Targeting Innate and Adaptive Immune Responses to Cure Chronic HBV Infection

Adam Gehring[1,2] and Ulrike Protzer[3,4]

[1] Toronto Centre for Liver Disease and Toronto General Hospital Research Institute, University Health Network, Toronto, Canada
[2] Department of Immunology, University of Toronto, Toronto, Canada
[3] Institute of Virology, Technical University of Munich / Helmholtz Zentrum München, Munich, Germany
[4] German Center for Infection Research (DZIF), Munich partner site, Munich, Germany

Correspondence to:

Adam J. Gehring, PhD
Toronto Medical Discovery Tower, 10-356
101 College Street
Toronto, Ontario, Canada M5G 1L7
Tel: +1 (416) 634-7095
adam.gehring@uhnresearch.ca

Prof. Ulrike Protzer, MD
Institute of Virology
Trogerstr. 30, D-81675 München, Germany
Tel: +49 (89) 4140-6886
protzer@tum.de; protzer@helmholtz-muenchen.de

Abstract

Fewer than 1% of chronic hepatitis B virus (HBV) infections per year are cured with antiviral treatment. This creates a need for long-term treatment, which poses challenges for patients and health systems. Since cure is accompanied by recovery of antiviral immunity, a combination of direct-acting antiviral agents and immunotherapy are likely to be required. Extensive efforts have been made to identify determinants of the failed immune response to HBV in patients with chronic infection. We review mechanisms of immune dysfunction in patients with chronic HBV infection, immunotherapy strategies in development and the challenges associated with successful implementation of immunotherapy.

Keywords: drug, HBsAg, vaccine, inflammation, HBV DNA

There is no finite, curative treatment for the more than 250 million individuals living with persistent hepatitis B virus (HBV) infection.[1] HBV infection has become a treatable disease, in that nucleos(t)ide analogues (NUCs) can suppress replication of the virus to a level below detection in the blood. Although this strategy has been effective at reducing progressive pathology associated with chronic infection, patients remain at risk for hepatocellular carcinoma (HCC)[2, 3] and face the stigma of chronic infection and the economic and personal burdens of long-term treatment. In addition, fewer than 5% of patients worldwide have access to antiviral drugs and, even in developed countries, treatment indication is limited to those who have developed inflammatory liver disease.

Limitations of NUCs include the fact that they act at the stage of reverse transcription—a late step in HBV replication. The viral persistence form, covalently closed circular (ccc)DNA, remains untouched in the nucleus of infected cells.[4] Therefore, integration of HBV-DNA may continue, hepatitis B surface antigen (HBsAg) secretion persists, and less than 1% of patients per year will actually be cured of HBV infection (for review, see [5]). This necessitates long-term treatment. When NUC therapy is withdrawn, most patients have a rebound in HBV DNA, with some developing liver inflammation [6] and potentially life-threatening flares. A finite treatment that produces long-term control, and ultimately a cure of HBV infection, is therefore the goal[7]. This will likely require a coordinated therapy targeting the virus and boosting immunity to clear the infected reservoir of hepatocytes.

HBV cure

The scientific community has established definitions of HBV cure to benchmark the progress of novel therapies.[8] A, sterilizing cure is defined as eradication of HBV including intrahepatic cccDNA and integrated HBV-DNA. A functional cure is sustained, undetectable HBsAg and HBV DNA in serum (with or without detectable antibodies against HBsAg) and resolution of liver injury after

completion of a finite course of treatment with a decreased in risk of HCC over time. Lastly, partial cure is defined as detectable HBsAg but persistently undetectable HBV DNA in serum after completion of a finite course of treatment.[8]

In attempts to cure HBV infection, it is important to remember that HBV does not only persist via cccDNA—it also integrates into the host genome.[9, 10] This integrated DNA does not replicate HBV but can still express HBV antigens, including HBsAg. In addition, cccDNA persists decades after resolution of HBV infection,[11] which can account for the reactivation of HBV infection when patients with resolved infection are immunosuppressed. However, this also shows that HBV infection is fully controlled by an immune response, with no or minimal risk of liver disease, and that HBsAg remains undetectable despite the persistence of integrated HBV genomes. Therefore, the goal of HBV therapy is to achieve a functional cure, similar to that achieved in individuals who spontaneously control acute HBV infection and maintain that control life long, without antiviral therapy. HBV infection is required for formation and spread of hepatitis delta virus (HDV), so a successful immune therapy for hepatitis B is expected to also lead to a cure of HDV infection.

Immune Control

The efficiency of the HBV-specific immune response is evident during resolution of acute infection, in which nearly 100% of hepatocytes are infected.[12] Patients who resolve acute HBV infection have a robust response of CD4+ and CD8+ T cells against the virus. These cells produce antiviral cytokines and provide co-stimulation to B cells. B cells produce anti-HBs, which clear antigen and virus from the circulation and prevent or limits re-infection, along with antibodies against hepatitis B e antigen (HBeAg) and HBV core antigen (HBcAg).

The dichotomy between patients who resolve acute infections and those with chronic HBV infection is apparent in magnitude and function of their immune responses. HBV-specific T cells are detected at significantly lower frequencies in patients with chronic vs acute infections. HBV-

specific T cells are present in all phases of chronic HBV infection but their low frequency makes them difficult to detect without in vitro expansion.[9, 13-16] This is in contrast to patients with other persistent virus infections, such as HIV, CMV, or EBV infections[17]—in these patients, virus-specific T cells are easily detected in blood samples. In addition, an effective B-cell and neutralizing antibody responses are lacking.[18]

Despite the scarcity of immune effector cells and an insufficient immune response in patients with chronic HBV infection, there is evidence that activation of immunity can clear the virus. A high frequency of activated, intrahepatic HBV-specific CD8+ T cells, combined with peripheral cytotoxic T cells that produce cytokines and expand, correlates with HBV control. The benefit of increased frequencies of HBV-specific T cells is supported by a cross-sectional study investigating the magnitude of T-cell responses. Patients that control HBV after long-term NUC therapy had T-cell frequencies similar to those of patients who resolved acute infections[19]. Higher frequencies of HBV-specific T cells in the blood might also correlate with controlled HBV rebound in patients who stopped taking NUCs.[20] Interestingly, T cell programmed cell death 1 (PDCD1, also called PD-1) expression correlated with a limited increase in alanine aminotransferase (ALT) in patients who stopped taking NUCs, [21] suggesting that the activity of hepatic T cells is regulated to preserve liver function.[22]

Interferons (IFNs), as well as other cytokines secreted by immune cells, can control HBV replication. T cells and natural killer (NK) cells can control, and even eliminate, HBV without cytolysis (without destruction of infected cells) by secreting cytokines.[12, 23] Studies in HBV-infected chimpanzees revealed loss of cccDNA when IFN gamma-producing CD8+ T cells infiltrate the liver, but before levels of liver enzymes in the serum increase.[12, 24] Cytokines can block HBV replication at transcriptional and post-transcriptional steps (for review, see [25]) and reduce cccDNA stability by inducing enzymes that edit and subsequently digest it.[26, 27] Thus, cytokines can eliminate cccDNA from infected cells without destroying them.[28]

In addition to T cells, B cells are required for control of chronic HBV infection. Antibodies that deplete B cells reactivate HBV in up to 60% of patients with chronic infection, potentially leading to severe flares. Even in individuals who resolved infection years earlier can have HBV reactivation upon B cell depletion.[29]

There is also robust evidence from patients receiving liver transplants that restoration of anti-HBV immune responses can clear the virus from an infected liver. Transplantation of livers from patients with chronic HBV infection into a donor with preexisting HBV immunity can cure the infection.[30] Alternatively, patients with chronic HBV infection that were given bone-marrow transplants, to reconstitute their immune system, could control HBV in the chronically infected liver. [31, 32] Therefore, boosting magnitude and quality of the virus-specific immune response is a rational strategy for therapy.

There is a continuous balance between immune control and immune tolerance in patients with chronic HBV infection, and sufficient recovery of the immune response can fully control HBV. A major effort has been invested to identify distinct features of immune dysfunction in patients with chronic HBV infection with the goal of restoring immunity capable of purging HBV from the chronically infected liver.

T cells

HBV-specific T cells are required for immune control of HBV infection. Their antiviral functions mediate resolution of acute HBV infections [33], and has been demonstrated in experiments with in vitro co-cultured cells,[27, 34, 35] HBV-transgenic mice,[23] mice with humanized livers,[36] and infected chimpanzees.[12, 37] Therefore, most research into immune dysfunction in patients with chronic HBV infection have focused on activities of T cells—particularly CD8+ T cells.

In patients with chronic HBV infection, virus-specific T cell failure can be caused by antigen-specific deletion, lack of expansion and arming of effector T cells, or functional adaptations. Ineffective activation of HBV-specific T cells in the liver, due to factors such as lack of co-stimulation by hepatocytes, can make them susceptible to antigen-specific deletion. This contributes to the low frequency of T cells in patients with chronic HBV infection.[38, 39] HBV-specific T cells from chronic patients have increased expression of the apoptotic protein BCL2 like 11 (BCL2L11, also called BIM) compared to T cells from patients with resolved acute HBV infection.[40] Expression of BIM sensitizes HBV-specific effector T cells to activation-induced apoptosis. In addition, exhausted HBV-specific T cells express death receptors, making them susceptible to tumor necrosis factor (TNF) superfamily member 10 (TNFSF10, also called TRAIL)-dependent destruction by NK cells.[41]

In the HBV-specific T cells that remain, proliferation and antiviral function are restricted. In patients with chronic infection, persistent antigen exposure results in T-cell exhaustion (inactivation) and increased expression of inhibitory receptors.[42] HBV-specific T cells express inhibitory receptors such as PD-1, cytotoxic T-lymphocyte associated protein 4 (CTLA4), and hepatitis A virus cellular receptor 2 (HAVCR2, also called TIM3).[13, 43, 44] Amino acid restriction by myeloid-derived suppressor cells as well as mitochondrial dysfunction of HBV-specific T cells are examples of metabolic defects that reduce T-cell function.[45, 46] This functional immune tolerance, such that T cells no longer recognize or respond to HBV antigens, is a key obstacle of effective immunotherapy strategies [47]

There are multiple networks that suppress T-cell activation, making it challenging to restore T-cell responses to HBV by targeting a single pathway. Activation of an immune response to HBV may require coordinated effective co-stimulation of T cells, blockade of the inhibitory signals, inducing an environment that facilitates T cell expansion, or totally circumventing T-cell dysfunction via adoptive T cell therapy. IL12 increases proliferation and cytokine production in

HBV-specific T cells and synergizes with checkpoint blockade to further increase the magnitude of T cell expansion.[41] T-cell co-stimulation and modification of the liver environment can provide a niche that supports intrahepatic expansion of virus-specific T cells.[48] These concepts have been incorporated into therapeutic strategies that involve potent adjuvants, monoclonal antibodies, or pattern recognition receptor agonists that alter the liver environment.

NK cells

Innate effector cells, such as NK cells, gamma delta T cells, and mucosal-associated invariant T cells can contribute to disease pathogenesis but these cells are not specific for HBV and little is known about their activities toward the virus. The cytolytic activity of NK cells has been associated with hepatic inflammation, and NK cells can kill activated T cells,[41, 49] reducing numbers of HBV-specific T cells. NK cells can contribute to control of HBV replication in mice, but their antiviral activity is suppressed by IL10 and transforming growth factor beta (TGFB) in patients with chronic HBV infection.[50, 51]

NK cells are highly enriched in human liver compared to the blood, giving them the potential to reduce the T-cell mediated immune response to HBV or serve as targets for innate immunotherapy. NK cells can be activated to produce IFN gamma following activation of toll-like receptor 8 (TLR8) on hepatic immune cells.[52] Therefore, altering the intrahepatic environment with pattern recognition receptor agonists could overcome the inhibitory effects of IL10 and TGFB in patients to harness the antiviral potential of NK cells. However, strategies that activate non-virus specific cells, such as NK cells, could have side effects such as induction of widespread inflammation.

B cells and antibodies

Antigen binding to the B cell receptor induces B-cell differentiation into plasma cells and stimulates antibody secretion. Antibodies against HBsAg are biomarkers of disease resolution and antibodies against HBeAg are used to differentiate the stages of chronic HBV infection. The production of antibodies to HBV after mitogen stimulation of B cells from vaccinated patients, or from patients who resolved HBV infection, is greater than B cells from patients with chronic HBV infection.[53, 54] However, frequencies of circulating HBs-specific B cells are independent of the HBV infection status. B cells from patients with chronic HBV infection have an atypical phenotype and are functionally altered, in that they express PD-1, which reduces their antibody production.[55, 56] Although most antibodies are complexed by HBsAg and HBeAg,[57-59] circulating immune complexes do not have pathologic effects in most patients with HBV infection—in contrast to patients with chronic HCV infection, who can develop cryoglobulinemia as an important extrahepatic complication.[60, 61] The presence of immune complexes in patients with chronic HBV infection correlates with the timing of HBeAg seroconversion and overlaps with a B-cell gene expression signature, identified by transcriptome analysis.[62]

Beyond the production of antibodies, we have little understanding of the phenotypes or function of HBV-specific B cells, such as their capacity to serve as antigen-presenting cells and their cytokine profiles. Although checkpoint inhibitors, such as antibodies against PD-1, might be used to induce an anti-HBV immune response,[55, 56] an optimal strategy to enhance the B cell response in in patients with chronic infection remains unclear. Further studies are needed to determine how B cells might be activated for treatment of chronic HBV infection.

The liver microenvironment

The liver is a vital organ important for carbohydrate, protein and lipid metabolism, clearance of toxins and bacterial components. Immune cells in the liver are uniquely regulated to avoid inappropriate activation of responses to local antigens and organ damage.[22, 63] This environment

exacerbates weaknesses in the HBV immune response through the presence of regulatory populations of liver resident macrophages, regulatory T cells, regulatory B cells and myeloid derived suppressor cells (MDSC). These cells express ligands for the inhibitory receptors PD-1 and TIM-3 and produce immunosuppressive cytokines IL-10 and TGF-β (Fig 1).[63]

Because of the slow blood flow in liver sinusoids, circulating immune cells have ample opportunity to interact with hepatocytes and sinusoidal lining cells such as Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells—all of which can inactivate T cells. These cells express inhibitory ligands and produce anti-inflammatory cytokines such as IL10 and TGFB, which prevent antigen-specific activation of T cells and maintain the immune-tolerant environment of the liver.[22] Although hepatocytes are shielded from the blood stream, T cells can gain access to hepatocytes through the fenestrated endothelium[63], where antigens are presented in the absence of effective co-stimulation. This process, however, does not expand the pool of memory T cells and increases T-cell susceptibility to activation-induced cell death or TNF-associated apoptosis.[38, 40, 41] The liver environment therefore contributes to the low abundance of HBV-specific T cells and regulation of the environment through immunotherapy is likely to play a key role in restoring immunity to control HBV.

Immune Therapy

The immune response fully controls HBV after decades of infection in 0.5% to 1% of patients each year.[64] HBsAg is cleared, antibodies against HBsAg become detectable in serum, and virus replication ceases even without treatment—accompanied by life-long T-cell and antibody-mediated immunity.[11] The immediate aim of immunotherapy is to restore HBV immunity to this state, establish long-term control of the virus without antiviral treatment, and significantly increase the proportion of patients with a functional cure.

Stimulating the innate immune system

IFN-based therapies are considered to be immune-modulatory strategies. IFN-alpha 2, used in therapeutic regimens, has antiviral effects,[5, 8, 65] but also affects the activation status of different immune cells. Although IFN-alpha 2 can cure HBV infection, it produces many side effects, limiting its efficacy as a stand-alone drug. However, new antiviral drugs are using IFN-α in combination therapies, as the only approved immunomodulator, which may improve response rates and shorten the treatment window for IFN-α.

The innate immune response is not antigen-specific, but produces antiviral and T-cell polarizing cytokines, presents antigens to T cells, and alters the liver microenvironment to promote an immune response over tolerance. To increase the antiviral activity of innate immune cells, new agents have been designed to activate pattern recognition receptors, such as TLRs or cytoplasmic nucleic acid sensors (Table 1). Although HBV replication does not induce production of cytokines by hepatocytes,[66-68] the virus is sensitive to cytokines that inhibit distinct steps of the HBV life cycle (for summary, see ref [68]). Stimulation of pattern recognition receptors leads to secretion of IFN or inflammatory cytokines such as TNF, IL1, IL6, IL12, and IL18. [65, 69] In mice, the antiviral effects are mediated mainly by type I IFNs, which control HBV transcription and RNA and capsid stability.[66, 70, 71] Type I IFNs, type III IFNs, lymphotoxin beta, and TNF cause loss of HBV cccDNA from the nucleus of infected cells.[26, 27]

Beyond the capacity of pattern recognition receptors to stimulate production of cytokines with antiviral activity, their activation promotes antigen presentation and affects the liver microenvironment. TLR signaling controls programs of phagosome maturation in professional antigen presenting cells to ensure the selection of pathogen-derived antigens for presentation by MHC class II.[72] In addition, non-parenchymal liver cells, particularly Kupffer cells, as well as monocytes, can be stimulated to alter the liver environment to facilitate the antiviral response.[48,

[73] Activation of an innate immune response can therefore lead to the production of cytokines with antiviral activity, increase antigen presentation, and alter the liver microenvironment. However, cytokines such as IFN alpha, IFN beta, and TNF can also induce immune tolerance in patients with chronic infection [74, 75], which may limit sustained immune control.

Targeting TLRs

TLRs are expressed either at the cell surface or on endosome membranes, where they recognize molecular patterns associated with invading pathogens. All but TLR3 require the signaling adapter protein MyD88. Different TLRs can lead to activation of transcription factors such as NF-kB and interferon regulatory factor 3 (IRF3) and IRF7, which regulate the patterns of cytokines produced by each type of cell (Fig 2).

TLRs are highly expressed on myeloid cells (dendritic cells, monocytes, macrophages) but, with the exception of TLR1, TLR2, TLR3, and TL4, are expressed at low levels on human hepatocytes.[76] Stimulation of TLR1, TLR2, TLR3, and TLR4 on hepatocytes has direct antiviral effects against HBV.[77] In contrast, mice and woodchuck hepatocytes express TLR7 and TLR9, which likely increases their sensitivity to agents targeting these receptors. In HBV-transgenic mice, injection of ligands that activate TLR3, TLR4, TLR5, TLR7, or TLR9 reduces HBV replication.[69] In woodchucks, activation of TLR7 and TLR9 reduce replication of woodchuck hepatitis virus.[78, 79] TLR-induced production of TNF by inflammatory monocytes can increase lymphoid cell aggregates in mice that support intrahepatic expansion of activated T cells.[48] The combination of TLR agonists and a therapeutic vaccine eliminated lymphocytic choriomeningitis virus, as well as HBV, from infected mice.

Based on positive results from preclinical studies, orally available, small-molecule agonists of TLR7 and TLR8 have been developed by Gilead (GS-9620, GS-9688) and Roche

(RO6864018) and are being tested in phase 1 and 2 trials (Table 1). After absorption in the intestine they are expected to activate TLRs on dendritic cells and macrophages in the intestine and liver. In woodchucks with woodchuck hepatitis virus infection, GS-9620 reduced levels of virus and HBsAg, resulting in anti-HBs seroconversion and significant reductions in levels of cccDNA.[78] In HBV-infected chimpanzees, short-term oral administration of GS-9620 provided long-term suppression of serum and liver HBV DNA, activated type I and II interferon signatures, induced aggregates, increased numbers of B cells and CD8+ T cells in liver, and resulted in the clearance of some HBV-infected cells.[80] GS-9620 is safe in humans,[81] and in treatment-naïve and NUC-treated patients with HBV infection, the agent induced transient expression of ISG15 in peripheral blood mononuclear cells but had no antiviral effects—even at the highest dose tolerated.[82]

AIC649, an inactivated *Parapoxvirus ovis* particle, activates complement and TLR-dependent and -independent signaling in myeloid cells to promote a T-helper 1 cell-mediated response.[83] In woodchucks, AIC649 reduced levels of HBsAg [79]. The agent displayed good safety data in a phase 1 trial of patients with chronic HBV infection.

Riboxxol is a synthetic 50-base pair double-stranded RNA that activates TLR3. Riboxxol had long-lasting antiviral effects in cultured primary human hepatocytes, controlled HBV replication, and reduced the level of cccDNA.[77] Further development of agents that activate TLRs is underway. Their efficacy and toxicity must be carefully assessed to improve their therapeutic index.

Stimulation of cytoplasmic nucleic acid sensors

The RNA helicases DExD/H-box helicase 58 (DDX58, also called RIG-I) and interferon induced with helicase C domain 1 (IFIH1, also called MDA5) are RNA sensors in the cytoplasm that induce production of cytokines by activating IRF3 and NF-kB (Fig 2). RIG-I can also activate the

inflammasome and induce apoptosis.[84] Double-stranded RNA is the ligand for MDA5[85] and 5' triphosphorylated viral RNA is the ligand for RIGI.[85-87]

Activation of RIG-I induces an antiviral response against HBV.[88] Interestingly, small interfering (si)RNAs can be transformed into RIG-I ligands by addition of a 5'-triphosphate to the siRNA directed against HBV. This siRNA becomes bi-functional and inhibits HBV replication with greater efficacy than unmodified siRNAs in cultured cells and transgenic mice.[88] An oral small molecule nucleic acid hybrid (SB-9200) activates RIG-I and nucleotide binding oligomerization domain containing 2 (NOD2) that may have a direct antiviral effect on the HBV polymerase and stimulate IFN production. In woodchucks, SB-9200 produced a dose-dependent reduction in woodchuck hepatitis virus DNA and surface antigen that rebounded following treatment withdrawal.[89] The more effective scheme, NUC therapy following SB-9200, is currently being evaluated in phase 2 clinical trials.

The transmembrane protein 173 (TMEM173, also called STING) senses the presence of cytoplasmic DNA via cyclic di-nucleotides. These are generated by GMP-AMP synthase, which dimerizes upon binding of cytoplasmic DNA.[90, 91] There is evidence that hepatocytes do no express STING, but non-parenchymal cells such as macrophages do respond to STING activation.[92] STING agonists are being evaluated in preclinical studies.[93]

Activating an adaptive immune response

Activation of an adaptive immune response appears to be a key to controlling and curing HBV infection. Agents developed to restore antigen-specific immune responses against tumors might be effective against HBV infection. Strategies include antibodies that target molecules on the surface of infected cells, checkpoint inhibitors that reactivate antigen-specific T cells, cell therapy with genetically modified T cells, and therapeutic vaccines. Cancer immunotherapy studies have

demonstrated the power of boosting effector T-cell responses but also the severe side effects. However, researchers are identifying methods to limit and control the side-effects of immunotherapy.

Therapeutic vaccines

A therapeutic vaccine against HBV needs to overcome mechanisms of HBV-specific immune tolerance. Since natural resolution of HBV infection is accompanied by life-long T-cell and antibody mediated immunity, these vaccines should induce HBV-specific B- and T-cell immune responses. Previous vaccine approaches largely fell into categories of protein-based, protein–antibody complex-based, or peptide-based vaccines and DNA-based vaccines. Development of vaccines for treatment of chronic HBV infection have advanced substantially. DNA vaccines, such as Inovio's INO-1800 or Janssen's JNJ-64300535, use electroporation devices to deliver the vaccine into intradermal DC. The TG-1050 vaccine, being developed by Transgene, expresses HBV antigens from an adenoviral vector (Table 1). Adjuvants, such as IL-12 expression, or viral vector-induced Th1 cytokine profiles capitalize on previous data showing the third signal in T cell activation is a critical component to overcoming multiple mechanisms of T cell exhaustion.

Preclinical studies of vaccines for treatment of HBV infection have produced promising results. However, translation of previous vaccines to patients induced low-level T-cell and B-cell responses and have not shown efficacy in clinical trials.[46, 94-96]. Suboptimal vaccine design could be a reason. Development of single-component vaccines is much easier than parallel development of the multiple components needed for a heterologous prime-boost, so most approaches have concentrated on homologous prime-boost vaccination. Natural immunity, however, most likely requires a heterologous prime-boost vaccination.[95] Different components are needed to induce broad, epitope-specific and multi-functional effector T and B cells. To achieve this, vaccines will have to deliver antigens covering multiple genotypes and provide the co-

stimulation (signal 2) and cytokine profile (signal 3) to induce a durable adaptive immune response to eradicate HBV-infected hepatocytes and prevent de novo infection of uninfected hepatocytes. However, all therapeutic vaccine strategies face the same major hurdle for successful immunotherapy: breaking T-cell tolerance in the presence of high levels of HBV antigens. Reduction of the viral antigen load should therefore be considered a key factor for success of therapeutic vaccination. Although nucleoside analogues do not reduce expression of viral antigens, agents such as siRNAs or nucleic acid polymers, which lower antigen loads, are being tested and could be given as pre-treatment before therapeutic vaccines.[94]

Activation of B cells

Induction of functional B cell responses and production of antibodies against HBV antigens is required for resolution of acute HBV infection and an important indicator of HBV cure. Strategies to activate an innate immune response are likely to also affect B cells. TLR agonists induce polyclonal B cells to expand in culture and may be able to (re-)activate B cells. Co-stimulation of CD4+ T cells by therapeutic vaccines or checkpoint inhibitors (antibodies against PD-1 or PD -ligands)[55, 56] might increase the HBV-specific B-cell response in patients with chronic infection.

Checkpoint inhibitors

PD-1 is the inhibitory receptor expressed by most HBV-specific T cells [43], so agents are in development to disrupt the interaction between PD-1 and its ligand, PD-L1. Agents that block this interaction increase numbers and function of HBV-specific T cells [13] and increase production of antibodies by B cells in culture.[55, 56] In woodchucks infected with woodchuck hepatitis virus, a combination of antiviral agents, DNA vaccines, and antibodies against PD-L1 resulted in loss of hepatitis surface antigen and virus DNA.[97] Preclinical evidence therefore supports that immune

checkpoint inhibitors could activate T-cell responses to HBV in patients and contribute to HBV cure. Other immune inhibitory receptors, such as CTLA4 and TIM3, might also inactivate HBV-specific T cells, but targeted therapies are not in clinical development.

Trials testing agents that block PD-1 in patients with HCC and hepatitis B might provide insight into safety and efficacy of checkpoint inhibitors in patients with chronic HBV infection, although those studies are not designed to assess antiviral efficacy.[98, 99] There is hesitation to use checkpoint inhibitors in patients with hepatitis due to the liver toxicity profile of antibodies against PD-1 or PD-L1. Overriding regulatory signals from the liver microenvironment might increase risk of further immune-pathology, because loss of PD-L1 causes severe autoimmune liver damage in mice.[100] Interim reports from a phase 1/2 study indicated that the PD-1 inhibitor nivolumab, given to more than 100 patients with liver cancer related to chronic HBV or HCV infection, did not induce severe hepatic toxicity.[101] Further careful studies of using PD-1/PD-L1 blocking agents as monotherapy, and in combination with therapeutic vaccines, is warranted.

T-cell redirection

Immune reconstitution through transplantation of bone marrow from donors with immunity to HBV into patients with chronic infection can clear HBV from a liver.[102-104] However, allogeneic stem cell transplantation is limited by severe side effects, such as graft vs host disease and a high mortality. Reconstituting immunity using autologous T cells genetically engineered to express HBV-specific receptors might be used to treat chronic HBV infection, prevent HBV-related complications, or for treatment of HBV-related HCC (Fig 3).[105, 106]

T cells can be engineered to express chimeric antigen receptors (CARs) against HBV antigens on the surface of hepatocytes [14] or T-cell receptors cloned from HBV-reactive cells.[107, 108] T cells that express CARs eliminated HBV-infected cells from autologous primary hepatocyte

cultures [14] and were safe in mice.[109] TCR-transduced T cells have even been administered to patients in pilot studies and have, thus far, proven to be safe.[110] Cytolytic and non-cytolytic antiviral effects of T cells that express CARs or engineered TCRs were observed in mice with humanized livers,[36, 111] so this approach might be developed for treatment of patients with HBV-related HCC or to cure chronic HBV infection.

Challenges to Immunotherapy

We now understand many of the mechanisms that suppress the anti-HBV immune response and are using this knowledge to develop immune-based therapies. Immunotherapy strategies aim to restore the magnitude and function of HBV-specific T cells, stimulate the innate immune response to produce antiviral and inflammatory cytokines, and/or circumvent T-cell dysfunction using T cells with engineered TCRs or CARs. For the design and implementation of a successful immunotherapy, knowledge gaps remain. The largest single gap being that immunotherapy has not consistently cured a significant number of CHB patients in which to study a successful immune response.

It can be argued that IFN alpha is an effective immunotherapy, since it can cure HBV infection in 15%–20% of patients over time,[5, 8] but there is no evidence that directly links an HBV-specific immune response to IFN's antiviral effect. IFN alpha might activate CD8+ T cells and NK cells early after treatment initiation, but that does not correspond with rapid decreases in HBV DNA or HBsAg. [112, 113] There is no apparent effect on HBV-specific T cells and flares associated with IFN alpha therapy are unpredictable, sometimes happening a significant amount of time after the 48-week treatment period.[114]

All immunotherapies face a clinical community that is wary of immune-pathologies, given the safety of NUCs. The current generation of immunotherapy drugs were developed with a better

fundamental understanding of immune dysfunction during chronic viral infections and were supported by solid preclinical data. If the new immunotherapies are found to cure higher proportions of patients, data and mechanistic immunological knowledge that regulates viral control and toxicity will be important refine the immunological targets that provide the greatest efficacy. Studies involving these drugs are mainly in phase 1 and 2 trials, so there are few data available on immune responses in the participants. However, based on data from preclinical studies, it's unlikely that any of these drugs will be a magic bullet so studying patients that do and do not respond to these agents is important.

Pattern recognition receptor agonists reduce levels of viral DNA and antigen, and were able to control HBV in animal models, [69, 78-80, 88] but have so far shown limited clinical efficacy. In addition, their lack of specificity, and the broad range of cytokines produced, fuel clinical concerns about toxicity. Knowing the distribution of pattern recognition receptors in the human liver as well as the cytokine or gene expression profile that correlates with virus control would help guide development of second-generation pattern recognition receptors agonists. Most agonists of pattern recognition receptors are given orally, so interactions between the intestine, liver, and microbiome might affect their efficacy. The microbiome affects responses to infectious diseases and inhibitors of PD-1 in patients with cancer.[115-119] This complexity, however, means that the microbiome, liver, and blood will need to be studied in treated patients, to identify variables that regulate the antiviral response.

Agents designed to activate an adaptive immune response provide greater specificity, but also carry limitations. Therapeutic vaccines have showed limited clinical efficacy.[95] Even with improvements in vaccine delivery, adjuvants, and immunogenicity, it is not clear whether boosting the T-cell response alone is sufficient to clear HBV infection.[120] This may be a consequence of antigen mismatch between the vaccine and the genotype of HBV in the infected patients, failure to restore T-cell polyfunctionality, an insufficient magnitude of activation for virus control, or rapid

counter-regulation of T-cell activation. These hurdles might be overcome by an optimized vaccine design, combination with checkpoint inhibitors [97] or drugs that reduce levels of HBV antigen.[94, 121] Synthetic RNAs might also be included in therapeutic vaccines.[122] The success of checkpoint blockade may be reduced by co-expression of inhibitory receptors such as CTLA-4 and TIM-3. Adoptive transfer of redirected T cells may be able to control or even eliminate HBV infection ([111] and Wisskirchen, Kah, Dandri and Protzer, unpublished), but require handling of patient cells ex vivo, under good manufacturing practice conditions, which are rarely available. Each strategy has potential to cure HBV but better methods are needed to measure the ex vivo frequency and functions of HBV-specific B and T cells to understand their success or failure.

The immune system is not like the HBV reverse transcriptase enzyme or capsid, which can be targeted directly to disrupt the HBV life cycle. Patients' immune responses vary based on genetics, age, sex, environment, and microbiome. Multiple immune cell populations simultaneously respond to and integrate signals to induce responses, and are compartmentalized among liver, and blood. This complexity has made it difficult to identify biomarkers of HBV-control in patients. However, there are promising results from preclinical studies, and good safety profiles from early-stage clinical studies. With good safety data, it is tempting to give these agents to patients and analyze effects on HBV response before undertaking immunological studies. However, information on whether the immune system responds to these agents as predicted and on the magnitude of the response is critical to provide insight into either a positive or negative effect on HBV replication to improve immune-based drugs.

Future Directions

Chronic HBV infection is characterized by quantitative and functional defects of the virus-specific responses of T and B cells. Virus-specific T cells are virtually undetectable in blood samples from many patients with chronic HBV infection and are exposed to a network of inhibitory mechanisms

that suppress an HBV-specific immune response. Although antibody responses are used as markers of successful immune control, B-cell responses to the virus are poorly understood. Generation or amplification of HBV-specific B and T cells should be a goal of immunotherapy for HBV infection and it is important that immunological studies are incorporated into clinical trials to identify biomarkers associated with HBV cure.

Figure Legends

Figure 1. Mechanisms of Immune Suppression in Liver. The liver has many signaling pathways, activated by environmental and contact-dependent factors, that regulate the immune response. TGFB, IL10, and arginase are produced by Kupffer cells, hepatic stellate cells (HSC), T-regulatory (Treg) cells, and myeloid-derived suppressor cells. HBV-specific CD8+ T cells can be deleted in a contact-dependent manner by NK cells, or their functions can be suppressed by inhibitory ligands, such as PD-L1, expressed by liver endothelial sinusoidal cells (LSECs), dendritic cells, Kupffer cells, and HSC. Due to the complexity of the mechanisms that suppress the T-cell–mediated immune response against HBV, agents that target a single molecule or pathway are not likely to be effective.

Figure 2. Agonists of pattern recognition receptors.
Hepatocytes express only the pattern recognition receptors TLR2, TLR3, TLR4, and RIG-I, whereas non-parenchymal cells such as monocytes, macrophages, and dendritic cells express the full range of pattern recognition receptors. TLRs localized to endosomes signal through MYD88 (TLR7, TLR8, TLR9) or TRIF (TLR3), resulting in activation the transcription factors NF-kB, IRF3, and/or IRF7. TLR-induced activation of NF-kB leads to expression of inflammatory cytokines, whereas activation of IRF3 and IRF7 lead to expression type 1 and type 3 IFNs. RIGI and STING remain in the cytosol and activate NF-kB, IRF3, and IRF7. Signaling pathways activated by different pattern recognition receptors overlap. Therefore, differences observed in cytokine profiles and therapeutic efficacy will likely result from differences in pattern recognition receptors by parenchymal vs non-parenchymal cells. The distribution of pattern recognition receptors has not been completely defined in human liver but is likely to affect immune responses to HBV-infected hepatocytes and cytokine production by other cells. It is important to increase our understanding of receptor distribution and the origins of cytokines with antiviral

activity and the potential to minimize inflammatory signals if we are to develop therapeutics that induce innate immune responses against HBV.

Figure 3. Strategies to Engineer T Cells to Respond to HBV. Preclinical and pilot studies are underway to test effects of T cells with engineered HBV-specific TCRs or chimeric antigen receptors (CARs). CARs combine an HBV antigen-specific antibody domain and T-cell signaling components, allowing T cells to recognize HBV and become activated without the requirement for HLA-restriction. These cells might be used to treat large populations of HBV-infected individuals, but are also susceptible to neutralization or exhaustion, due to the large amounts of circulating antigen. However, T cells with CARs might be combined with agents that reduce production of HBV antigens. It is also possible to engineer T cells to express TCRs with specificity for HBV, based on TCR genes isolated from patients who have resolved HBV infection. TCRs recognize peptides generated from HBV antigens that are presented on MHC-I or MHC-II. An advantage to this approach is that these TCRs would therefore not interact with HBV antigens in the circulation but can recognize all HBV antigens produced by infected hepatocytes or tumor cells. A disadvantage is that the TCRs are HLA-restricted, but this obstacle can be largely overcome with a relatively small library of TCRs that cover the most frequent HLA alleles in the population.

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