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REVIEW

Contribution of Toll-like receptors to the control of hepatitis B virus infection by initiating antiviral innate responses and promoting specific adaptive immune responses

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It is well accepted that adaptive immunity plays a key role in the control of hepatitis B virus (HBV) infection. In contrast, the contribution of innate immunity has only received attention in recent years. Toll-like receptors (TLRs) sense pathogen-associated molecule patterns and activate antiviral mechanisms, including intracellular antiviral pathways and the production of antiviral effector interferons (IFNs) and pro-inflammatory cytokines. Experimental results from *in vitro* and *in vivo* models have demonstrated that TLRs mediate the activation of cellular signaling pathways and the production of antiviral cytokines, resulting in a suppression of HBV replication. However, HBV infection is associated with downregulation of TLR expression on host cells and blockade of the activation of downstream signaling pathways. In primary HBV infection, TLRs may slow down HBV infection, but contribute only indirectly to viral clearance. Importantly, TLRs may modulate HBV-specific T- and B-cell responses *in vivo*, which are essential for the termination of HBV infection. Thus, TLR agonists are promising candidates to act as immunomodulators for the treatment of chronic HBV infection. Antiviral treatment may recover TLR expression and function in chronic HBV infection and may increase the efficacy of therapeutic approaches based on TLR activation. A combined therapeutic strategy with antiviral treatment and TLR activation could facilitate the restoration of HBV-specific immune responses and thereby, achieve viral clearance in chronically infected HBV patients.
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INTRODUCTION

Hepatitis B virus (HBV), a hepatotropic non-cytopathic DNA virus, affects millions of people worldwide and is one of the major causes of fatal liver diseases, such as cirrhosis and hepatocellular carcinoma.[1] The outcome of acute HBV infection is determined by the strength of the specific host immune response against HBV. It is well accepted that cell-mediated immune responses play a major role in viral clearance during acute HBV infection. Patients with chronic HBV infection usually fail to develop adequate HBV-specific immune responses.[2] Although the central role of adaptive immunity in controlling HBV infection is well established, the contribution of innate immunity in this regard is largely unexplored.

Pattern recognition receptors (PPRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors, are essential for sensing invading pathogens, initiating innate immune responses, limiting the spread of infection and promoting efficient adaptive immune responses.[3] Due to the lack of cell cultures and small animal models that efficiently supporting the full HBV infection cycle, the role of PPRs in HBV infection and pathogenesis can only be partially studied.[4] While the interaction between HBV and TLRs has been examined in a number of studies, the role of RLRs and NOD-like receptors in HBV infection is largely unknown. RLRs can sense viral RNAs in the cytoplasm and play an important role in recognition of hepatitis C virus (HCV) RNA.[5] Unlike HCV, the HBV genome replicates in the nucleocapsid and may evade detection by RLRs. Recent studies have indicated that TLR expression in peripheral immune cells, liver immune cells, and hepatocytes changed during chronic HBV infection accompanied by impaired TLR functions.[6–9] Meanwhile, TLR-mediated innate immune responses have been shown to inhibit HBV replication in hepatocytes and animal models.[10–14] Stimulation of innate immune responses with TLR agonists may further

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improve the immunotherapeutic effect of combination strategies against the hepadnaviral infection. A great number of reviews have already described the basic features of the TLR system and related signaling pathways.[15,16] Therefore, we will only focus on the interaction between TLR and HBV in the present review.

INTERACTION BETWEEN TLRS AND HBV

Despite numerous studies of the interaction between TLRs and HBV, no convincing evidence is available that HBV proteins and viral RNAs and DNA are truly recognized by TLRs. TLR2 was found to recognize viral lipoproteins and glycoproteins and lead to the activation of immune cells. Cooper *et al.*[17] reported that full-length HBcAg triggers the production of proinflammatory cytokines, such as TNF-α, IL-6 and IL-12p40, in macrophages in a TLR2-dependent manner *in vitro*. This finding was challenged by other scientists, as recombinant HBcAg was derived from a bacterial expression system with risk of contamination.[18,19] In contrast, Lee *et al.*[20] indicated that the HBV nucleocapsid does not activate TLR2 but activates TLR7 with packaged single-stranded RNA (ssRNA) as a TLR7 agonists. However, these findings were not confirmed in other systems. It has been shown that HBV interacts with hepatic non-parenchymal cells (NPCs) and induces the production of IL-6, though it is not clear how hepatic NPCs sense HBV.[21] However, in a mouse model based on hydrodynamic injection, persistent HBV replication in the mouse liver does not induce measurable TLR activation, such as IL-6 or interferon (IFN) production, after an initial cytokine induction.[22]

HBV is claimed to be a 'stealth virus' in the early phase of infection because it does not induce IFNs and IFN-stimulated genes (ISGs) in acute HBV-infected chimpanzees[23] or patients.[24] This fact could be explained by two possibilities: inability of HBV to activate PPRs or viral inhibition of PPR signaling pathways. Accumulating evidence supports the latter hypothesis that HBV is able to interfere with PPR signaling pathways. It has been consistently shown that TLR expression and function are reduced during HBV infection. Using hepatocytes and Kupffer cells (KCs) isolated from liver biopsies of patients with chronic hepatitis B (CHB), Visvanathan *et al.*[6] first described significantly decreased TLR2 expression on hepatocytes, KCs, and peripheral monocytes in patients with HBeAg-positive CHB compared with HBeAg negative CHB and controls. HBeAg, when expressed by transduction through a recombinant baculoviral vector, was able to inhibit the induction of TNF-α in hepatic cell lines.[6] Subsequently, HBeAg was shown to suppress TIR-mediated activation of NF-κB-dependent and IFN-β promoters through disruption of homotypic TIR–TIR interaction, which is critical for TLR-mediated signaling,[25] and also through inhibition of IL-1β-mediated NF-κB activation in hepatocytes.[26] Consistently, Chen *et al.*[7] also reported that TLR2 mRNA and protein levels were significantly decreased in PBMCs from CHB patients based on real-time PCR and flow cytometric analysis. Impaired cytokine production was observed in PBMCs from CHB patients after challenged with TLR2 ligand, but correlating with the plasma HBsAg levels. HBsAg not only directly blocks the TLR2-mediated JNK-MAPK pathway but also indirectly induces monocytes to release IL-10.[27,28] Thus, TLR2 expression and function apparently represent important targets of viral inhibition.

There are experimental findings supporting the view that HBV is able to block the host IFN responses mediated by TLRs. Plasmacytoid dendritic cells (pDCs), the professional producers of type I IFNs, play a pivotal role in innate and adaptive immune responses against viral infections. TLR9 is a PRR for viral and bacterial DNA motifs and stimulates type I IFN secretion by pDCs *via* IRF7 activation.[15] During chronic HBV infection, pDCs display reduced TLR9 expression and an impaired ability to secrete IFN-α after *ex vivo* stimulation with TLR9 ligands.[8,29,30] HBV virions were able to directly inhibit TLR9 transcription through downregulation of TLR9 promoter activity, by blocking the MyD88-IRAK4 axis and Sendai virus targeting IRF7 to suppress IFN-α production.[8] Along the same lines, Xu *et al.*[31] demonstrated that HBsAg can inhibit TLR9-mediated IRF7 expression and nuclear translocation through upregulation of SOCS-1 expression. HBsAg, HBeAg and HBV virions may also inhibit the activation of liver NPCs by TLR3 ligands.[32] Coculture of hepatic NPC cell supernatants containing HBsAg, HBeAg and HBV virions resulted in abrogation of TLR-induced antiviral activity, correlating with decreased activation of IRF-3, NF-κB and ERK1/2 in NPCs. Our recent data suggested that HBsAg may trigger IL-10 production in hepatic cells and thereby, attenuate the TLR3-mediated activation of NPCs.[33]

Additionally, HBV proteins, such as polymerase and HBx protein, could interfere with intracellular signaling pathways, preventing IFN responses in hepatocytes. HBV polymerase was reported to inhibit TLR3-mediated IFN-β induction in human hepatocytes through interference with IRF3 activation.[34] Another recent study also demonstrated that HBV polymerase suppressed TNF-α, TLR3 or TLR4-induced NF-κB signaling by inhibiting the activity of IKKs *via* interaction with Hsp90β in hepatoma cells.[35] The RLR-mediated signaling pathway senses intracellular viral RNAs to activate IFN promoter stimulator 1 (IPS-1), IRF3 and NF-κB, leading to the production of type I IFN and pro-inflammatory cytokines.[36] HBx protein was reported to inhibit RIG-I-mediated IFN-β induction by promoting ubiquitin-dependent degradation of IPS-1 in hepatoma cells.[37–39] HBV polymerase was shown to suppress RIG-I-induced IFN-β induction by interference with IRF3 phosphorylation and nuclear translocation and inhibiting the interaction between TBK1/IKKe and DDX3 in human hepatocytes.[34,40] The mechanisms employed by HBV to counteract innate immune system are summarized in Table 1.

These findings suggest that HBV evolves to escape from the surveillance of the host innate immune system by targeting PRR signal pathways. However, when considering these results in the context of natural HBV infection, we need to pay more attention, because the majority of experiments were performed either under conditions of over-expressing a single viral protein or in cells that are not normally infected by HBV. Other factors

Table 1 The mechanisms employed by HBV to counteract the TLR system

Experimental model	*Mechanism*	*HBV protein involved*	*Functional outcome*	*Reference*
Human PBMCs, liver samples from CHB patients	Reducing the expression of TLR2 or TLR9 on innate immune cells	HBeAg, HBsAg and HBV virions	Reduced induction of inflammatory cytokines of IFN after TLR stimulation	6–8,29,30
Hepatoma cell lines	Blocking the homotypic TIR–TIR interaction	HBeAg	Suppressed TIR-mediated activation of NF-κB dependent and IFN-β promoters	25
Human pDCs from CHB patients	Inhibition of TLR9-mediated expression and activation of IRF7	HBV virions, HBsAg	Inhibition of IFN induction	8,31
Murine NPCs	Suppressing activation of IRF-3, NF-κB and ERK1/2, triggering production of IL-10	HBsAg, HBeAg and HBV virions	Reduced TLR3-mediated induction of inflammatory cytokines and IFN	32,33
Hepatoma cell lines	Blocking the interaction of TBK1 and DDX3, inhibition of activation of IRF3	HBV polymerase	Inhibition of IFN production in response to RIG-I and TLR3	34
Hepatoma cell lines	Interaction with Hsp90β and inhibiting activity of IKKs	HBV polymerase	Suppressed TNF-α, TLR3 or TLR4-induced NF-κB signaling	35

Abbreviations: HBV, hepatitis B virus; IFN, interferon; pDC, plasmacytoid dendritic cell; TLR, Toll-like receptor.

besides HBV proteins may regulate TLR expression and function in patients. For example, the reduced expression or function of individual TLRs during chronic HBV infection may not only be due to the replication of HBV, but also be attributed to the different inflammatory environments present in healthy and chronically HBV-infected subjects. Two recent studies showed decreased expression of TLR signaling molecules, such as MyD88, IRAK1 and IRAK4, in PBMCs from CHB patients compared with healthy controls.[41,42] One study showed increased IL-12 and decreased IL-6 levels in the serum of CHB patients compared with that of healthy controls. However, it was not clear whether there is a correlation between the inflammatory cytokines and expression of TLR signaling molecules.[42] During chronic HCV infection, TLR2 upregulation in peripheral blood monocytes and liver tissue was correlated with increased levels of serum TNF-α and hepatic inflammation.[43,44] These results supported the hypothesis that the changed inflammatory environment may lead to reduced expression or function of individual TLRs in CHB patients. Our recent studies suggest that TLR expression and function may significantly change during the different phases of the natural history of chronic HBV infection (our unpublished results). Regardless, due to the lack of small animal models supporting efficient HBV infection, the interaction between HBV components and TLRs has not been confirmed directly *in vivo*. Thus, the issue of HBV–TLR interaction requires further investigation.

ACTIVATION OF TLR-MEDIATED INNATE IMMUNE RESPONSES INHIBITS HBV REPLICATION

Regardless of the fact that TLR-mediated innate immunity is not or is only weakly activated by HBV infection, experimental activation of the TLR system in hepatic cells and in the liver could suppress HBV replication *in vitro* and *in vivo*[10–14] (Figure 1). Early experiments in HBV transgenic mice demonstrated the ability of TLR3 ligand poly(I:C) to induce IFN-α-dependent suppression of HBV replication.[45] Later, Isogawa *et al.*[10] reported that a single dose intravenous injection of ligands for TLR3, TLR4, TLR5, TLR7 or TLR9 led to a non-cytolytic inhibition of HBV replication in a type I IFN-dependent manner in HBV transgenic mice. Hepatic NPCs, including KCs and liver sinusoidal endothelial cells (LSECs), play a key role in the TLR-mediated inhibition of HBV.[11] The supernatants of TLR3- or TLR4-activated KCs and TLR3-activated LSECs efficiently inhibited HBV replication in an immortalized murine hepatocyte cell line derived from HBV transgenic mice (HBV-Met). The antiviral effect of TLR3 was largely mediated by IFN-β, whereas the factor for the TLR4 effect remained undefined.[11] Myeloid dendritic cells (DCs) respond to a broad range of TLR ligands, including those for TLR1/2, -3, -4, -7 and -9, to produce antiviral cytokines, which in turn inhibit HBV replication in HBV-Met cells.[11] Thus, TLR activation in hepatic NPCs and extra-hepatic DCs could significantly reduce HBV replication through secretion of type I IFNs or other antiviral cytokines.

In addition to the induction of type I IFNs and pro-inflammatory cytokines, TLR stimulation leads to the activation of intracellular signaling pathways that directly inhibit HBV replication. By over-expression of the TLR adapters MyD88 and TRIF, or the RLR adapter IPS-1, Guo *et al.*[13] clearly demonstrated the ability of intracellular TLR or RLR signal pathways to inhibit HBV replication in hepatoma cells. The over-expression of these adaptors does not lead to the release of antiviral cytokines by transfected hepatoma cells, as HBV replication was not affected by treatment with culture media harvested from cells transfected with each of the three adaptors. In this experimental setting, NF-κB activation was found to play a

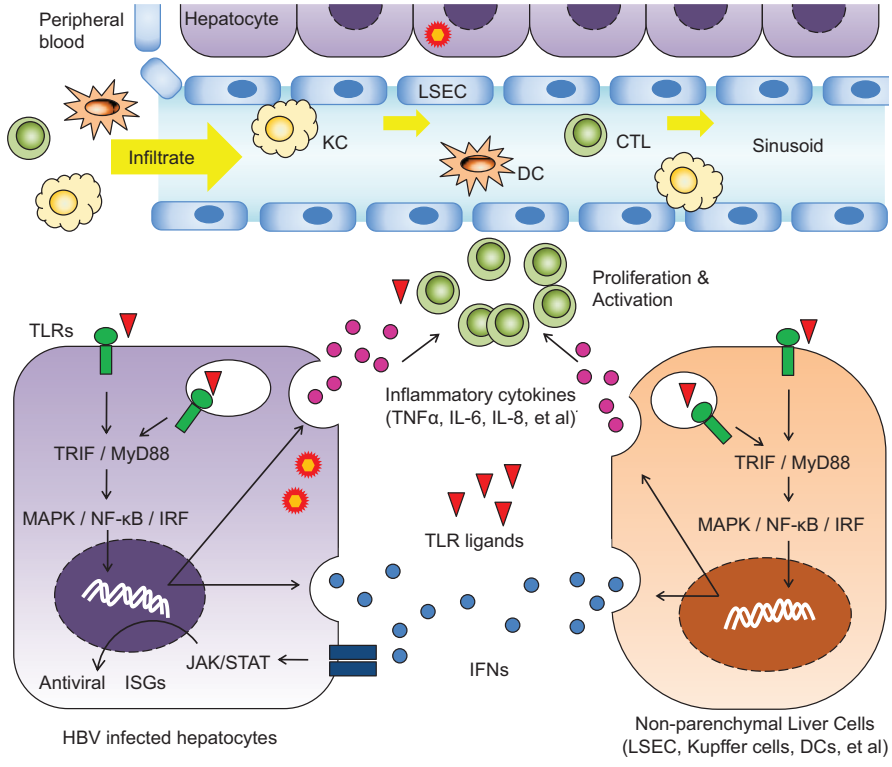

Figure 1 TLRs and activation of antiviral innate and adaptive immune responses in HBV infection. TLRs are expressed in hepatocytes and hepatic non-parenchymal cells, including LSECs, Kupffer cells, DCs and other cell types. Stimulation of TLRs by their respective ligands leads to the activation of downstream MyD88/TRIF-dependent signaling pathways in hepatic cells and the production of pro-inflammatory cytokines, chemokines and IFNs. Inhibition of HBV replication can be achieved by direct or indirect modes: (1) the intracellular MAPK- and NF-κB-dependent signaling pathways trigger antiviral mechanisms; (2) IFNs and other as yet unknown antiviral factors stimulate the expression of ISGs and other antiviral actions in hepatocytes. However, chemokines and inflammatory cytokines recruit specific T cells into the liver, promote T cell proliferation, and enhance the antiviral functions of HBV-specific $CD8^+$ T cells. Therefore, TLRs inhibit HBV in the liver by activating both innate and adaptive responses. DC, dendritic cell; HBV, hepatitis B virus; IFN, interferon; LSEC, liver sinusoidal endothelial cell; TLR, Toll-like receptor.

key role in adaptor-induced antiviral responses.[13] Consistently, TLR2 ligands inhibited HBV or woodchuck hepatitis virus (WHV) replication in human hepatoma cells or primary woodchuck hepatocytes through activation of intracellular signaling pathways.[12] TLR2-mediated antiviral action is dependent on the presence of adaptor molecules, such as TAK1, IRAK1/4 and TRAF6, and the downstream MAPK and PI-3k/Akt pathways. Silencing the expression of adaptor molecules or blocking the MAPK and PI-3k/Akt pathways with chemical inhibitors abolished the TLR2-mediated antiviral effect and significantly enhanced HBV replication.[12] Similarly, TLR4 stimulation with LPS led to a pronounced reduction of WHV replication intermediates without IFN induction in primary woodchuck hepatocytes.[46] TLR2 and TLR4 share the cellular MyD88-dependent signaling pathway in mammalian cells and apparently utilize the same intracellular mechanisms to inhibit hepadnaviral replication.

The reports mentioned above demonstrated the ability of TLRs to mediate indirect and direct antiviral activities against HBV. However, TLR-mediated antiviral effects in human hepatoma cells and woodchuck primary hepatocytes were rather low compared with that of potent nucleos/tide analogues. The antiviral effect of TLR ligands in HBV transgenic mice was transient and largely dependent on the induction of IFN production. Therefore, recent results did not conclusively answer the important question about the relevance of TLR-mediated activation of antiviral mechanisms for the control of HBV infection. The innate arm of the immune system usually serves as the first line of defense against microbial invasion and slows down the spread of infection but frequently is overcome by viral evasion strategies. Innate immune responses are generally not long-lasting but rather transient and therefore, essential but not sufficient to control viral infection. It is not yet clear whether a constant or repeated stimulation of TLR-mediated responses is effective over a long period, which may be required for the treatment of chronic HBV infection. Thus, another TLR-mediated function to promote specific adaptive immune responses will need more attention in this context, as discussed in the next section.

ACTIVATION OF TLR-MEDIATED SIGNALING PATHWAYS MODULATES HBV-SPECIFIC IMMUNE RESPONSES

Appropriate HBV-specific T- and B-cell responses are required to control and terminate HBV infection.[47,48] Particularly, strong and multispecific $CD8^+$ T-cell responses against HBV proteins are required to clear HBV by both noncytolytic and

cytolytic mechanisms.[49–51] However, HBV-specific CD8$^+$ T-cell responses are characteristically weak, transient and barely detectable in patients with chronic HBV infection.[52,53] Therefore, therapeutic restoration of HBV-specific CD8$^+$ T-cell responses in chronically infected HBV patients represents an attractive strategy to terminate HBV persistence.

The liver is recognized as an immunologically privileged organ with a unique composition of innate immune cells. TLRs and RLRs are expressed not only in bone marrow-derived immune cells, such as KCs and hepatic DCs, but also in liver-resident cells, such as hepatocytes, LSECs and hepatic stellate cells.[54] Activation of TLRs and RLRs in hepatic cells leads to an induction of type I IFN and a variety of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, IL-12 and IL-18, which play important roles in inhibiting HBV replication,[10,11] but also modulate specific immune responses (Figure 1). Priming of naïve CD8$^+$ T cells in the liver usually leads to tolerance instead of immunity, accompanied by poor effector functions and apoptosis of activated CD8$^+$ T cells due to an increased expression of the pro-apoptotic protein Bim.[55,56] Consistently, Bim was upregulated on HBV-specific CD8$^+$ T cells during chronic HBV infection and contributed to the deletion of HBV-specific CD8$^+$ T cells.[57] LSECs are unique liver-resident antigen-presenting cells that are capable of antigen cross-presentation and induction of CD8$^+$ T-cell tolerance under non-inflammatory conditions[58] or promotion of CD8$^+$ T-cell immunity under certain conditions, such as viral infection.[59] Recently, we examined functional maturation of LSECs by TLR ligand stimulation, demonstrating that pretreatment of LSECs with P3C (TLR1/2 ligand) but not poly(I:C) (TLR3 ligand) or LPS (TLR4 ligand) reverted their suppressive properties to induce specific T-cell immunity.[60] IL-12, which was produced at a low but sustainable level after TLR2 stimulation, was identified as one essential mediator for LSEC-mediated CD8$^+$ T-cell immunity.[60] In chronically HBV-infected patients, it was demonstrated that IL-12 but not IFN-α is capable of rescuing the anti-viral function of exhausted HBV-specific CD8$^+$ T cells.[61] Therefore, we speculate that activation of the TLR-mediated signaling pathway may lead to an enhancement of HBV-specific T- or B-cell responses in the liver.

Huang *et al.*[62] found that TLR9 activation induced intrahepatic aggregates of myeloid cells that enabled the population expansion of Ag-specific cytotoxic effector CD8$^+$ T cells without causing immunopathology. Importantly, such intrahepatic expansion of DNA vaccine-induced CD8$^+$ T cells controlled chronic HBV infection in a mouse model that was established by the transfer of the HBV genome using an adenovirus vector. In another HBV-persistent mouse model based on hydrodynamic injection of an HBV-genome containing plasmid pAAV/HBV1.2, HBV-specific CD8$^+$ T-cell and anti-HBs antibody responses were impaired. Administration of a dually functioning vector containing both an immunostimulating ssRNA and an HBx-silencing short hairpin RNA induced systemic adaptive immune responses against HBV, including IFN-γ-secreting HBV-specific CD8$^+$ T cells and anti-HBs antibody. The TLR7 signaling pathway and IFN-α/β receptor were found to be important for CD8$^+$ T-cell activation and HBV clearance in this model.[63] The same group showed that unmethylated CpG ODNs derived from the HBV genome (HBV-CpG) induced robust secretion of IFN-α by pDCs in a TLR9-dependent manner. Nanoparticles containing HBV-CpG activated DCs, NKs and T cells *in vivo*, exerting a strong immunostimulatory effect on lymphocytes. Combined with rHBsAg immunization, administration of nanoparticles containing HBV-CpG led to the clearance of HBV and induced an anti-HBsAg response in HBV carrier mice.[64] Along the same lines, Wu et al. demonstrated that intrahepatic administration of TLR3 ligand poly(I:C) could recruit CD8$^+$ T cells into the liver and clear HBV in an IFN- and CXCR3-dependent manner.[65] These studies indicate that TLR agonists may be used as immunomodulatory agents for the treatment of chronic HBV infection by augmenting the HBV-specific T- or B-cell responses. The mechanisms of TLR-mediated activation of innate immunity or modulation of adaptive immunity to control HBV infection are summarized in Table 2.

Recent evidence has also demonstrated that different cell types of the adaptive immune system, such as effector T cells, also express TLRs.[66] Several groups have shown that TLR2 is expressed on activated and memory CD4$^+$ and CD8$^+$ T cells and serves as a costimulatory molecule. TLR2 agonists, such as Pam3CSK4, stimulate activated T cells, thus promoting their proliferation, differentiation and effector function *in vitro* and *in vivo*.[67–69] It has been shown that TLR2 engagement on CD8$^+$ T cells increased T-bet transcription in a MyD88–Akt–mTOR- and protein kinase C-dependent manner.[68] Consistently, Pam3CSK4 application *in vivo* with transferred tumor Ag-specific CD8$^+$ T cells results in enhanced therapeutic efficacy in tumor models.[70,71] The expression of TLR3 and TLR9 on human CD8$^+$ T cells was also demonstrated by some studies. TLR stimulation directly promoted IFN-γ production in CD8$^+$ T cells.[72–74] Thus, the molecular mechanisms underlying TLR-mediated T-cell proliferation and functional differentiation may be further explored for the treatment of chronic HBV infection.

USING TLR AGONISTS FOR IMMUNOTHERAPY IN CHRONIC HBV INFECTION

Based on the findings mentioned above, TLR activation not only induces innate immune responses to limit the replication of HBV in hepatocytes, but could also modulate the adaptive immune response against HBV. Thus, it is rational to discover and develop TLR agonists as antiviral agents as well as adjuvants for preventive and therapeutic vaccination against HBV infection. Before the discovery of TLRs, a TLR3 agonist named polylysine and carboxymethylcellulose-modified polyriboinosinic : polyribocytidylic acid was recognized as an IFN inducer in chimpanzees.[75] Chronically HBV-infected chimpanzees showed transient changes of infection markers when treated with polyriboinosinic : polyribocytidylic acid. Serum Dane particle-associated DNA polymerase, HBeAg and HBsAg, and intrahepatic hepatitis-B surface and core antigens were

Table 2 The mechanisms of TLR-mediated inhibition of HBV through activation of innate immunity and modulation of adaptive immunity

Experimental model	*Mechanism*	*TLRs involved*	*Antiviral effects*	*Reference*
HBV transgenic mice	Induction of IFNs	TLR3, TLR4, TLR5, TLR7 or TLR9	Non-cytolytic inhibition of HBV replication in the liver *in vivo*	10,45
HBV Met cell line	Induction of IFN-β and undefined factors from NPCs	TLR3 and TLR4	Inhibition HBV replication *in vitro*	11
Hepatoma cell lines	Activation of NF-κB signal pathway	Over-expression of TLR adaptors MyD88, TRIF or IPS-1	Inhibition HBV replication *in vitro*	13
Hepatoma cell lines, PWHs	Activation of NF-κB, MAPK and PI-3k/Akt signal pathways	TLR2 or TLR4	Inhibition HBV or WHV replication *in vitro*	12,46
Ad-HBV mouse model	Induction of intrahepatic aggregation of myeloid cells	TLR9	Enhanced expansion of HBV-specific $CD8^+$ T cells induced by DNA vaccination	62
pAAV/HBV1.2 mouse model	Induction of type I IFNs	TLR7	Induction of IFN-γ-secreting HBV-specific $CD8^+$ T cells and anti-HBs antibody response	63
pAAV/HBV1.2 mouse model	Inducing robust secretion of IFN-α by pDCs	TLR9	Clearance of HBV and induction of anti-HBs antibody response	64
pAAV/HBV1.2 mouse model	Activation of ISGs and recruits $CD8^+$ T cells into the liver	TLR3	Clearance of HBV and induction of anti-HBs antibody response	65

Abbreviations: HBV, hepatitis B virus; IFN, interferon; PWH, primary woodchuck hepatocyte; TLR, Toll-like receptor.

transiently diminished during treatment.[76] This is the first report demonstrating that TLR activation inhibits HBV replication *in vivo*. The other TLR3 agonists, such as mismatched double-stranded RNA and Ampligen, induce IFN-like activity and had an antiviral effect in DHBV-infected ducks.[77,78] Recently, TLR7 ligand GS-9620 has been examined for its antiviral effect in the woodchuck and chimpanzee models. Interestingly, a 4-week treatment with GS-9620 resulted in a sustained, marked reduction in serum WHV DNA and WHsAg levels and in the induction of anti-WHs antibody responses as well as a markedly decreased incidence of hepatocellular carcinoma in chronic WHV-infected woodchucks.[79] Furthermore, GS-9620 induced an increase in serum IFN-α in a dose-dependent manner and triggered ISG expression in PBMCs and the liver in chimpanzees. Short-term oral administration of GS-9620 resulted in a long-term suppression of serum and liver HBV DNA, as well as a reduction in serum levels of HBsAg and HBeAg in 3 chronically HBV-infected chimpanzees.[14] In contrast, TLR9 ligand CpG-containing ODNs have been tested in the woodchuck model for treatment of chronic hepatitis B, but failed to show sufficient therapeutic effect when applied alone (Lu M *et al*., manuscript in preparation). Recently, the TLR8 ligand ssRNA40 was found to selectively activate liver-resident innate immune cells to produce high levels of the antiviral cytokine IFN-γ not only in healthy human livers, but also in chronically HBV- or HCV-infected livers. These results demonstrated the therapeutic implications of TLR8 agonists for the treatment of chronic liver infections.[80] Therefore, TLR3, 7 and 8 agonists are promising drug candidates for the treatment of chronic HBV infection if their toxicity can be reduced to a tolerated range.

During chronic HBV infection, the function of innate immunity is impaired due to reduced TLR expression and disturbance of TLR and RLR signaling pathways. However, recent studies have suggested that antiviral treatment in chronic HBV patients can restore the expression of TLRs in PBMCs.[9,12,81] Huang et al. demonstrated that chronic HBV patients had lower TLR3 and TLR9 mean fluorescence intensities on PBMCs, liver Kupffer cells and hepatocytes. Hepatic TLR3 and TLR9 mRNA were also significantly reduced in patients. However, pegylated-IFN or entecavir treatment may induce a sustained virological response that is correlated with a restoration of TLR3 and TLR9 expression.[9,81] In the WHV-infected woodchuck model, we also found that TLR2 expression in PBMCs was negatively correlated with WHV viral loads during acute WHV infection, and increased if WHV loads were suppressed in woodchucks by antiviral treatment.[12] However, these studies did not address the question of whether the functionality of TLRs was also restored by antiviral treatment. Further studies are needed to clarify this question. Regardless, these studies provide a rationale for combination strategies to restore TLR expression and functionality by antiviral treatment followed by TLR stimulation, which could improve the effect of immune modulation in chronic HBV infection.[82]

A potential use for TLR ligands as adjuvants for prophylactic and therapeutic vaccines has been considered for a long time.

The rational design of specific TLR agonists may increase potency and tolerability of new adjuvants and provide the opportunity to meet the stringent safety criteria for a new vaccine formulation.[83] Monophosphoryl lipid A, a chemically modified derivative of the lipid A moiety of TLR4 agonist LPS, is considerably less toxic but has similar immunostimulatory activity.[84] The AS02 and AS04 adjuvant system, which consists of monophosphoryl lipid A adsorbed on either aluminum or oil, has been evaluated in prophylactic vaccines against HBV.[85] The AS04-adjuvant HBV vaccine (Fendrix) boosted the anti-HBs antibody response with an acceptable safety profile not only in liver-transplant candidates,[86] who had a suboptimal response to double doses of recombinant hepatitis B vaccine,[87] but also in healthy non-responder individuals.[88] The AS02-adjuvant HBV vaccine (Supervax) induced more rapid, enhanced, and persistent protection with fewer doses than the currently-licensed AS04-adjuvant HBV vaccine.[89] Two TLR9 agonists of class B CpG-ODNs called CPG7909 and 1018 ISS in combination with recombinant HBsAg have been tested in clinical trials. The addition of CPG 7909 to the conventional HBV vaccine (Engerix-B) resulted in higher anti-HBs antibody titers and an enhanced late affinity maturation process to increase the avidity of anti-HBs antibody.[90,91] Furthermore, a two-dose regimen of recombinant HBsAg and 1018 ISS (Heplisav) rapidly produced higher anti-HBs titers with generally well-tolerated adverse events, compared with the standard three-dose regimen of Engerix-B.[92–94] Such a vaccine formulation may be used for patients with an impaired immune system, as it is more effective in the hypo-responsive population, such as HIV-infected patients, than conventional HBV vaccines.[95–97] The potential therapeutic application of TLR agonists is summarized in Table 3.

CONCLUDING REMARKS

While the central role of adaptive immunity in controlling HBV infection is well established, the contribution of innate immunity in this regard has been appreciated only in recent years. The development of new *in vitro* methods of HBV infection will soon shed some light on the recognition of HBV by PRRs.[98–100] Previous studies indicated that HBV infection apparently does not activate innate immunity in chimpanzees and patients. HBV proteins, such as HBsAg, HBx and HBV polymerase, are associated with the inhibition of TLR or RLR signaling pathways and lead to impaired IFN production. However, it remains to be clarified whether the inhibition of TLR or RLR signaling pathways by HBV is the cause of the lack of early interferon and other innate responses *in vivo*. Whether the innate immune responses would control HBV infection if initiated in the early phase of HBV infection must be determined. It is well documented that the activation of TLR-mediated signaling pathways not only exhibits direct inhibition of HBV replication, but also enhances HBV-specific T-cell and B-cell responses. Although the expression of TLRs on host cells is downregulated by HBV infection, inhibition of HBV replication by antiviral treatment could partially restore TLR expression in patients. Thus, a treatment strategy with a combination of viral suppression, activation of TLR-mediated innate immunity and restoration of HBV-specific immune responses is needed to achieve effective long-term control of HBV infection and cure chronically HBV-infected patients.

Table 3 Using TLR agonists for immunotherapies of chronic HBV infection

Experimental model	*Mechanism*	*TLRs involved and ligands*	*Effects on HBV*	*Reference*
Chronically HBV-infected chimpanzees, DHBV-infected ducks	Induction of IFNs or IFN-like activity	TLR3: polyICLC, Ampligen	Transient diminish of serum and intrahepatic HBeAg and HBsAg	75–78
Chronically WHV-infected woodchucks	Induction of IFNs	TLR7: GS-9620	Sustained and marked reduction of serum WHV DNA and WHsAg levels	79
Chronically HBV-infected chimpanzees	Induction of IFNs and expression of ISGs	TLR7: GS-9620	Long-term suppression of serum and liver HBV DNA, reduction of serum HBsAg and HBeAg	14
Chronically HBV-infected livers from patients	Activation of liver-resident innate immune cells to produce high levels of IFN-γ	TLR8: ssRNA40	The antiviral effects were not tested *in vivo*	80
Health controls and HBsAg non-responders	Induction of IFNs and pro-inflammatory cytokines	TLR4: MPLA (AS04 and AS02 adjuvants)	Inducing more rapid, enhanced and persistent anti-HBs antibody response	86–89
Health controls and immunocompromised individuals	Induction of IFNs and pro-inflammatory cytokines	TLR9: CPG7079 or 1018ISS	Induction of high titers of anti-HBs antibody with higher affinity, effective in immune-compromised individuals	90–97

Abbreviations: DHBV, duck hepatitis B virus; HBV, hepatitis B virus; IFN, interferon; ISG, IFN-stimulated gene; MPLA, monophosphoryl lipid A; polyICLC, polyriboinosinic : polyribocytidylic acid; PWH, primary woodchuck hepatocyte; TLR, Toll-like receptor.

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