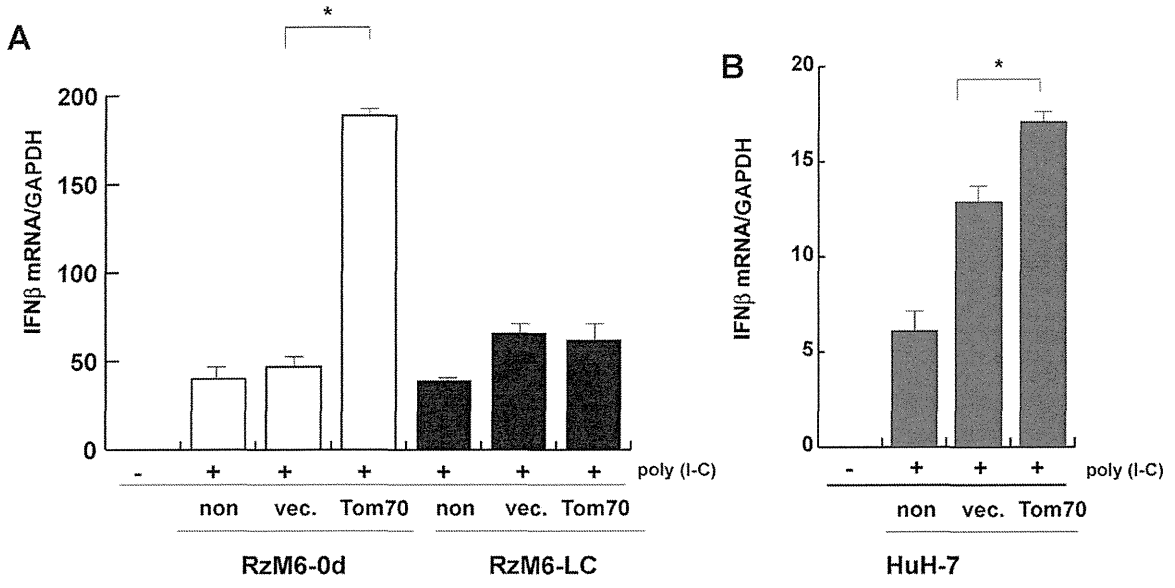

Fig. 2. Tom70-induced IFN synthesis was impaired by HCV. (A) RzM6-0d cells and LC cells were transfected with mock-vector, control pcDNA vector (vec.), or pcDNA-Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA using Gene expression assay kit (GE-Healthcare). Poly(I–C) (GE Healthcare) (5 μg) was transfected with RNAi Max reagent (Invitrogen) and IFN-βmRNA was measured after 6 h of poly(I–C) treatment. Vertical bars indicate S.D. $^*p < 0.05$. (B) HuH-7 cells were transfected with mock-vector, control vector, or Tom70 expression vector, and the amount of IFN-β mRNA was measured by RTD-PCR and normalized to the amount of GAPDH mRNA. Vertical bars indicate S.D. $^*p < 0.05$.

control siRNA did not have a significant effect on Tom70 protein expression.

We next examined the effects of HCV JFH-1 (Wakita et al., 2005) infection on Tom70 expression (Fig. 1B). Infection with HCV significantly increased the level of Tom70 protein expression. We also examine the role of Tom70 in HCV replication (Fig. 1C and D). Silencing of Tom70 by siRNA decreased the HCV replication in a dose dependent manner.

Thus, HCV induces Tom70 expression, and Tom70 is involved in viral replication.

It was recently shown that Tom70 recruits TBK1/IRF3 to mitochondria by binding to Hsp90 and inducing IFN-β synthesis (Liu et al., 2010). Therefore, we examined the effects of Tom70 overexpression on IFN synthesis and modification by HCV (Fig. 2). Level of IFN-β mRNA synthesis was quantitated by real-time detection (RTD) PCR. Overexpression of Tom70 by transfection of pcDNA6-Tom70 (Takano et al., 2011a) induced IFN-β mRNA synthesis in the absence of HCV after poly(I–C) treatment (RzM6-0d cells). However, the Tom70-mediated induction of IFN-β mRNA transcription was impaired in the presence of HCV (RzM6-LC cells) (Fig. 2A). Overexpression of Tom70 induced IFN-β mRNA synthesis in HuH-7 cells (Fig. 2B). Induction of IFN-β mRNA was lower in HuH-7 cells than HepG2 based RzM6 cells, which might be due to the defect in IFN induction system in HuH-7 cells (Preiss et al., 2008).

We have further addressed the mechanism of impairment of IFN-β mRNA transcription by HCV.

To identify the viral protein that was responsible for the induction of Tom70, we examined the Tom70 protein expression levels in HCV core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B protein-expressing cells (data not shown), and Tom70 protein expression level was highest in the NS3/4A-expressing cells than was observed in cells expressing other proteins (Fig. 3A, data not shown), indicating an effect of HCV NS3/4A protein on Tom70 expression.

The expression vector of Myc- and His-tagged Tom70 was transfected into the empty control or NS3/4A-expressing cells and immunoprecipitated with anti-Myc antibody (Suppl. Fig. 1A). Results showed that Myc-Tom70 was precipitated in both cells (right panel) and NS3 protein was specifically precipitated by anti-Myc antibody in the NS3/4A-expressing cells (left panel). NS4A protein could not be detected (data not shown).

We next stained the NS3/4A-expressing cells with anti-NS3 and -Tom70 antibodies, and observed with confocal microscopy (Suppl. Fig. 1B). The signal of NS3 protein was clearly merged with that of Tom70, strongly supporting the possibility that the NS3 protein co-localizes with the Tom70 protein.

To clarify the effect of Tom70 on NS3, we transfected NS3/4A-expressing cells with the siRNA of Tom70 (Fig. 3A). Silencing of Tom70 decreased the level of NS3 protein in cells, but did not influence the levels of the MAVS and NF-κB proteins. These results suggest the possibility that Tom70 may increase the stability of NS3 protein in cells.

Tom70 reportedly interacts with MAVS during viral infection (Liu et al., 2010). Therefore, we examined the MAVS protein in cells expressing either the control empty or NS3/4A lenti-virus vector (Fig. 3B). Cleavage of MAVS (indicated as ΔMAVS) was observed in NS3/4A protein-expressing cells, as was reported previously (Meylan et al., 2005). Overexpression of Tom70 did not have a significant effect on the MAVS expression level and did not prevent MAVS cleavage by NS3. IRF-3 phosphorylation was suppressed in NS3/4A-expressing cells and was not influenced by Tom70 overexpression. The induction of IFN-β was impaired in NS3/4A-expressing cells, even in the presence of Tom70 overexpression (Fig. 3C). These data may indicate that MAVS exists upstream of Tom70 and that cleavage of MAVS by NS3/4A impaired the downstream signaling activation of IRF-3 phosphorylation (Suppl. Fig. 2).

Mitochondria provide a substantial platform for the regulation of IFN signaling. The MAVS adapter protein is a member of the family of RIG-I like receptors (RLRs), which links the mitochondria to the mammalian antiviral defense system (Seth et al., 2005). Proteomic studies have demonstrated that MAVS interacts with Tom70 (Liu et al., 2010). This interaction was accelerated by Sendai virus infection and synergized with ectopic expression of Tom70 to significantly increase the production of IFN-β (Liu et al., 2010). The results of the present study revealed that infection with HCV induced Tom70 expression, but the presence of HCV impaired IFN

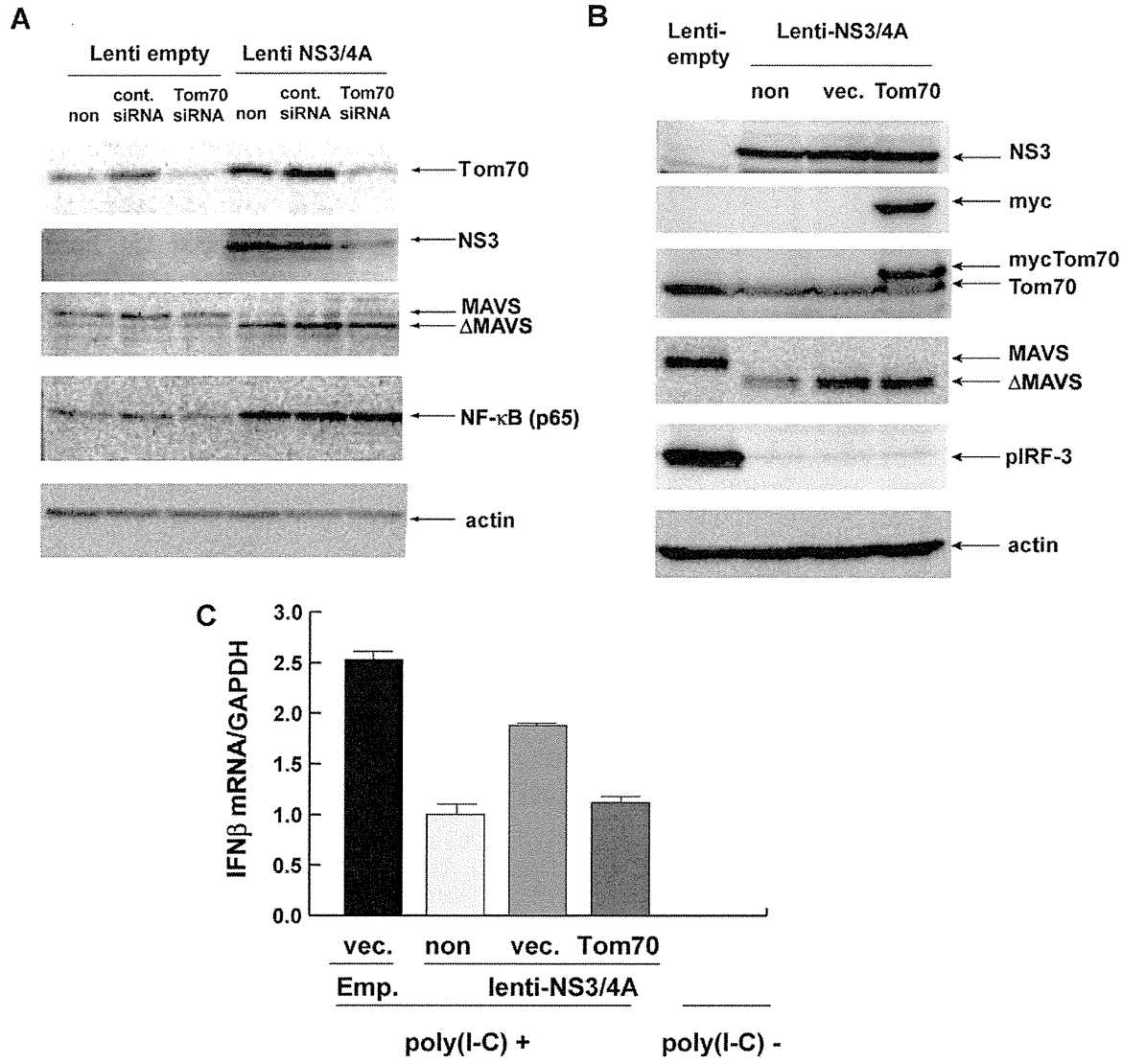

Fig. 3. Silencing of Tom70 decreased the level of NS3 and cleavage of MAVS by NS3/4A impaired IRF-3 phosphorylation even in the presence of Tom70. (A) Empty or NS3/4A-lenti virus vector expressing HepG2 cells were transfected with control siRNA and Tom70 siRNA or mock-transfected (non) as a control. MAVS, NS3, Tom70, and actin proteins were detected by western blot. (B) Empty or NS3/4A-expressing HepG2 cells were transfected with control pcDNA vector (vec.) and pcDNA6 (Invitrogen)-Tom70 or mock-transfected (non) as a control. NS3, Tom70, phosphorylated IRF-3, MAVS, and actin proteins were examined by western blot. (C) IFN-β mRNA was measured by RTD-PCR and normalized with GAPDH mRNA amount in empty or NS3/4A expressing cells with transfection of mock (non), pcDNA-vector (vec.) or pcDNA-Tom70 (Tom70). Poly(I–C) was treated, as described in the legend of Fig. 2.

induction. It has been reported that the C-terminal transmembrane domain (TM) of MAVS interacts with the N-terminal transmembrane domain of Tom70 (Liu et al., 2010). The HCV NS3 protein cleaves MAVS at residue 508 (Meylan et al., 2005), which should impair the interaction of MAVS and Tom70. This may attenuate the downstream signaling pathway (TBK-IRF3) and the induction of IFN synthesis (Suppl. Fig. 2). In our study, the level of NF-κB protein was not significantly influenced by Tom70 in the presence or absence of NS3. This may indicate that other pathways, such as TLR3 and downstream pathways, might compensate to maintain the NF-κB protein expression level in the absence of the MAVS-Tom70 signaling pathway.

Infection with HCV induced expression of Tom70, but the activation of the IFN signaling pathway was abrogated by the HCV NS3 protease. These findings indicate that recovery of the MAVS-Tom70 pathway may be a means to increase the efficacy of IFN therapy against HCV infection.

Recently, we observed that overexpression of Tom70 increased the resistance to the TNFα-induced apoptotic response (Takano et al., 2011a), indicating that Tom70 overexpression might contribute to the apoptotic resistance of HCV-infected cells and the establishment of persistent HCV infection. Thus, Tom70 might be a novel target for the regulation of HCV infection.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.009.

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INVITED REVIEW ARTICLE

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IMMUNOREGULATION OF HEPATITIS B VIRUS INFECTION –RATIONALE AND CLINICAL APPLICATION–

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ABSTRACT

Hepatitis B virus (HBV) is susceptible to the cellular immune responses, especially to the signal of interferon (IFN)-γ. The action of IFN-γ is pleiotropic, and causes downregulation of HBV in protein, RNA, and possibly DNA levels. Therefore, therapeutic vaccination to induce cellular immune responses to HBV is a promising approach for controlling chronic HBV infection. A number of clinical trials with this approach have been conducted to date, however, they have not been as successful as initially expected. T-cell exhaustion induced by the excessive HBV antigens caused by persistent infection is thought to be one of the main causes of poor responses to therapeutic vaccination. In this review, the mechanisms behind immunoregulation of HBV replication and immunodysfunction during chronic HBV infection are summarized, and novel approaches to improve the efficacy of therapeutic vaccination, from basic research to clinical trials, are introduced.

Key Words: chronic hepatitis B, interferon-γ, adaptive immune response, therapeutic vaccination, T-cell exhaustion

INTRODUCTION

Hepatitis B virus (HBV) is a type of the hepadnavirus which is spread by contact with infected blood and body fluids, and causes acute and chronic necroinflammatory liver diseases.[1-3] Since HBV is a noncytopathic virus, inflammation in the liver is mediated by host immune responses to the HBV-infected hepatocytes. HBV infection in immunocompetent adults results in a self-limited, transient liver disease, and subsequent viral clearance is achieved in more than 95% of adults, whereas more than 90% of neonates exposed to HBV at birth become persistently infected.[1-3] Even in persistently infected individuals, hepatitis B e antigen (HBeAg)/antibody (Ab) seroconversion (SC) with marked reduction of HBV replication, associated with biochemical and histologic regression of liver inflammation, occurs in the majority of patients in their natural course. If a reduction of HBV replication does not occur, the infected individuals are under a greater risk of developing cirrhosis (LC), liver failure, and hepatocellular carcinoma (HCC).[1-3]

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Chronic liver diseases associated with chronic HBV infection are serious public health problems worldwide. It is estimated that 370 million people are chronically infected with HBV, and that up to 1.2 million people die every year due to the complications of HBV-related chronic liver diseases, such as LC and HCC.[4-6] HBV infection has been the most significant factor associated with the development of liver cancer, which is one of the most malignant cancers; the second most frequent cause of cancer death in men, and the sixth leading cause of cancer death in women.[4-6] HBV infection accounts for about 60% of all HCC occurrences in developing countries and about 23% of cancer occurrences in developed countries; the corresponding percentages for hepatitis C virus (HCV) infection are 33% in developing countries and 20% in developed countries.[6] As of 2008, a total of 177 countries (91%) had introduced the HBV vaccine for their national infant immunization schedules.[7,8] This efficient vaccination method decreases the incidence of HBV infection; however, chronic HBV infection remains a serious problem in countries with a higher prevalence of chronic HBV infection, such as those in Asia and sub-Saharan Africa, where the prevalence exceeds 8% of their populations.[6,9] Even in Japan, where the HBV carrier rate is about 1% and is gradually decreasing, thanks to the success of a national program for immunoprophylaxis of perinatal HBV transmission, the number of HBV-related HCC is not decreasing[10,11]. It is obvious that there is an urgent need for the effective treatment control and termination of HBV infection.

It is considered that permanent and profound suppression of viral replication is beneficial for preventing complications of chronic hepatitis B (CHB), as persistent HBV viremia has been proven to be the most important predictor of progression to LC, hepatic failure, and development of HCC.[12] Therefore, the initial goal of the treatment is to suppress active viral replication and to subsequently restrain the activity of hepatitis.

Currently, CHB patients are treated mainly with antiviral reagents, i.e., nucleoside or nucleotide analogues (NAs). NAs target the reverse transcriptase of HBV and are potent inhibitors of viral replication. NA treatment usually results in a rapid decline of serum HBV DNA levels, and long-term therapy results in reduction in hepatic fibrosis, hepatic decompensation, and liver-related mortality.[13-15] Induction of NAs opened a new era in the treatment of CHB, providing a safe, effective and well-tolerated therapeutic option. However, at the same time, there is a drawback of NA treatment, which is not negligible. Since the complete eradication of HBV infection is rarely achieved with this treatment, NA must be administered for an extremely long period of time. And long-term treatment is occasionally associated with an increased risk for development of viral resistance to the drugs, which eventually results in recurrence and progression of the disease.[16-18]

Interferon (IFN)-α/β is another option for CHB treatment. It has a direct antiviral effect, but is thought to act mainly by augmenting host immune response specific to the HBV-related antigens.[15,19,20] Because of its immunomodulatory mechanism of action, IFN-α/β requires only a limited treatment period, and brings out a durable response even after the discontinuation of the treatment if the endpoint response is favorable.[21] In addition, recently approved pegylated IFN (Peg-IFN)-α shows a slightly increased efficacy compared to standard IFN-α/β.[15,22-25] However, the response rate by IFN therapy is not necessarily high, despite the use of Peg-IFN-α (sustained response rate: 10 to 30%),[15,22-25] and IFN-based therapy is associated with a wide spectrum of adverse events, including flu-like symptoms, decrease of white blood cells and platelets, and depression. In addition, IFN is contraindicated in patients with decompensated LC.

As seen in the case of IFN treatment, the key factor for obtaining permanent control over HBV is to induce effective virus-specific immune responses which are strong enough and effectively regulate viral replication. In other words, therapeutic vaccination is a promising candidate for the treatment of chronic HBV infection.

Currently, the only available vaccine for HBV is the hepatitis B surface antigen (HBsAg) vaccine, which is usually used for the prophylaxis of HBV infection, as mentioned above. The HBsAg vaccine elicits a level of anti-hepatitis B surface antigen antibody (HBsAb) high enough to protect against the infection of newly intruding HBV in more than 90% of vaccinees, if properly administered.[26] However, therapeutic use of this HBsAg vaccine has not brought satisfactory results for controlling viral replication at present.[27,28] Therefore, a new vaccine or novel strategy is definitely needed to achieve sustained control of chronic HBV infection.

In this review, the mechanisms of immune responses that control HBV replication, their application to therapy, and current and future approaches for developing a vaccination strategy to achieve sustained control or eradication of HBV, are described.

Immune responses that control HBV replication

In general, the immune responses that terminate viral infection work in several steps. During the early phases of acute viral infection, natural killer (NK) cells and natural killer T-(NKT) cells are the first lines of defense, and the activation of these cells helps to reduce the viral load through the secretion cytokines, such as IFN-γ.[29-32] In the liver, the frequencies of NK and NKT cells are higher than in other organs,[33] and these cells mediating innate immunity are thought to play certain roles in the pathogenesis of HBV infection. Indeed, during the early phases of acute HBV infection, the activation of NK cells helps to reduce the viral load through the action of IFN-γ.[34] However, this activation is rapidly suppressed by interleukin (IL)-10 at the peak of viremia, indicating that the roles of NK cells on HBV regulation are rather limited.[35] In fact, in chimpanzees infected by HBV, the innate immune cells, such as NK and NKT cells, do not significantly contribute to the pathogenesis of viral hepatitis during the symptomatic phase of acute or chronic HBV infection.[36] Therefore, it is assumed that a crucial role in the pathogenesis of HBV infection and the control of HBV replication is played by the adaptive immune response primed after the initial response by the innate immunity.

HBV transgenic mouse hepatitis model for analysis of liver pathogenesis

The roles of specific immune responses on the pathogenesis of HBV infection and viral clearance have been well examined in the hepatitis model with HBV transgenic mouse (Fig. 1). The adoptive transfer of HBV-specific cytotoxic T-lymphocyte (CTL) lines and clones into immunologically tolerant HBV transgenic mice produced liver disease with hepatocyte necrosis and inflammation that is histologically very similar to acute viral hepatitis in human.[1,3, 37-43]

The initial step in the disease process after adoptive transfer is apoptosis caused by the direct attack of CTLs against viral antigen-positive hepatocytes. Apoptosis occurs immediately after the transfer of CTLs to mice and is necessary for further steps in the disease process. The direct CTL-target cell interaction results in the appearance of widely scattered apoptotic hepatocytes.[37-39,42] After the initial step of direct CTL killing, many host-derived inflammatory cells, such as polymorphocnuclear neutrophils (PMNs), antigen-nonspecific T-cells, NK cells, and macrophages, are recruited around apoptotic hepatocytes and virus-specific CTLs, and form necroinflammatory foci, which resembles the histology observed in acute hepatitis in men.[37-39,42] This process, from hepatocyte apoptosis to necroinflammatory foci, is shown to be associated with the action of activated platelets,[43] cytokines such as IFN-γ and the tumor necrosis factor (TNF)-α,[37,40,41] chemokines such as CXCL9 and CXCL10,[44] and matrix metalloproteinases (MMPs) produced by PMNs (Fig. 2).[45]

Mechanisms of HBV elimination by adaptive immune response

In the same HBV transgenic mouse model, the mechanisms of viral elimination by adaptive

immune response is examined in detail. As mentioned above, HBV-specific CTLs directly kill HBV-expressing hepatocytes, and the recruited host-derived inflammatory cells also cause damage

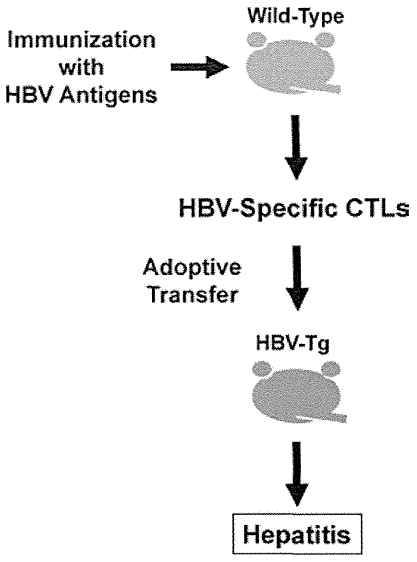

Fig. 1 HBV transgenic mice hepatitis model. Nontransgenic (B10.D2; H-2^d) mice were immunized with either recombinant vaccinia virus or HBsAg protein. Spleens were harvested and stimulated in vitro with irradiated HBsAg-expressing P815 cells. Primed spleen cells were restimulated and expanded in vitro, and cloned by limiting dilution method. HBsAg-specific CTL clones or cell lines were intravenously injected to HBV transgenic mice (1.3.32; inbred B10.D2, H-2^d). Liver inflammation and viral clearance were observed in HBV transgenic mice.

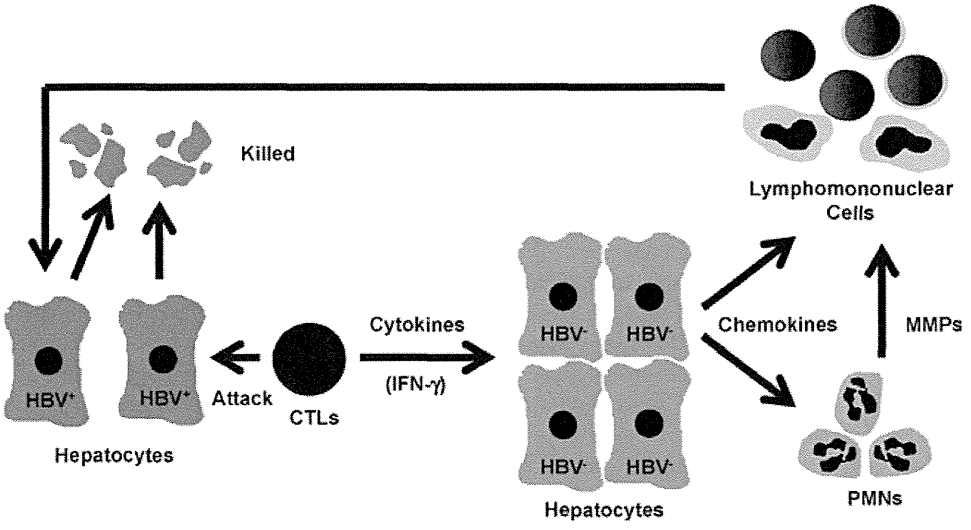

Fig. 2 Mechanisms of CTL-induced liver disease and viral clearance. HBV-specific CTLs kill antigen-expressing hepatocytes via Fas lingad- and perforin-mediated pathways, and produce antiviral cytokines, such as IFN-γ, that inhibit HBV replication noncytopathically in a greater number of adjacent cells. IFN-γ activates hepatpcytes to produce chemokines that recruit antigen-nonspecific polymorphonuclear cells (PMNs) and antigen-nonspecific lymphomononuclear cells (e.g., NK cells, T-cells, and macrophages) into the liver. Production of matrix metalloproteinases (MMPs) by PMNs also promotes migration of antigen-nonspecific lymphomononuclear cells. These antigen-nonspecific inflammatory cells amplify liver disease initiated by CTLs. The figure is modified from that of ref #3.

to infected hepatocytes, thus reducing the viral load to some extent (Fig. 2). However, more importantly, IFN-γ produced by activated CTLs following antigen recognition is responsible for most of the antiviral potential of the CTL-mediated immunity.[40,41,47] The action of IFN-γ on HBV elimination is thought to be pleiotropic. It is indicated that IFN-γ hinders the assembly of pregenomic HBV RNA-containing nucleocapsid protein in a proteasome- and kinase-dependent manner.[48-50] Also, IFN-γ causes destabilization of HBV RNAs in the nucleus by a La-dependent mechanism. Briefly, HBV RNAs are stabilized in the nucleus by the binding of the full length of La, which is known as an autoantigen of Sjögren's syndrome. However, IFN-γ produced by activated CTLs induces proteolytic cleavage of La, and the subsequent binding of cleaved La to HBV RNAs results in the destabilization of HBV RNAs, i.e., facilitating the destruction of HBV RNAs by nuclear RNases.[51-55] IFN-γ also effectively downregulates replication intermediates of HBV DNA in a nitric oxide (NO)-dependent manner, while the effect of IFN-γ on covalently closed circular DNA (cccDNA) in the nucleus is not yet clarified (Fig. 3).[36,56,57] Thus, with all

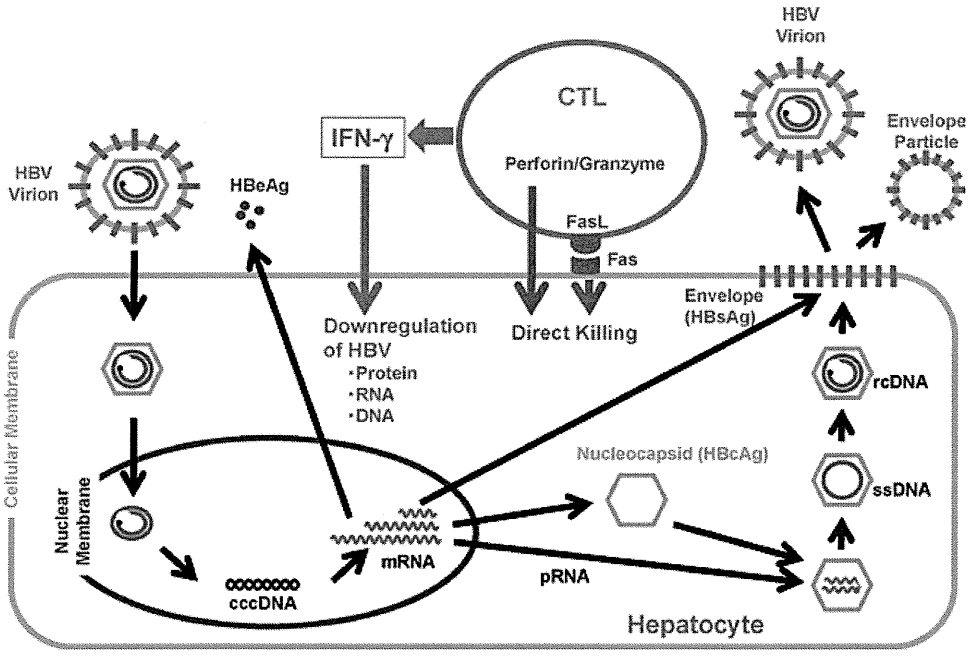

Fig. 3 HBV life cycle and CTL-mediated viral clearance. The process of HBV entry into hepatocyte is poorly understood, however, it is thought to be a receptor-mediated process. Once the virion enters the cytoplasm, it is uncoated; the uncovered nucleocapsid is transported to the nucleus. Following nucleocapsid disassembly, the second strand of the open circular viral genome is completed, and the ends of each strand are ligated. This process results in production of a covalently closed circular DNA (cccDNA), which is the transcriptional template of the virus. Transcription by viral polymerase leads to production of viral RNAs which are transported from the nucleus to the cytoplasm. Transcripts are translated into corresponding proteins, such as envelope proteins, nucleocapsid proteins, HBeAg, polymerase protein, and X protein. The envelope proteins traverse the ER membrane as integral membrane proteins. HBcAg and polymerase proteins assemble around pregenomic RNA (pRNA) to form HBV RNA-containing nucleocapsid, within which RNA is reverse transcribed to produce the first single-strand viral DNA (ssDNA). ssDNA serves as the template for second-strand DNA synthesis, producing nucleocapsid containing a partially double-stranded, relaxed circular DNA molecule (rcDNA). At the ER membrane, it interacts with the envelope proteins that trigger an internal budding reaction. This reaction results in the formation of virion which is transported out of the cell. HBV-specific CTLs recognize and cause death to the HBV-infected cells via Fas lingad- and perforin/granzyme-induced pathways. Activated CTLs produce cytokines, such as IFN-γ, which causes profound, pleiotropic downregulation of the virus noncytopathically in adjacent infected cells.

these mechanisms, CTLs and IFN-γ produced by CTLs bring about a very profound downregulation of HBV, most of which is accomplished without destroying hepatocytes.

Helper T-cells (Ths) also have the potential to eliminate HBV, since they are important producers of IFN-γ. In the HBV transgenic mouse model, it was indicated that HBV-specific Ths also cause HBV downregulation in an IFN-γ-dependent manner, but less efficiently, compared to CTLs.[38] On the other hand, Ths are also thought to contribute to controlling HBV infection by assisting in the induction and maintenance of HBV-specific CTLs by regulating immune response.[38] Indeed, strong HBV-specific Th responses are usually associated with significant CTL responses in humans and chimpanzees that resolve HBV infection.[1,59]

Considering the mechanisms of HBV elimination by adaptive immune responses, it is obvious that therapeutic vaccination that induces HBV-specific CTLs and Ths has great potential as a strategy to terminate chronic HBV infection.

Alteration of immune response during chronic HBV infection

Despite the presence of immunological mechanisms of HBV elimination, the virus is rarely eradicated when the infection becomes chronic. The key features of chronicity of HBV infection are the impairment of immune responses related to the cellular composition of the liver, and the presence of excessive viral antigens caused by persistent infection.

The liver is composed of parenchymal hepatocytes and non-parenchymal cells, including biliary epithelial cells (BECs), hepatic stellate cells (HSCs), sinusoidal endothelial cells (LSECs), and Kupffer cells (KCs). Importantly, most of these cells do not express enough costimulatory molecules to induce functional activation of antigen-specific T-cells. Therefore, HBV antigen presentation by these cells tends to lead T-cells toward tolerance rather than activation.[60-62]

Unlike the cell populations mentioned above, dendritic cells (DCs) professional antigen-presenting cells (APCs) do express costimulatory molecules, and play an important role in the induction of functional T-cells. However, in patients with CHB, decreases in the number of circulating dentritic cells (DCs) and functional impairment of both myeloid (mDCs) and plasmacytoid DCs (pDCs) have been reported, and they may account for the dysfunction of the adaptive immunity.[63-65]

Chronic exposure to excessive antigens leads T-cells to progressive exhaustion, when they lose the ability to differentiate into memory cells and the effector function, e.g., proliferative capacity, cytotoxicity, and cytokine production (IFN-γ, TNF, etc.).[66-68] The exhausted T-cells express inhibitory receptors on their surface, such as programmed death-1 (PD-1), and the interaction between PD-1 and its ligand, PD-1 ligand 1 (PD-L1) are thought to be the major inhibitory receptor pathways involved in T-cell exhaustion.[66-68] Indeed, PD-1 is shown to be overexpressed on HBV-specific T-cells, which produce only a small amount of IFN-γ.[67,69] As T-cell exhaustion progresses along with prolonged exposure to high doses of antigens, it may finally result in the clonal deletion of HBV-specific T-cell populations. In addition, regulatory $Foxp3^{+}CD4^{+}$ T-cells (Tregs) are known to affect immune responses during chronic infections, and are thought to affect the exhaustion of antigen-specific T-cells.[67] However, the role of Tregs in chronic HBV infection is not clearly understood.[70-73]

Even though our understanding of the role and alteration of immune responses during chronic HBV infection has been greatly improved, many questions remain to be elucidated. It is important to continue attempting to answer these questions in order to develop new strategies of therapeutic vaccination.

Prophylactic HBsAg vaccines for therapeutic vaccination

The first series of therapeutic vaccine trials were conducted with commercially available

prophylactic HBsAg vaccines.[27,74-77] An initial pilot study demonstrated that HBsAg-vaccination induced cell-proliferated responses and IFN-γ production specific for HBsAg, and reduction of serum HBV DNA levels in some patients (7 out of 27 patients were positive for proliferative response to envelope protein).[27] Further studies also proved that HBsAg vaccination induced HBsAg-specific T-ell responses, a decrease of HBV DNA levels, and a significant increase in HBeAg/antibody (Ab) seroconversion (SC); however, most of these effects were temporal.[76,77] In addition, only a few patients showed HBsAg clearance and development of antibody against HBsAg in these studies.[76,77] Since the ultimate goal of CHB treatment is the clearance of HBsAg and the induction of HBsAb, which can neutralize viral particles, the use of HBsAg for therapeutic vaccination seems to be a reasonable choice. However, the results of these studies clearly indicate that the classical prophylactic vaccines do not have enough antiviral potential, and that development of novel vaccines or a strategy with greater efficacy is necessary.

Studies also showed that HBsAg vaccines are more effective when used in patients with low viral loads, suggesting the possibility that the presence of excessive viral antigens lessens the efficacy of vaccination. Therefore, combining antiviral drugs that can suppress viral replication and reduce the viral antigen load simultaneously through the vaccination is thought to be one of the better therapeutic approaches.

Combining antiviral drugs to vaccination

Lamivudine (LMV) is the first NA used for the treatment of HBV infection.[78,79] LMV itself does not have a direct immunomodulatory effect, however, LMV treatment is shown to restore the responses of HBV-specific T-cells during the first few months of the treatment period, and other NAs may have the same effect. This means that reducing the HBV antigen load by antiviral therapy itself may increase the responsiveness of HBV-specific T-cells,[80] probably by restoring the function of exhausted T-cells. Since the restoration of T-cell responses is weak, transient, and does not lead to an increase of sustained viral response, such as HBeAg/Ab SC,[81] there have been a number of attempts to combine NA administration to vaccination to gain continuous activation of HBV-specific T-cell responses (Fig. 4).

Combining LMV to vaccination is the most widely applied method of treatment, and is found to be well tolerated and safe, without serious adverse effects. This combination therapy is reported to give a significantly higher and earlier rate of viral suppression than LMV or vaccine monotherapy, and increases the rate of HBeAg/Ab SC to some extent.[82-84]

We also conducted a controlled trial composed of 53 patients with CHB, 33 with $HBeAg^+$, and 20 with $HBeAg^-$, in which participants randomly received either LMV monotherapy or the combination therapy of LMV and HBsAg vaccine. In the $HBeAg^-$ group, LMV treatment by itself was fairly effective, and no difference was seen with regard to HBV DNA levels, the rate and period of complicating viral breakthrough (BT), or breakthrough hepatitis (BTH) between the two groups. In the $HBeAg^+$ group, the decrease of serum HBV DNA was faster and the rate of HBV DNA negativation was higher in the combination therapy group, while the ratio of HBeAg/Ab SC did not differ between the two groups. Also, in $HBeAg^+$ patients, the rates of developing BT and BTH were significantly lower in the combination therapy group than in the monotherapy group. Furthermore, there was a tendency for the time period before developing BT to be longer in the combination therapy group than in the monotherapy group. However, most of the patients in both groups developed post-treatment relapse after the cessation of LMV in both groups.[85]

Type I IFN is used as an antiviral drug in the treatment of HBV infection, but has the potential to enhance HBV-specific immunity, as mentioned. In general, it enhances both T- and B-cell response by upregulating the expression of human leukocyte antigen (HLA) class I on the

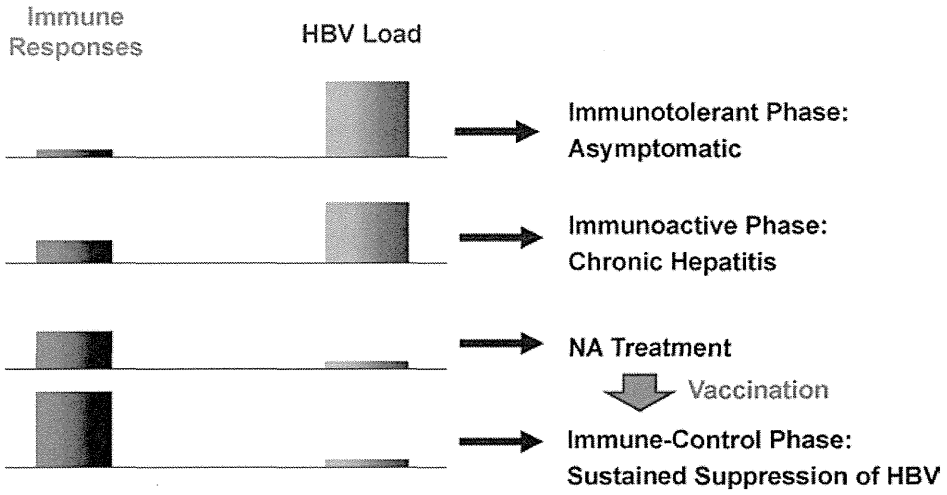

Fig. 4 Balance between immune response and viral load in chronic HBV infection. In patients with perinatally acquired HBV infection, immunotolerant phase is the first phase of infection, when patients are asymptomatic without significant immune responses and with higher viral load. The second phase is the immunoactive phase, when patients have liver inflammation with slight increase of immune responses and slight decrease of viral load. If spontaneous HBeAg/Ab seroconversion (SC) does not occur, liver damage progresses from chronic hepatitis to cirrhosis. Nucleoside or nucleotide analogue (NA) treatment can reduce viral load and is thought to increase immune responses probably by restoring the function of exhausted T-cells. Since increase of immune responses is partial and transient, therapeutic vaccination to increase immune responses is recognized as a promising measure to achieve sustained control of HBV replication.

surface of APCs. It thereby promotes CTL responses by augmenting and maintaining the production of IgG from plasma cells.[19,20] Indeed, in haemodialysis patients whose immune responses were impaired, combining IFN-α to a prophylactic HBsAg vaccine was reported to improve the efficiency of the vaccination.[86] There are a few reports describing the efficacy of the combination therapy of type I IFN and HBsAg vaccine for the treatment of CHB. In these reports, combination therapy was reported to accelerate the reduction of HBV DNA and to improve the HBeAg/Ab SC rate compared to IFN monotherapy, but without significant differences.[87,88]

In the studies of therapeutic vaccination combined with either NA or IFN, there is a difficulty when generalizing the obtained results, since treatment protocols, backgrounds of the subjects, and the criteria for defining virological responders vary so much. In addition, only a few reports have analyzed cellular or humoral immune responses specific to HBV, and no obvious association was found between HBsAg-specific T-cell responses and any clinical or virological parameters, such as HBeAg/Ab SC and HBsAg clearance, in most of the studies. The protocol of treatment should be deliberately reconsidered, and the immunological analysis should be carefully planned for further improvement of its efficacy.

Recently, an in vitro study demonstrated that the 50-end triphosphate hepatitis B virus X gene (HBx)-short interfering RNAs (siRNAs) exerted significantly stronger inhibitory effects on HBV replication, with a retinoic acid-inducible gene-I (RIG-I) and in a type I IFN-dependent manner. Moreover, its efficacy was confirmed in HBV carrying mice.[89] While there is the unsolved problem of how to deliver siRNAs as a drug, they may be candidates for antiviral drugs that can be combined with vaccination as well as NAs.

Use of adjuvants

Adjuvants have been used for decades to improve the immune response to vaccine antigens.

The use of adjuvants with vaccine is aimed at enhancing, accelerating, and prolonging the specific immune response towards the desired response to vaccine antigens.[90] Therefore, adjuvants are suitable for the purpose of therapeutic vaccination for HBV infection.

The major means by which adjuvants exert themselves are through the upregulation of antigen/adjuvant uptake to the antigen presentation cells (APCs), or the potentiation of immune responses, both quantitatively and qualitatively. Alum, which is used for prophylactic HBsAg vaccine, oil emulsion, and immune stimulating complexes (ISCOMs) (including Quillaia saponins, or liposomes) are used for the former purpose, and monophosphoryl lipid A (MPL) of Toll-like receptor (TLR) 4 agonist, CpG DNA of TLR9 agonist, poly I:C of TLR3 agonist, Montanide-ISA 51, Montanide-ISA 720, MF59, or QS-21 of saponin derivatives, are used for the latter purpose.[91] Most of the adjuvants listed above have been tested for their ability to enhance the immune responses to HBV antigens, and have been proven effective. Clinical trials for the application of the vaccines which include some of these adjuvants, mainly for prophylaxis of HBV infection, are in progress or have been completed, such as "HEPLISAV," comprised of the HBsAg and TLR9 agonist.[92] Therapeutic use of the vaccines containing these adjuvants for the treatment of CHB is expected to be effective, however, we must wait for the results of appropriate clinical trials.

There have been attempts to use cytokines, such as IL-2 and IL-7, as adjuvants. IL-2 is a strong Th1-inducing cytokine, and is expected to increase the efficacy of therapeutic vaccination by augmenting Th1 responses that help the induction and maintenance of antigen-specific CTLs. Several trials, including one for HBV infection, have evaluated the efficacy of IL-2 as an adjuvant of therapeutic vaccine.[93] However, the use of IL-2 for treatment should be conducted carefully because there is a potential concern that IL-2 administration may increase Tregs, which constitutively express the IL-2 receptor. IL-7 is essential for the homeostatic proliferation of T cells in vivo, and may increase the pool of naïve T-cells potentially stimulated by vaccination.[94] A clinical trial combining antiviral treatment with HBsAg vaccine and multiple IL-7 injections is currently underway.

Blocking the PD-1/PD-L1 inhibitory signals on exhausted T-cells by anti-PD-L1 antibodies may be an option for enhancing the efficacy of vaccination. Indeed, it has been suggested that restoration of intrahepatic HBV-specific T-cell responses in CHB patients was possible by blocking PD-1/PD-L1 signals in vitro.[69,95]

In the transgenic mouse model, an anti-CD40 agonistic antibody was reported to inhibit HBV replication noncytopathically by a process associated with the recruitment of dendritic cells, macrophages, T-cells, and NK cells into the liver, and the induction of inflammatory cytokines, such as IFN-γ.[96] The antibody itself shows an antiviral potential by activating APCs nonspecifically, however, it may also be a candidate of an adjuvant for vaccination, since stimulation of CD40 on DCs and B-cells has been proven to augment cell-mediated and humoral immunity.[97]

We have recently demonstrated that alpha-galactosylceramide (α-GalCer), a ligand for NKT cells, significantly enhanced the induction and proliferation of HBsAg-specific CTLs by HBsAg vaccination in the mouse model. Even in HBV transgenic mice which are deeply tolerant to HBV antigens, co-administration of HBsAg and α-GalCer successfully induced HBsAg-specific CTLs.[98] Although this approach could not clear HBsAg from transgenic mice despite the induction of HBsAg-specific CTLs, we believe that it is one of the promising approaches to augment the efficacy of vaccination.

Other forms of vaccine candidates

Several types of vaccines with different compositions have been proposed with the aim of increasing vaccination efficacy.

There have been a number of attempts to use peptide-based vaccines targeting T-cell epitopes

for the treatment of viral infections as well as cancers, since they are easily produced in large amounts and can be safely administered to patients. A lipopeptide-based vaccine named "Theradigm-HBV," the HLA-A2.1-restricted, dominant CTL epitope, HBV core Ag (HBcAg) aa18–27, linked to the universal Th epitope tetanus toxoid (TT) has been tested for its ability to induce CTL responses in healthy individuals and CHB patients. "Theradigm-HBV" was capable of inducing HBV-specific CTL responses in a dose-dependent fashion even in CHB patients; however, the clinical effects, such as HBV DNA decrease, were not satisfactory.[99,100] While the efficacy of peptide-based vaccine may be further improved with the use of an appropriate adjuvant, the design of the vaccine construct should be carefully considered. Since the problem of HLA restriction is inevitable in peptide-based vaccine, an HLA allele targeted to the binding of epitope peptide should be selected in order to cover a large population of a relevant country or region. In addition, the emergence of vaccine escape variants should be considered, especially in the case of a viral vaccine. We have previously shown that the CTL response to individual viral epitopes can be markedly polyclonal and multispecific, and that mutational inactivation of a single site of the epitope will not usually lead to viral escape.[101] Even though a viral mutation which can evade the CTL attack may emerge, simultaneous use of plural epitope peptides may reduce the risk of disabling vaccine efficacy.

HBsAg-HBsAb immune complexes (IC) with alum as the adjuvant have been developed, targeting DCs[102]. In vitro study has demonstrated that IS stimulated DCs to secrete a large amounts of IL-12, a key cytokine of $CD4^+$ T-cell differentiation into type 1 T helper (Th1) cells that can promote cell-mediated immunity.[103] In the phase II trial, a significant virological effect, such as a higher HBeAg/Ab SC rate compared to the control group, was observed in the IC-treated group.[102] However, the immune responses that could explain this therapeutic effect, were not analyzed. It makes the evaluation of the efficacy of this vaccine rather difficult, as with other vaccine trials.

It is known that during acute hepatitis, the HBcAg and the polymerase are specific targets of the immune response as well as envelope antigens.[1,104] Therefore, the use of a combination of recombinant HBsAg and HBcAg, known as "NASVAC," is a promising therapeutic vaccine candidate for treating CHB patients. These antigens form virus-like particles, and they work together in the development of cellular and humoral immune responses. In addition, this novel vaccine could be administered in a non-invasive way, i.e., intranasally.[105,106] Clinical trials are underway, and the results are awaited.

DNA-based vaccines have been tested for both prophylactic and therapeutic purposes, and encouraging results have been reported in the animal models. The advantage of a DNA vaccine is that it can induce both humoral and cellular immune responses. The phase I trial administering a DNA vaccine which encodes HBV envelope proteins in CHB patients showed favorable results.[107] DNA vaccination led to the induction of IFN-γ-secreting T-cells and CTLs specific to envelope antigens, and to an increase in an NK cell subset known to produce abundant cytokines.[108] The clinical trials with DNA vaccination are increasing, and most of them have shown promising results. However, these trials should be carefully conducted, since there are concerns about potential side effects, such as possible integration of plasmid DNA into the host genome, that result in mutations.

Few studies have reported the use of autologous DCs pulsed with HBV antigens for therapeutic purposes. One of them is a pilot study with a small number of CHB patients that evaluated the safety of HBsAg-pulsed autologous DCs. The administration of DCs was safe with no exacerbation of liver damage; however, the number of patients was too small to evaluate the efficacy of vaccination.[109] Another recent trial was conducted using autologous DCs pulsed with HLA-A2-restricted peptides derived from HBcAg (aa18–27), with re-injection of middle envelope

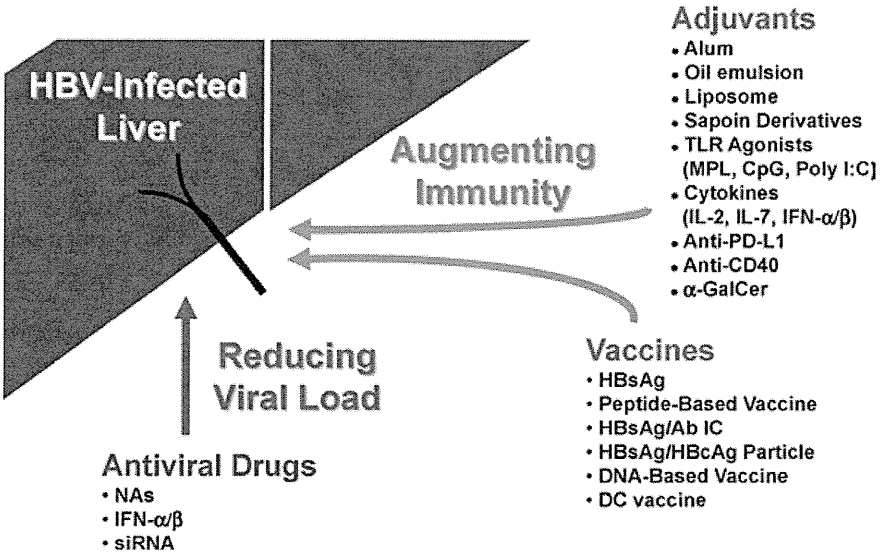

Fig. 5 Summary of new strategies for treatment of chronic HBV infection. Strategies for CHB immunotherapy consist of novel vaccines, adjuvants, and combination of these to antiviral drugs. Details are described in the text.

protein in CHB patients.[108] The treatment was more effective in the patients with $HBeAg^-$ or with a low viral load, and some of them had cleared HBsAg. However, in that study, the immune response-mediated mechanisms accounting for the observed serologic and virological responses were not reported, raising questions about the specificity of this DC-mediated therapy.[110]

CONCLUSION

Therapeutic vaccination is a reasonable approach for the treatment of chronic HBV infection, considering the natural course of HBV infection and the susceptibility of HBV to the signal of IFN-γ. And there have been a number of attempts at therapeutic vaccination, including those using conventional prophylactic vaccine, those combining antiviral drugs with vaccine, those using vaccine with novel adjuvant, or those using newly-developed vaccines as introduced in this review (Fig. 5). The efficacies of current therapies are still lower than initially expected. However, the efficacy may be improved thanks to the progress made in understanding the underlying mechanism of immunodysfunction in HBV carriers, and the development of new therapeutic tools for immunostimulation. The efforts to improve the efficacy of vaccine therapy should be continued in order to establish an ideal therapeutic vaccination for the treatment of CHB. While most of these therapeutic vaccines have been safely used without serious complications, it should be kept in mind that we should be always careful about possible complications, such as exacerbation of hepatitis due to the excessive enhancement of immune responses.

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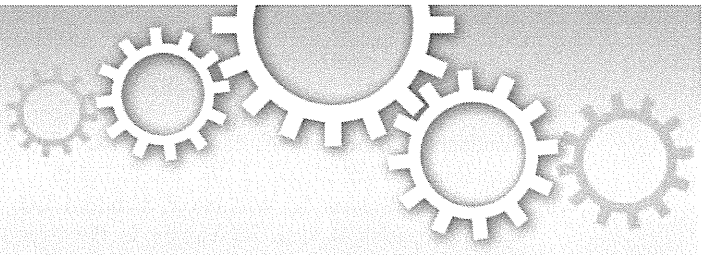

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High susceptibility to lipopolysaccharide-induced lethal shock in encephalomyocarditis virus-infected mice

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Secondary bacterial infection in humans is one of the pathological conditions requiring clinical attention. In this study, we examined the effect of lipopolysaccharide (LPS) on encephalomyocarditis virus (EMCV) infected mice. All mice inoculated with EMCV at 5 days before LPS challenge died within 24 h. LPS-induced TNF-α mRNA expression was significantly increased in the brain and heart at 5 days after EMCV infection. $CD11b^+/TLR4^+$ cell population in the heart was remarkably elevated at 5 days after EMCV infection, and sorted $CD11b^+$ cells at 5 days after EMCV infection produced a large amount of TNF-α on LPS stimulation *in vivo* and *in vitro*. In conclusion, we found that the infiltration of $CD11b^+$ cells into infected organs is involved in the subsequent LPS-induced lethal shock in viral encephalomyocarditis. This new experimental model can help define the mechanism by which secondary bacterial infection causes a lethal shock in viral encephalomyocarditis.

Polymicrobial infectious diseases show the involvement of 2 or more microbes, including viruses, bacteria, fungi or parasites, and these microbes act synergistically to mediate complex disease processes. In particular, a bacterial infection superimposed over an acute viral infection such as influenza is well known as the aggravation factor in the infectious disease[1,2]. Lipopolysaccharide (LPS), the outer membrane of gram-negative bacteria, causes systemic inflammatory response syndrome, endotoxic shock, disseminated intravascular coagulation and multi-organ failure[3,4]. LPS is recognized by Toll-like receptor 4 (TLR4)-expressing immunocompetent cells, mainly monocytes and macrophages, and induces the production of inflammatory cytokines through NF-κB activation[3]. Toxic effects of LPS are partially induced by the release and action of macrophage-derived inflammatory cytokines. Especially, the mass production of tumor necrosis factor-α (TNF-α) causes septic shock with an abrupt reduction in blood pressure, leading to rapid aggravation of the disease condition[5,6]. It is known that LPS-induced lethal shock is caused by a large quantity of TNF-α produced by immune cells activated by some kind of pre-stimulation[7,8].

Encephalomyocarditis virus (EMCV), which is a single-stranded RNA virus and a member of *Cardiovirus* in the Picornaviridae family, causes acute myocarditis and encephalitis in various animal species[9,10]. In some reports, including our previous study, the death of mice by EMCV infection of high density (500 plaque-forming units (pfu)) occurred after 6 days, and the mice that survived for 12 days subsequently recovered. Additionally, remarkable inflammation in the brain and heart occurred at around 6 days after EMCV infection, and thereafter the inflammation reduced as the days progressed. Hence, these results suggested that inflammatory cells reach the activating stage around 6 days after EMCV infection[11–13]. It was also demonstrated that various cells under the activating stage augment reactivity to LPS stimulation[14–16]. Previous studies showed that TNF-α is produced in large quantities by subsequent LPS stimulation during adenovirus infection[17], lymphocytic choriomeningitis virus, and varicella-zoster virus infection[18], but the original source of TNF-α is not elucidated. Moreover, it is unknown how LPS affects in the viral encephalomyocarditis. The aim of our study is to examine the effect of LPS