In Situ Liver Expression of HBsAg/CD3-Bispecific Antibodies for HBV Immunotherapy

Robert L. Kruse,1,2,3,4 Thomas Shum,1,3,4 Xavier Legras,1,2 Mercedes Barzi,1,2 Frank P. Pankowicz,1,2 Stephen Gottschalk,1,3,5,6,7 and Karl-Dimiter Bissig1,2,3,8,9

1Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX 77030, USA; 2Center for Stem Cells and Regenerative Medicine, Baylor College of Medicine, Houston, TX 77030, USA; 3Translational Biology and Molecular Medicine Program, Baylor College of Medicine, Houston, TX 77030, USA; 4Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA; 5Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030, USA; 6Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA; 7Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA; 8Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA; 9Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA

Current therapies against hepatitis B virus (HBV) do not reliably cure chronic infection, necessitating new therapeutic approaches. The T cell response can clear HBV during acute infection, and the adoptive transfer of antiviral T cells during bone marrow transplantation can cure patients of chronic HBV infection. To redirect T cells to HBV-infected hepatocytes, we delivered plasmids encoding bispecific antibodies directed against the viral surface antigen (HBsAg) and CD3, expressed on almost all T cells, directly into the liver using hydrodynamic tail vein injection. We found a significant reduction in HBV-driven reporter gene expression (184-fold) in a mouse model of acute infection, which was 30-fold lower than an antibody only recognizing HBsAg. While bispecific antibodies triggered, in part, antigen-independent T cell activation, antibody production within hepatocytes was non-cytotoxic. We next tested the bispecific antibodies in a different HBV mouse model, which closely mimics the transcriptional template for HBV, covalently closed circular DNA (cccDNA). We found that the antiviral effect was noncytopathic, mediating a 495-fold reduction in HBsAg levels at day 4. At day 33, bispecific antibody-treated mice exhibited 35-fold higher host antibodies only recognizing HBsAg.10 Redirected T cells were shown to reduce cccDNA from infected primary hepatocytes in vitro10 and mediated transient viral reduction in an HBV transgenic mouse model.11 While CAR-T cells represent a potential therapy against HBV, T cell products currently have to be produced for each patient individually, limiting their potential utility as a readily available therapeutic. To develop an “off-the-shelf product” to redirect T cells to HBsAg-positive hepatocytes, we investigated the use of bispecific antibodies that recognize HBsAg and CD3, which is expressed on almost all T cells.

INTRODUCTION

Hepatitis B virus (HBV) is a partially double-stranded DNA virus with tropism to the liver, infecting over 300 million people chronically worldwide and causing cirrhosis and liver cancer in a significant number of these patients.1 Once chronically infected, very few HBV patients develop antibodies (Abs) against and clear hepatitis B surface antigen (HBsAg), which serves as a clinical biomarker for functional cure.2 There is no effective treatment for chronic HBV patients; a 5-year treatment course with entecavir, a reverse transcriptase inhibitor, results in HBsAg seroconversion in only 1.4% of patients.3 These antiviral inhibitors suppress serum HBV DNA levels but have no effect on covalently closed circular DNA (cccDNA), the episomal transcriptional template of HBV. This molecule is very stable once formed in the hepatocyte, and cccDNA has been shown to persist for years.4 Pegylated interferon (IFN)-α is also approved for HBV therapy but has shown efficacy only in a minority of patients and is not well tolerated.5

In patients who clear HBV during the acute infection, the CD8-positive T cell response is crucial.6 This immune response is, in part, noncytopathic, relying primarily on secreted cytokines, IFN-γ and tumor necrosis factor (TNF)-α, to mediate cccDNA degradation.7 However, the frequency of HBV-specific T cells is low in chronically infected HBV patients,8 and their functionality is impaired.9 Given the paucity of antiviral T cells in the host, T cells have been redirected to attack HBV-infected hepatocytes using chimeric antigen receptors (CARs) specific for HBsAg.10 Redirected T cells were shown to reduce cccDNA from infected primary hepatocytes in vitro10 and mediated transient viral reduction in an HBV transgenic mouse model.11 While CAR-T cells represent a potential therapy against HBV, T cell products currently have to be produced for each patient individually, limiting their potential utility as a readily available therapeutic. To develop an “off-the-shelf product” to redirect T cells to HBsAg-positive hepatocytes, we investigated the use of bispecific antibodies that recognize HBsAg and CD3, which is expressed on almost all T cells.
Bispecific antibodies targeting CD3 to direct T cells to cell surface antigens were originally reported over 30 years ago and have shown promising antitumor activity in numerous preclinical models. However, only blinatumomab, a bispecific Ab that targets CD3 and CD19, expressed on B cell malignancies, has received Food and Drug Administration (FDA) approval so far.14 Current bispecific Ab approaches were originally reported over 30 years ago12,13 and have shown promising antitumor activity in numerous preclinical models. How- ever, only blinatumomab, a bispecific Ab targeting CD3 and CD19, expressed on B cell malignancies, has received FDA approval so far.14 Current bispecific Ab approaches are challenged by a complicated manufacturing pro- cess, short half-lives requiring continuous infusions, and side effects secondary to systemic T cell activation.15 These hurdles could be overcome through in situ expression of bispecific Abs from DNA or RNA templates in patient tissues, but there have been few reports on such strategies.16–18 The liver absorbs major fractions of gene therapy vectors, nanoparticles, or liposomes, allowing gene constructs to be delivered more readily than in any other organ. Expression of bispecific Abs in the liver should result in increased Ab concentrations in the liver, as judged by bioluminescence imaging, in a process resembling acute HBV infection.28 To evaluate if in vivo expression of HBs-Fc has antiviral activity, we adapted a murine model that allows for measuring the clearance of HBV using non-invasive bioluminescence imaging, which correlated with serum HBsAg and HBV DNA levels.25 Bioluminescence of a reporter gene has previously been shown to be a sensitive readout for CD8 T cell responses in the liver against a co-delivered antigen gene.26,27 Briefly, we generated a plasmid encoding the HBV genome and a GFP-2A-firefly luciferase (GFP-2A-ffLuc) expression cassette, both under the transcriptional control of identical HBV core promoters (pHBV-ffLuc; Figure S1A). HTV injection of pHBV-ffLuc into NOD scid gamma (NSG) mice resulted in luciferase expression in the liver as judged by bioluminescence imaging, confirming the functionality of pHBV-ffLuc (Figures S1B and S1C). The introduction of HBV plasmid DNA by HTV injection into immunocompetent mice results in immune clearance over 2 weeks, in a process resembling acute HBV infection.28 To evaluate if expression of HBs-Fc induces clearance of HBV, pHBV-ffLuc was co-injected with pCAG.HBs-Fc, a pCAG plasmid encoding an Ab specific for an irrelevant antigen29 (EGFRvIII; pCAG.EvIII-Fc), or a control plasmid. Keeping the total amount of DNA injected consistent, co-injection of 5 μg pHBV-ffLuc with 15 μg pCAG.HBs-Fc resulted in a significantly lower luciferase signal in comparison to co-injection with 15 μg pCAG.EvIII-Fc or control plasmid (Figure 2A). In addition, pHBV-ffLuc/pCAG.HBs-Fc co-injected mice had significantly lower levels of serum HBsAg (Figure 2B) compared to the other treatment groups, indicating that HBs-Fc has antiviral activity in vivo.

Herein, we have developed an approach to express in situ a bispecific Ab to redirect T cells to HBsAg. Our results in transfection-
To evaluate the contribution of Fc receptor-mediated phagocytosis or cell killing to the observed antiviral activity of our bispecific Abs, we replaced the wild-type human IgG1 Fc domain in HBs-Fc-CD3 and EvIII-Fc-CD3 with a mutated human IgG4 Fc (mFc) domain that does not bind to Fc receptors (HBs-mFc-CD3; EvIII-mFc-CD3) (Figure 4A). We compared the antiviral activity of pCAG.HBs-mFc-CD3 to pCAG.EvIII-mFc-CD3 in our model. Both bispecific Abs had antiviral activity as judged by bioluminescence imaging (Figure 4B). HBs-mFc-CD3 had significantly greater antiviral activity at day 4 (10-fold) than EvIII-mFc-CD3 (Figure 4B), while as shown previously using the non-mutated Fc (Figure 3B), the HBs-Fc-CD3 had only 5-fold greater activity than the corresponding EvIII-Fc-CD3 at the same time point. We confirmed this observation with additional replicates (Figure S3) and thus selected HBs-mFc-CD3 and EvIII-mFc-CD3 as a control for our subsequent experiments.

Bispecific Antibodies Act Early after Injection and in a CD3-Dependent Manner in the HBV Model

We sought to explore the kinetics of HBs-mFc-CD3 action over the course of acute clearance. We co-injected 5 μg pHBV-flLuc and 15 μg pCAG.HBs-mFc-CD3 or control, measuring bioluminescence each day for the first 4 days. We found that a 13-fold difference between control and HBs-mFc-CD3 treatment had already occurred at day 1 post-injection (Figure S4A), with a similar difference maintained subsequently. This matches the published kinetics of gene expression after HTV injection, which peaks at 8 hr post-injection, and suggests that ongoing bispecific Ab production and/or antiviral T cell activation after day 1 is minimal.

We also investigated the requirements for T cell signaling by substituting a different moiety for T cell stimulation, replacing the CD3 targeting portion. For this purpose, we utilized the extracellular domain of the mouse CD80 protein, which can interact with CD28 expressed on T cells. We cloned this portion at the N terminus of HBs-Fc or EvIII-Fc, respectively (pCAG.HBs-Fc-CD3; pCAG.EvIII-Fc-CD3; Figure 3A).

Including an Anti-CD3 Domain in HBs-Fc Enhances the Antiviral Activity In Vivo

Having established that pCAG.HBs-Fc has anti-HBV activity in vivo, we next determined if inclusion of an scFv specific for murine CD3, which activates T cells, further enhances its antiviral activity. We generated pCAG expression plasmids encoding HBs-Fc-CD3 or EvIII-Fc-CD3 bispecific Abs by inserting the murine CD3-binding scFv from monoclonal antibody (mAb) 145-2C1110,31 at the C terminus of HBs-Fc or EvIII-Fc, respectively (pCAG.HBs-Fc-CD3; pCAG.EvIII-Fc-CD3; Figure 3A).

Keeping the total amount of DNA injected consistent, 5 μg pHBV-flLuc was injected by HTV injection in combination with 15 μg control plasmid or plasmids encoding the respective Ab. In this experiment, we compared pCAG.HBs-Fc, pCAG.HBs-Fc-CD3, EvIII-Fc, and pCAG.EvIII-Fc-CD3. Inclusion of a CD3-specific scFv enhanced the antiviral activity of pCAG.HBs-Fc 30-fold (p < 0.05) as judged by bioluminescence imaging (Figure 3B). Representative bioluminescence images of mice at day 4 post-injection are shown, and there was a significant difference between pCAG.EvIII-Fc and pCAG.EvIII-Fc-CD3 at this time point (p < 0.05), indicating that bispecific Abs induce unspecific T cell activation (Figure 3C). Unspecific T cell activation was confirmed with a pCAG plasmid encoding an Ab specific for the irrelevant antigen EphA2 and CD3 (pCAG.EphA2-Fc-CD3; Figure S2).32

In Vivo Expression of HBs-mFc-CD3 Does Not Result in Hepatocyte Toxicity

Having demonstrated that unspecific T cell activation contributes to the antiviral activity of HBs-mFc-CD3, we next explored unspecific hepatotoxicity triggered by HBs-mFc-CD3 expression. We first evaluated liver transaminase elevation that might result from bispecific Ab expression, knowing that the HTV procedure itself results in transient hepatocyte injury and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increases.34 No difference between the conditions was observed at day 4, with AST levels still mildly elevated at this time point post-injection (Figure S5A). Since it is difficult to separate bulk damage from the HTV procedure versus direct transgene toxicity by examining transaminases alone, we sought to more precisely study toxicity in individual transgene-modified...
hepatocytes. Toward this goal, we developed a new hepatotoxicity assay based on bioluminescence imaging to evaluate whether transfected hepatocytes persisted. Transgenic ROSA26-LoxP-STOP-LoxP-ffLuc (Rosa-Luc) mice were injected by HTV injection with pCMV-NLS-Cre and pCAG.HBs-mFc-CD3 or control plasmids. The injection results in co-delivery of plasmids to the same hepatocytes; expressed Cre recombinase in transfected cells of Rosa-Luc mice induces ffLuc expression, and the resulting bioluminescence signal correlates with the number of viable, transfected hepatocytes in vivo (details in the Materials and Methods).

HBs-mFc-CD3 expression did not reduce the bioluminescence signal on day 4 or 8 post-injection versus control mice (Figure S5B), indicating that HBs-mFc-CD3 is non-toxic in hepatocytes. These findings were confirmed using standard histological examination (H&E staining) of liver sections from mice co-injected with pHBV-ffLuc and control or pCAG.HBs-mFc-CD3 on day 4 post-injection (Figure S5C). Likewise, no histomorphological changes indicating toxicity were noted when transfecting previously used Ab constructs (Figure S6). These histological stains also did not demonstrate a notable increase in lymphocyte levels among the different test constructs at day 4, indicating that additional T cells were not recruiting into the liver, but rather tissue-resident T cells in the liver were likely activated (Figure S6).

In Vivo Expression of HBs-mFc-CD3 Has Antiviral Activity in a Recombinant cccDNA HBV Mouse Model

Finally, we wanted to explore the ability of HBs-mFc-CD3 to induce cccDNA clearance in vivo, which more closely mimics HBV transcriptional templates in human cells than plasmids carrying the HBV genome. We adapted previously reported HBV murine models that utilize recombinases to generate a recombinant cccDNA-like (rcccDNA) molecule lacking bacterial DNA.35,36 In our system, we constructed a floxed HBV genome with an NLS-Cre recombinase cassette driven by a cytomegalovirus (CMV) promoter in cis on the same plasmid (pCLX; Figure S7A; R.L.K, M.B., X.L., F.P.P., B. Bissig-Choisat, C. Whitten-Bauer, B.L. Slaagle, U. Garaigorta, K.D.B., unpublished data). In the floxed, unexcised state without Cre recombinase, there is no detection of HBV antigens, since viral transcripts and/or proteins are interrupted by the LoxP sequences preventing expression (Figures S7B and S7C). After HTV injection of pCLX, Cre expression and resultant rcccDNA formation yields a high level of HBsAg production and HBV core expression 1 week post-injection, demonstrating the functionality of our model (Figures S7B and S7C). When pCLX is introduced into Rosa-Luc mice, the Cre recombinase also induces ffLuc expression. Thus, bioluminescence imaging serves as a non-invasive means to monitor viable HBV transfected cells and, as in our previous Rosa-Luc experiments, can be used to monitor hepatotoxicity of our bispecific Ab therapy.

To determine the antiviral activity and safety of HBs-mFc-CD3 in our rcccDNA model, we co-injected pCLX with pCAG.HBs-mFc-CD3, pCAG.EvIII-mFc-CD3, or control plasmid. On day 4 post-injection,
we measured 495-fold and 30-fold lower HBsAg levels in pCAG.HBs-mFc-CD3-injected mice compared to mice receiving control plasmid and pCAG.EvIII-mFc-CD3, respectively (Figure 5A). pCAG.HBs-mFc-CD3 therapy not only reduced HBsAg levels, but it also induced HBsAg Abs earlier and at higher levels than pCAG.EvIII-mFc-CD3 and controls (Figure 5B). The antiviral activity of pCAG.HBs-mFc-CD3 therapy was also confirmed by immunofluorescence in a subset of animals on day 4 post-injection, showing less HBV core expression within individual hepatocytes compared to the pCAG.EvIII-mFc-CD3 and control groups (Figure 5C). We used bioluminescence imaging to determine the safety of pCAG.HBs-mFc-CD3 therapy. On day 4 post-injection, there was a 3.3-fold reduction in the bioluminescence signal in pCAG.HBs-mFc-CD3-injected mice compared to mice receiving control plasmid (Figure 5D), and long-term follow-up revealed a further decline in the bioluminescence signal of pCAG.HBs-mFc-CD3-injected mice. At days 4, 20, and 26 post-injection, there were significant differences between pCAG.HBs-mFc-CD3- and pCAG.EvIII-mFc-CD3-injected mice (p < 0.05, Figure 5E).

**DISCUSSION**

Here we developed a novel therapeutic strategy using bispecific Abs for HBV immunotherapy. We describe the production of bispecific Abs in the liver leading to local T cell activation, modulating the immune response in the organ. This differs from recombinant protein strategies, which do not specifically accumulate at tissue sites unless additional targeting moieties are included, or from using cell-based carriers for delivery.25 Our strategy utilizes the recruitment of naive T cells against HBV, as opposed to relying on exhausted, dysfunctional HBV-specific T cells26 to mediate antiviral effects.

We demonstrated that Abs can be expressed in hepatocytes and retain their functionality. As expected, the HBs-Fc Ab was specific and effectively mediated an antiviral effect, similar to the parent monoclonal Ab.19,22 While the addition of CD3-specific scFv led to an even more potent antiviral response. This antiviral effect peaked at day 1 post-injection, and we were unable to detect significant levels of bispecific antibodies in the serum at day 4, likely due to activation and/or engagement with mouse T cells preventing high-level accumulation. Delivering co-stimulation through the CD80 ectodomain instead of CD3-specific scFv did not elicit high-level antiviral responses. Interestingly, the bispecific Ab targeting CD3 and an unrelated antigen had significant anti-HBV activity in vivo, indicating that T cell activation occurred independent of HBsAg binding. Fc receptor cross-linking did not play a role in enhancing antiviral effects or in facilitating T cell activation,37 since the construct with mutated Fc receptor binding sites, HBs-Fc-CD3, had similar anti-HBV activity to HBs-Fc-CD3. Antigen-independent secretion of IFN-γ has been observed previously in vitro with the bispecific Ab format used in this study.38 In addition, antigen-independent T cell activation by bispecific Abs secreted from cell lines has also been reported.39 While the exact mechanism of antigen-independent T cell activation by bispecific Abs is not completely understood, self-aggregation, resulting in cross-linking of CD3, is the most likely explanation.

In this study, we utilized a novel procedure for dual monitoring of hepatocyte viability and HBV markers, adapting recombination methods to generate cccDNA-like molecules similar to previous reports,35,36 while also leveraging recombination of host cell reporter genes. Our study was able to replicate other reports that initial T cell effects against HBV in the liver are noncytopathic, since the HBsAg levels were reduced to a much larger extent in the bispecific Ab-treated groups compared to bioluminescent radiance, reflecting hepatocyte viability. Beyond activating T cells, HBs-mFc-CD3 may have also reduced serum HBsAg levels in the cccDNA model via the ability of Abs to block HBsAg secretion from hepatocytes through their engagement with the neonatal Fc receptor.40 A later decrease in radiance in groups treated with bispecific Abs was observed, similar to the pattern of final clearance of infected cells in chimpanzees.6 In conjunction with the higher levels of HBsAg IgG Ab production, this indicates that bispecific Abs increased the host adaptive immune response versus the control group.

Our study differs from previous gene therapy reports expressing an individual cytokine, IFN-γ, in the liver to yield antiviral activity.21,42
When tested in a surrogate woodchuck model, adenoviral delivery of IFN-γ and TNF-α genes to the liver did not result in an immediate reduction in the woodchuck hepatitis virus (WHV), but rather the later adaptive response against adenovirus by T cells decreased WHV viral loads, suggesting that expressing individual cytokines alone may not be sufficient.43 Thus, activating T cells through CD3, which results in the production of not only IFN-γ but also other proinflammatory cytokines such as TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF), 44 might be more effective. Our targeted approach to HBV is also distinct from the infusion of a recombinant T cell receptor (TCR)-like Ab to deliver IFN-α to HBV-infected hepatocytes47,48 and from gene therapy with a Apo-A1/IFN-α fusion protein.49 In addition, our strategy targeting HBsAg and CD3 format differs from previous protein-based bispecific Ab strategies targeting two different epitopes on HBV, rather than a chronic HBV mouse model, which can be established in immunocompetent mice with AAV or low-dose adenoviral delivery of HBV genomes. Future work will seek to treat in chronic HBV models with translatable delivery systems. In these studies, it will be important to evaluate the kinetics and expression of bispecific antibodies from these other vector systems. We also plan to evaluate the exact cytokine profile induced by bispecific antibodies in the liver, as well as the effects on intrahepatic viral markers.

In conclusion, we developed a novel therapeutic approach to target HBV infection by expressing bispecific Abs in hepatocytes, leading to a local and predominantly noncytopathic immune response against HBV. Our approach may be of value for treating not only HBV but also other viral liver diseases.

The current study is limited in relying on HTV injection, which results in efficient co-delivery of plasmids to the same cells but cannot be applied to patients. To overcome this limitation, we are planning to explore clinical translatable liver gene delivery systems such as adeno-associated virus (AAV) or mRNA nanoparticles. In a recent study, mRNA nanoparticles effectively targeted the liver in mice and systemically secreted bispecific antibodies to engage Claudin-6 and CD3, triggering T cell cytotoxicity against subcutaneously injected tumors. This strategy could be applied to our approach to treat in situ liver disease. Another limitation is that HTV injection results in an acute model of HBV infection, rather than chronic HBV, which can be established in immunocompetent mice with AAV or low-dose adenoviral delivery of HBV genomes. Future work will seek to treat in chronic HBV models with translatable delivery systems. In these studies, it will be important to evaluate the kinetics and expression of bispecific antibodies from these other vector systems. We also plan to evaluate the exact cytokine profile induced by bispecific antibodies in the liver, as well as the effects on intrahepatic viral markers.

HBsAg, 47,48 as well as a strategy targeting HBx and CD3 for hepatocellular carcinoma.49

www.moleculartherapy.org

Figure 5. In Vivo Expression of HBs-mFc-CD3 in a Recombinant cccDNA Model of HBV Has Antiviral Effects and Induces Endogenous HBsAg Antibodies

Rosa-Luc mice were co-injected by HTV injection with 5 μg pCLX and 15 μg pCAG.HBs-mFc-CD3, pCAG.EvIII-mFc-CD3, or control plasmid (n = 4). (A) HBsAg serum levels were determined by ELISA at day 4 post-injection (mean ± SEM, n = 4; **p < 0.005, ***p < 0.0001). (B) The development of host HBsAg IgG Abs was determined by ELISA at the indicated time points (mean ± SEM, n = 4; *p < 0.05). (C) Tissue was harvested from mice at day 4 post-injection, and HBV core protein (red) expression was assessed by immunofluorescence (blue = DAPI; scale bars represent 50 μm). (D and E) Quantitative bioluminescence imaging data (radiance = photons/sec/cm2/sr) for all mice is shown on (D) day 4 post-injection and (E) during long-term follow-up (mean ± SEM, *p < 0.05, **p < 0.01). n.s., not significant.
MATERIALS AND METHODS

Plasmid Constructs

Generation of Ab Constructs

A codon-optimized minigene was synthesized by Integrated DNA Technologies (IDTDNA; Coralville, IA) containing the immunoglobulin heavy-chain leader peptide (MDWIWRILFLVGAATGAHS), the HBs-specific mAb 19.79.535 heavy chain, a glycine (G) serine (S) linker ([G4S]3), and the 19.79.5 light chain flanked by 5’ Xhol and 3’ BamHI sites. The human IgG1 Fc domain was PCR amplified from a plasmid encoding a CAR containing an IgG1 Fc hinge with PCR primers possessing 5’ BamHI and 3’ NotI. The minigene and PCR product was cloned into pCAG by three-way ligation to create pCAG-EvIII-Fc. Cloning was confirmed by sequencing (Lone Star Labs, Houston, TX).

Generation of Bispecific Ab Constructs

The 145-2C1135 scFv specific for murine CD3 was PCR amplified from pRV2011.145-2C11-1D3-Thy1.1 with 5’ EcoRV and 3’ NotI sites. Four-way ligation was performed with 5’ Xhol leader-HBs or EvIII scFv-3’ BamHI, 5’ BamHI-Fc-3’ EcoRV, 5’ EcoRV-145-2C11 scFv-3’ NotI, and pCAG digested with Xhol and NotI to generate pCAG.HBs-Fc-CD3 and pCAG.EvIII-Fc-CD3. pCAG.HBs-mFc-CD3 and pCAG.EvIII-mFc-CD3 were generated in a similar fashion except a codon-optimized minigene (IDTDNA, Coralville, IA) encoding the human IgG4 Fc with mutated Fc binding sites33 and flanking 5’ BamHI and 3’ EcoRV sites was used instead of 5’ BamHI-Fc-3’ EcoRV. pCAG.CD80-mFc-HBs was generated by synthesizing the extracellular domain including the leader sequence of murine CD80 (B7.1) protein between 5’ Xhol and 3’ BamHI sites. Separately, PCR amplification of HBs scFv added 5’ EcoRV and 3’ NotI sites. The four-way ligation reaction with 5’-Xhol-CD80-3’ BamHI, 5’ BamHI-mFc-3’ EcoRV, and 5’ EcoRV-HBs-3’ NotI and pCAG digested with Xhol and NotI was performed to generate pCAG.CD80-mFc-HBs. Cloning was confirmed by sequencing (Lone Star Labs, Houston, TX).

Generation of pCAG

The pCAG vector was constructed from the pCIG vector (containing the hybrid promoter CMV enhancer chicken beta actin [CAG] promoter, rabbit beta globin 3’ UTR, polyadenylation sequence, internal ribosome entry site [IRES]-NLS-GFP, and SV40 origin of replication) by removing IRES-NLS-GFP, leaving Xhol and NotI sites for inserting transgenes (Figure 1A).

Generation of pHBV-ffLuc

pHBV-ffLuc was generated by inserting a GFP-2A-ffLuc expression cassette by PCR cloning (provided by Dr. Inder Verma, Salk Institute, San Diego, CA) into the Smal and Sacl sites of pSP65awy1.3 (provided by Dr. Stefan Wieland, University of Basel, Switzerland). pSP65awy1.3 encodes an over-length HBV genome from genotype D, subtype ayw, GenBank V01460. The Smal site is located at the 3’ end of the over-length HBV genome and the PCR primers for GFP-2A-ffLuc subcloning were designed so that GFP-2A-ffLuc translation is in frame with core protein translation at the 3’ end of the HBV genome (see Figure S8 for more sequence information). Cloning was confirmed by sequencing (Lone Star Labs, Houston, TX).

Bioluminescence Imaging

The IVIS system (Xenogen Corp., Alameda, CA) was used for bioluminescence imaging. Mice were anesthetized with isoflurane and injected intraperitoneally with 200 µL of 7.5 mg/mL luciferin solution (GoldBio, Olivette, MO). Luciferin was allowed to circulate for 10 min post-injection, and mice were placed ventral side up and imaged promptly thereafter. Luminescence signals were quantified using Living Image 4.2 software (Caliper Life Sciences, Hopkinton, MA) with a region of interest (ROI) circling the area over the liver.

HBsAg ELISA

HBsAg levels were determined as previously described.56 Briefly, serum HBsAg levels were evaluated with commercially sold ELISA kits according to the manufacturer’s instructions (International Immuno Diagnostics, Foster City, CA). Quantification of serum HBsAg was made by comparing serial dilutions of known standards (Alpha Diagnostic International, San Antonio, TX). HBsAg levels were reported in nanograms per milliliter, consistent with the standards utilized. The conversion ratio to international units per milliliter is not provided by the manufacturer, but many kits have conversions of 1 or 10 ng/mL to 1 IU/mL. HBsAg as approximate guidelines.57

HBsAg IgG Antibody ELISA

Serum HBsAg IgG Ab levels were quantified by ELISA according to the manufacturer’s instructions (Alpha Diagnostic International, San Antonio, TX). HBsAg IgG Ab levels were reported as milli-international units per milliliter. The ELISA assay can detect both human and mouse immunoglobulin.

Transaminase Analysis

Serum ALT and AST were measured by the Comparative Pathology Laboratory (Baylor College of Medicine, Houston, TX) using the COBAS INTEGRA 400 plus analyzer (Roche Diagnostics, Indianapolis, IN).

Animal Experiments

All animal experiments followed a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. For all experiments using immunocompetent mice, the Rosa-Luc strain from Jackson Labs [FVB.129S6(B6)-Gt(ROSA)26Sortm1(Jls)] was utilized, in which the expression of the firefly luciferase (Luc) gene is blocked by a loxP-flanked STOP fragment placed between the Luc sequence and the Gt(ROSA)26So promoter. Even though most experiments did not utilize the endogenous luciferase reporter, using Rosa-Luc mice for all experiments reduced the risk of inter-strain variability. Rosa-Luc mice, aged 6–10 weeks, were selected for HTV injection. pCMV-Gaussia luciferase (Thermo
were stained with H&E.  

was washed with PBS 1

core antigen (Dako/Agilent, Santa Clara, CA). The primary Ab

Triton-100) using the primary Ab, rabbit anti-hepatitis B virus

in 4% PFA overnight, and serial sections of paraf

in PBS-T buffer (PBS 1

used for slide mounting. In other experiments, liver tissue was

work in this manuscript.

IgG ELISA quanti

SORTI (THCCC) (CPRIT no. RP150587), and the Diana Helis

grant R01HL134510, the Texas Hepatocellular Carcinoma Con-

National Institute of Allergy and Infectious Diseases (NIAID) grant

Basel) for providing pSP65-HBVayw1.3. K.B. is supported by Na-

help with animal experiments, and Stefan Wieland (University of

We thank Beatrice Bissig-Choisat (Baylor College of Medicine) for

CONFLICTS OF INTEREST

AUTHOR CONTRIBUTIONS

SUPPLEMENTAL INFORMATION

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software

(GraphPad Software, Inc., La Jolla, CA). Data measurements are

presented as the mean ± SEM. Mean differences were tested using

appropriate tests, including unpaired, parametric, one-tailed t tests.

The significance level used was p < 0.05, unless otherwise specified.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found

with this article online at http://dx.doi.org/10.1016/j.omtm.2017.

08.006.

AUTHOR CONTRIBUTIONS

R.L.K., S.G., and K.B. designed the experiments; R.L.K., T.S., and

F.P.P. performed in vivo experiments; M.B. did immunostaining;

X.I. did ELISA. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

R.L.K., T.S., S.G., and K.B. have a patent application filed covering the

work in this manuscript.

ACKNOWLEDGMENTS

We thank Beatrice Bissig-Choisat (Baylor College of Medicine) for

help with animal experiments, and Stefan Wieland (University of

Basel) for providing pSP65-HBVayw1.3. K.B. is supported by Na-

tional Institute of Allergy and Infectious Diseases (NIAID) grant

R01AI094409, National Heart, Lung and Blood Institute (NHBLI)

grant R01HL134510, the Texas Hepatocellular Carcinoma Con-

sortium (THCCS) (CPRIT no. RP150587), and the Diana Helis

Henry and Adrienne Helis Malvin Medical Research Foundations.

R.L.K. and T.S. were supported by NIH grant T32DK060445. T.S.

was supported by NIH grant ST32HL90233. The Dan L. Duncan

Cancer Center is supported by NIH grant P30CA125123. The In-

tegrated Microscopy Core at the Texas Medical Center Digestive

Disease Center is supported by NIH grant P30DK56338.

REFERENCES

1. El-Serag, H.B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma.

Gastroenterology 142, 1264.e1–1273.e1.

2. Liu, J., Yang, H.-L., Lee, M.-H., Lu, S.-N., Jen, C.-L., Wang, L.-Y., You, S.L., Iloeje,

U.H., and Chen, C.J.; REVEAL-HBV Study Group (2010). Incidence and determi-

nants of spontaneous hepatitis B surface antigen seroclearance: a community-based

follow-up study. Gastroenterology 139, 474–482.

3. Chang, T.-T., Lai, C.-L., Kew Yoon, S., Lee, S.S., Coelho, H.S.M., Carrilho, F.J.,

Poordad, F., Halota, W., Horsmans, Y., Tsai, N., et al. (2010). Entecavir treat-

ment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B.

Hepatology 51, 422–430.

4. Werle-Lapostolle, B., Bowden, S., Locarnini, S., Warnthorn, K., Petersen, J., Lau, G.,

Trepo, C., Marcellin, P., Goodman, Z., Delaney, W.E., et al. (2004). Persistence of

cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 126, 1750–1758.

5. Perrillo, R. (2009). Benefits and risks of interferon therapy for hepatitis B. Hepatology

49 (Suppl.), S103–S111.

6. Thimme, R., Wieland, S., Steiger, C., Ghizyeh, J., Reimann, K.A., Purcell, R.H., and

Chisari, F.V. (2003). CD8(+ T cells mediate viral clearance and disease pathogenesis

during acute hepatitis B virus infection. J. Virol. 77, 68–76.

7. Xia, Y., Stadler, D., Lucifora, J., Reisinger, F., Webb, D., Hölzl, M., Michler, T.,

Wisskirchen, K., Cheng, X., Zhang, K., et al. (2016). Interferon-γ and tumor necrosis

factor-α produced by T cells reduce the HBV persistence form, cccDNA, without

cytolysis. Gastroenterology 150, 194–205.

8. Boni, C., Fiscaro, P., Valdatta, C., Arnadei, B., Di Vincenzo, P., Giuberti, T., Laccabue,

D., Zerbini, A., Cavalli, A., Missale, G., et al. (2007). Characterization of hepatitis B virus

(HBV)-specific T-cell dysfunction in chronic HBV infection. J. Virol. 81, 4215–4225.

9. Park, J.-J., Wong, D.K., Wahed, A.S., Lee, W.M., Feld, J.J., Terrault, N., Khalili, M.,

Sterling, R.K., Kowdley, K.V., Bzowej, N., et al.; Hepatitis B Research Network

(2016). Hepatitis B virus-specific and global T-cell dysfunction in chronic hepatitis B.

Gastroenterology 150, 684–695.

10. Bohne, F., Chmielicewski, M., Ebert, G., Wiegmann, K., Kürschner, T., Schulze, A.,

Urban, S., Krönke, M., Abken, H., and Protzer, U. (2008). T cells redirected against

hepatitis B virus surface proteins eliminate infected hepatocytes. Gastroenterology

134, 239–247.

11. Krebs, K., Böttiger, N., Huang, L.R., Chmielicewski, M., Arzberger, S., Gasteiger, G.,

Jüger, C., Schmitt, E., Bohne, F., Aschler, M., et al. (2013). T cells expressing a chimeric

antigen receptor that binds hepatitis B virus envelope proteins control virus replica-

tion in mice. Gastroenterology 145, 456–465.

12. Staerz, U.D., Kanagawa, O., and Tabernero, J. (2015). A phase I trial of intravenous catumaxomab: a bispeci

ty monoclonal antibody targeting EpCAM and the T cell coreceptor CD3. Cancer

Chemother. Pharmacol. 76, 1453–1457.

13. Przepiorka, D., Ko, C.-W., Deisseroth, A., Yancey, C.L., Candau-Chacon, R., Chiu,

H.-J., Gehrke, B.J., Gomez-Broughton, C., Kane, R.C., Kirshner, S., et al. (2015).

FDA approval: blinatumomab. Clin. Cancer Res. 21, 4035–4039.

14. Mau-Sørensen, M., Dittrich, C., Dienstmann, R., Lassen, U., Büchler, W., Martinius,

15. Mau-Sørensen, M., Dittrich, C., Dienstmann, R., Lassen, U., Büchler, W., Martinius,

16. Compte, M., Nuñez-Prado, N., Sanz, L., and Alvarez-Vallina, L. (2013). In vivo secre-

tion of bispecific antibodies recruiting lymphocytic effector cells. Antibodies 2,

415–425.

Molecular Therapy: Methods & Clinical Development Vol. 7 December 2017 39
54. Dion, S., Bourgine, M., Godon, O., Levillayer, F., and Michel, M.-L. (2013). Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J. Virol. 87, 5554–5563.

55. Huang, L.R., Gäbel, Y.A., Graf, S., Arzberger, S., Kurts, C., Heikenwalder, M., Knolle, P.A., and Protzer, U. (2012). Transfer of HBV genomes using low doses of adenovirus vectors leads to persistent infection in immune competent mice. Gastroenterology 142, 1447–1450.

56. Billoud, G., Kruse, R.L., Carrillo, M., Whitten-Bauer, C., Gao, D., Kim, A., Chen, L., McCaleb, M.L., Crosby, I.R., Hamatake, R., et al. (2016). In vivo reduction of hepatitis B virus antigenemia and viremia by antisense oligonucleotides. J. Hepatol. 64, 781–789.

57. Locarnini, S., and Bowden, S. (2012). Hepatitis B surface antigen quantification: not what it seems on the surface. Hepatology 56, 411–414.

58. Kovacsics, D., and Raper, J. (2014). Transient expression of proteins by hydrodynamic gene delivery in mice. J. Vis. Exp. 87, 51481.