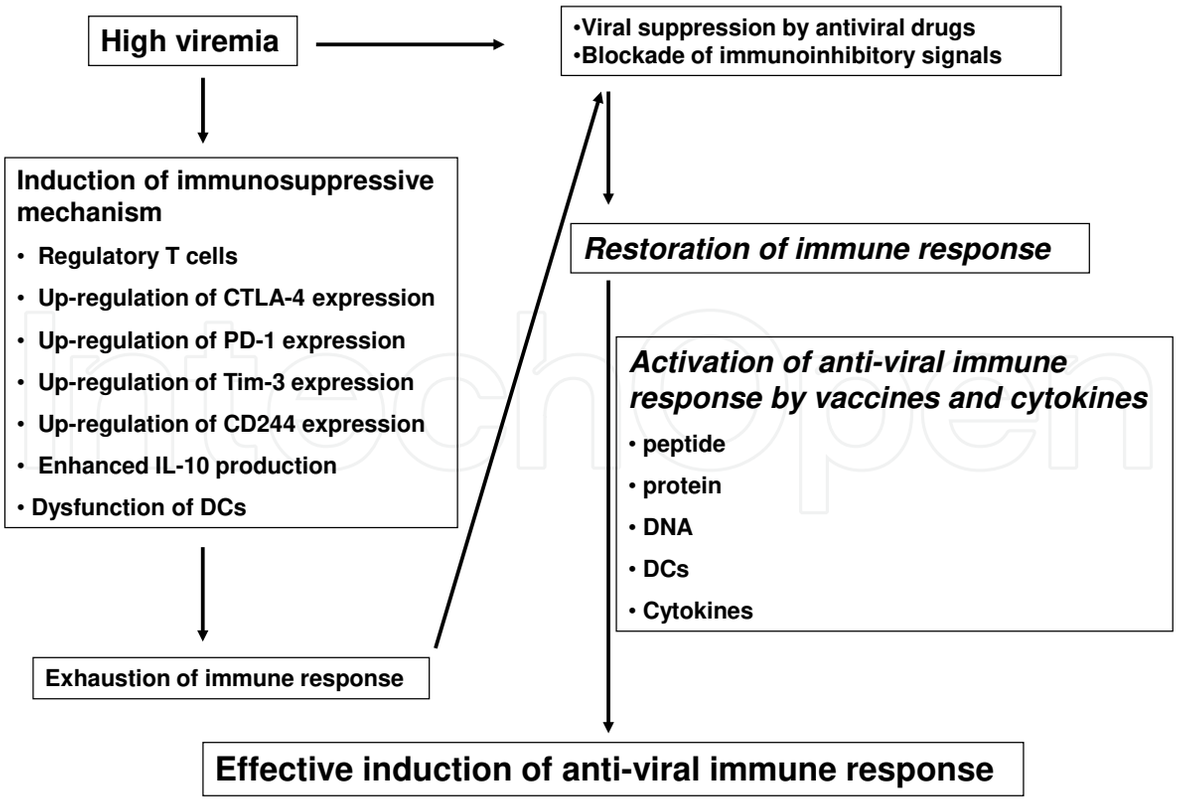


**Figure 1.**

express not only PD-1, but also CTLA-4 [146,147], CD244 [148] or Tim-3 [70,149], and blocking of these molecules in combination could be better than blocking any single molecule to achieve full activation of the exhausted T cells.

## 6. Conclusion

There have been several advances in immunotherapy and vaccine development both for prophylactic and therapeutic purposes in HBV and HCV infections and some of the data are promising. For therapeutic purposes, viral suppression, stimulation of antiviral immune response with vaccines with peptides, proteins, plasmid or DC, blockade of immunoinhibitory signals must be combined to achieve desirable antiviral effects (Fig.1). Further studies are required to explore the best protocols and their most efficient combinations to become a promising and practical treatment.

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