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Immunomodulation and RNA interference alter Hepatitis B Virus-specific CD8 T cell recognition of infected HepG2-NTCP

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List of abbreviations: Hepatitis B virus; HBV, RNA interference; RNAi, Tenofovir disoproxil fumarate; TDF, Toll-like Receptor; TLR, Conditioned Media; CM, short interfering RNA; siRNA, HBV Core antigen; HBcAg, HBV Surface antigen; HBsAg, Chronic Hepatitis B; CHB, Nucleos(t)ide analogues; NUCs, covalently closed circular DNA; cccDNA, Healthy Donor; HD, Peripheral Blood Mononuclear Cells; PBMCs, Standard Deviation; SD, Multiplicity Of Infection; MOI, T cell receptor; TCR, interferon alpha receptor 2; IFNAR2, Genome Equivalent; GE

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Abstract:

Background & Aims: CD8 T cells are essential in controlling Hepatitis B virus (HBV) infection. Viral control is dependent on efficient recognition of HBV-infected hepatocytes by CD8 T cells, which can induce direct lysis of infected hepatocytes. In addition, CD8 T cells produce IFN-γ, which mediates

non-cytopathic viral clearance. Innate immunomodulators and HBV-targeted RNA interference (RNAi) are being developed to treat chronic hepatitis B, but may modify HBV antigen presentation and impact CD8 T cell recognition, in addition to their primary mechanisms of action. Approach & Results: HBV infected HepG2-NTCP were treated with tenofovir disoproxil fumarate (TDF), Toll-like receptor (TLR) 7/8 agonists, TLR7/8 conditioned media (CM) collected from immune cells, or RNAi using short-interfering RNAs (siRNAs). The effect of these treatments on antigen presentation was measured through co-culture with CD8 T cells recognizing HLA-A0201 restricted epitopes, HBc18-27 or HBs183-191. Cytokine profiles of TLR7/8 CM was measured using cytometric bead array. TDF reduced viral replication, but not CD8 T cell recognition of infected cells. Direct exposure of infected HepG2-NTCP to TLR7/8 agonists had no impact on T cell recognition. Exposure of infected HepG2-NTCP to TLR7/8 CM enhanced HBV-specific CD8 T cell recognition through type 1 interferon (IFN) and IFN-γ dependent mechanisms. RNAi rapidly suppressed HBV DNA, HBV Core antigen (HBcAg), and HBV S antigen (HBsAg) expression, impairing recognition by HBV-specific CD8 T cells. Conclusions: Immunomodulation, and RNAi, but not nucleos(t)ide analogues, alter recognition of infected HepG2-NTCP by HBV-specific CD8 T cells. Understanding these changes will inform combination treatments for CHB.

Introduction:

Chronic hepatitis B (CHB) infection causes an estimated 887,000 deaths annually with a global prevalence of 292 million individuals (1). CHB is managed with nucleos(t)ide analogues (NUCs), suppressing HBV replication and liver inflammation. However, NUCs do not eliminate replicative covalently closed circular DNA (cccDNA) and do not suppress viral antigens. Consequently, NUCs do not restore antiviral immunity and are required indefinitely to prevent disease progression and virological relapse.

Novel therapeutics in clinical evaluation for the treatment of CHB, can suppress HBV replication and/or, antigen production, or induce cytokines with direct antiviral activity (2). siRNAs have been developed to target the overlapping transcriptome of HBV, suppressing expression of all transcripts (3). While reducing systemic antigen load may improve the ability to therapeutically boost T cell immunity, the impact on hepatocyte antigen presentation has not been investigated. Given that hepatocyte antigen presentation is a key driver of HBV-specific T cell exhaustion, eliminating hepatocyte antigen presentation may be key to therapeutic vaccination. In contrast, CD8 T cells are required to eliminate infected hepatocytes (4). Thus, understanding how antigen reduction impacts T cell recognition of hepatocytes is important for T cell stimulatory approaches and may help guide timing of therapy discontinuation with antigen reducing agents like siRNA.

In contrast to antigen reduction, immunomodulatory drugs targeting TLRs are being developed with the goal of driving intrahepatic immunity. This approach has demonstrated promising pre-clinical data in murine, woodchuck, and chimpanzee models (5–8). In humans, both TLR7 and TLR8 agonists have entered phase 2 clinical trials. TLR7 agonists induce type 1 interferons from plasmacytoid dendritic cells, while TLR8 agonists compounds induce IFN-γ production in T and NK cells, secondary to myeloid derived IL-12 and IL-18 (9,10). Both type 1 IFN and IFN-γ can mediate non-cytolytic clearance of HBV from the liver (9,11), but early data suggest cytokines alone are insufficient to clear HBV (12). In addition to their antiviral activity, type I IFN and IFN-γ may enhance antigen presentation by up-regulating MHC-1 and immunoproteasome component expression (9,13). Therefore, innate immunomodulators may drive HBV-specific CD8 T cell recognition of infected hepatocytes.

Data from animals and humans have highlighted the integral role for HBV-specific CD8 T cells in controlling infection (4,14,15). However, it remains to be defined how, or if, siRNAs and immunomodulatory drugs alter CD8 T cell recognition of infected hepatocytes. Given the essential

role for HBV-specific CD8 T cell responses in the control of HBV, identifying strategies which enhance HBV-specific CD8 T cell responses and understanding underlying mechanisms will inform future therapeutic strategies.

Methods:

Methods for transduced HBV-specific CD8 T cell production, HBV infection of HepG2-NTCP cells, TDF treatment and cytokine quantification in conditioned media can be found in the supplementary methods. HepG2-NTCP cells were donated by Prof. Dr. Stephan Urban and NTCP over expression was verified using qPCR.

Quantification of extracellular HBV rcDNA

Cell culture supernatant was centrifuged 3400 RPM for 5 minutes to remove debris and 100 ul was taken for DNA extraction. Cell culture supernatant was processed using a DNeasy Blood and Tissue kit (Qiagen) and HBV was quantified using qPCR (Supplementary table 2). Serial dilutions of HBV 1.3-mer WT replicon (Addgene) were used for quantification.

Immunofluorescence assay:

Cells were fixed with 4% PFA and permeabilized with 0.5% Triton X-100 before washing. Cells were stained with polyclonal rabbit anti HBcAg prior to secondary staining with donkey anti-rabbit AF647 (Biolegend). Nuclei were stained with DAPI. Cells were imaged using an EVOS FL Auto 2 (ThermoFisher Scientific) at 100x or 200x magnification. Images were overlayed and immunofluorescence was quantified using ImageJ.

HBV-specific CD8 T cell co-culture with HepG2-NTCP cells

$5x10^4$ infected and uninfected HepG2-NTCP were seeded into a flat bottom 96 well plate. $1x10^5$ HBV-specific CD8 T cells were seeded on top of HepG2-NTCP cells for 16 hours with Brefeldin A at 1 µg/ml. As a positive control, HepG2-NTCP cells were pulsed with 1 µM HBc18-27 or HBs183-191 for HBcAg-specific or HBsAg-specific CD8 T cells, respectively. Following overnight co-culture, HBV-specific T cells were stained for viability (Efluoro 506), CD8α, and IFN-γ before acquisition. T cell IFN-γ positivity was graphed using GraphPad Prism 9 software.

Conditioned media (CM) generation and treatment of HepG2-NTCP:

Fresh healthy donor (HD) peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient separation. PBMCs were resuspended in AIM-V supplemented with 2% human serum (LifeTech) and Primocin (Invivogen) at $1x10^6$ cells/ml. To generate CM, PBMCs were left untreated or stimulated with Imiquimod (Invitrogen) at 5 µg/ml or TL8:506 (Invitrogen) at 100 ng/ml for 24 hours. CM was harvested, centrifuged, and cryopreserved.

To model how immunomodulatory drugs alter recognition of HBV-infected hepatocytes, $5x10^4$ infected or uninfected HepG2-NTCP were seeded to 96 well plate for 24 hours. HepG2-NTCP were treated with TLR agonists alone or with CM for 24 hours before washing and co-culture with HBV-specific CD8 T cells as described above. To determine the phenotypic changes in co-receptor expression, HepG2-NTCP were treated with TLR agonists or CM for 24 hours and stained for CD80, CD86, HLA-A, and PD-L1 (Supplementary table 1). To identify changes in proteasome and immunoproteasome expression, HepG2-NTCP were treated with TLR agonists or CM for 24 hours

before RNA extraction using a RNeasy Micro kit (Qiagen) and cDNA was synthesized using a high-capacity cDNA synthesis kit (ThermoFisher Scientific). Expression of *PSMB5*, *6*, *7*, *8*, *9*, & *10* were quantified through TaqMan PCR and normalized to *GAPDH* (supplementary table 2). To determine roles for inflammatory cytokines, CM were diluted to 10% and treated with blocking antibodies for TNF-α and IFN-γ at 10 μg/ml for 30 minutes prior to treatment of infected HepG2-NTCP. To block type 1 interferon signalling, HepG2-NTCP cells were treated with IFNAR2 blocking antibodies at 5 μg/ml for 30 minutes prior to and during CM treatment. Following 24 hours of treatment with cytokine blocked CM, HepG2-NTCP cells were co-cultured with HBsAg-specific CD8 T cells as above. HepG2-NTCP viability was not altered by 24 hour treatment with TLR CM (not shown).

siRNA treatment of HepG2-NTCP:

HepG2-NTCP were cultured for 4 days post infection prior to transfection. siRNAs against *PSMB 8, 9, & 10* (Dharmacon) were used for immunoproteasome knockdown. Two HBV-specific siRNAs (siHBV 74 and siHBV 77) were synthesized as previously described (3). HepG2-NTCP were transfected with 50 nM of siRNA using Dharmafect 1 according to manufacturer's instructions (Dharmacon). 24 hours post transfection, cells were washed, re-seeded, and cultured up to 10 days post treatment. For immunoproteasome knockdown, HepG2-NTCP were cultured for 4 days post siRNA treatment before treatment with TLR-CM and co-culture as described above. For HBV-specific siRNA, supernatant was replenished daily, 100 ul of supernatant was taken for qPCR, and 400 ul were taken for HBsAg quantification (Abbott). HBV DNA was quantified as described above. To monitor changes in CD8 T cell recognition, siRNA treated HepG2-NTCP were co-cultured with HBcAg-specific CD8 T cells as described above. To investigate how TLR CM altered siRNA treated cells, siRNA treated cells 4 days post HBV siRNA transfection were treated with TLR CM and co-cultured with HBV-specific CD8 T cells as above. Transfection did not alter HepG2-NTCP viability within 10 days (not shown).

Results:

Rapid, multiplicity of infection (MOI)-dependent recognition of infected hepatocytes by HBV-specific CD8 T cells

We previously showed that hepatocytes are poor antigen presenting cells and the scale of HBV antigen expressed affects CD8 T cell functionality (16). Therefore, we defined HBV replication, antigen expression, and CD8 T cell recognition of HepG2-NTCP cells infected *in vitro* with HBV. We demonstrated that by day 4 post infection, there was an increase in HBV in the supernatant of infected HepG2-NTCP cells (Fig. 1A) and infected cells expressed HBV core antigen by 2 days post infection (Fig. 1B). To establish a renewable and consistent source for HBV-specific CD8 T cells, we utilized a retroviral T cell receptor (TCR) transduction system (17) to introduce CD8 T cell receptors specific for two HLA-A0201 restricted HBV epitopes HBc18-27 or HBs183-191 into HLA-A0201 negative PBMCs. TCR expression was confirmed using HLA-A0201 dextramer staining and IFN-γ production in response to co-culture with peptide pulsed T2 cells (supplementary Fig. 1A, B).

HBV-specific transduced CD8 T cells were co-cultured with HBV-infected HepG2-NTCP to define T cell activation induced by presentation of endogenous HBV antigens during infection. CD8 T cell activation was measured by staining for IFN-γ production. We demonstrated that HBcAg-specific CD8 T cells are activated by HBV-infected HepG2-NTCP cells by two days post infection and T cell activation remained stable up to 8 days post infection (Fig. 1C). Importantly, CD8 T cell responses against uninfected cells were negative, demonstrating that CD8 T cell activation is infection dependent. While HBcAg-specific T cells readily responded during co-culture, HBsAg-specific CD8 T cell responses were weak (Fig. 1C). When HepG2-NTCP cells were infected with increasing MOIs of HBV, increasing MOIs correlated with increasing CD8 T cell responses against both epitopes. However, the magnitude of CD8 T cell response was epitope dependent. HBcAg CD8 T cell responses were more robust compared to HBsAg-specific CD8 T cell responses across all MOI (Fig. 1D). In all cases, we observed that the positive control, peptide pulsed HepG2-NTCP, elicited CD8 T cell

responses, validating that HBV-specific CD8 T cells recognize their cognate antigen on HepG2-NTCP. These data validate this model to test the impact of different interventions on hepatocyte antigen presentation and suggest the efficacy of antigen presentation is epitope specific. Overall, this demonstrates that HepG2-NTCP and TCR transduced CD8 T cells serve as a model to measure the impact of hepatocyte manipulation on antigen presentation. For further experiments, an MOI of 1000 was utilized.

Nucleoside analogue therapy does not alter HBcAg-specific CD8 T cell recognition

Our first goal was to define the impact of NUC therapy on CD8 T cell recognition of infected and uninfected HepG2-NTCP. Nucleoside analogues reduce viral replication, but do not reduce HBV antigen levels in CHB patients. Infected and uninfected HepG2-NTCP cells were treated with 10 μM TDF, with replenishment of TDF every 48 hours. Immunofluorescence revealed no changes in HBcAg expression within 9 days of treatment (Fig 2A). In contrast, by 4 days post TDF treatment, HBV replication was suppressed to a maximum of 93% (Fig. 2B). Finally, when HBcAg-specific CD8 T cell responses were investigated, we found that TDF treatment had only a modest enhancement of CD8 T cell recognition of infected cells on day 5 post TDF treatment (Fig. 2C).

Immunomodulatory drugs enhance CD8 T cell recognition of HBV-infected hepatocytes

As NUC therapy did not reduce CD8 T cell recognition, we next tested how immunomodulatory drugs impact antigen presentation in infected HepG2-NTCP. TLR7/TLR8 agonists are being developed to treat CHB. CM was generated through 24h stimulation of fresh PBMCs with the TLR7 agonist Imiquimod or the TLR8 agonist TL8-506. When the cytokine composition of CM was investigated using a multi-analyte cytometric bead array, we found that relative to unstimulated CM, TLR7 and TLR8-stimulated CM were enriched in IFN-α2, IFN-β, IFN-γ, IFN-λ 1, IL-10, IL-12p40, soluble FasL, and TNF-α. Interestingly, TLR7-stimulated CM were distinct from TLR8-stimulated CM exclusively in IFN-α2. Despite elevated IFN-α2, we did not see that TLR7-stimulated CM were enriched in IFN-β. In

contrast, TLR8-stimulated CM contained more IFN-β, IFN-γ, IFN-λ 1, IL-12p40, soluble FasL, and TNF-α than TLR7-stimulated CM. All CM contained similar levels of IFN-λ 2/3 (Fig. 3A). We could not detect any cytokine production after TLR7 or TLR8-stimulation of HepG2-NTCP (data not shown).

To model how immunomodulatory drugs alter CD8 T cell recognition, we tested direct stimulation of HepG2-NTCP cells with TLR agonists and exposure to CM from PBMCs stimulated with TLR7 and TLR8 agonists. HepG2-NTCP were treated with CM for 24 h, washed, and co-cultured with HBcAg or HBsAg-specific CD8 T cells. Consistent with the lack of cytokine production by HepG2-NTCP, we did not detect changes in HBcAg and HBsAg-specific CD8 T cell activation after co-culture with infected and uninfected HepG2-NTCP cells treated with TLR7 or TLR8 agonists (Fig. 3B). However, exposure of HepG2-NTCP cells to TLR7 and TLR8-stimulated CM, induced significant increases in CD8 T cell activation (Fig. 3C). The effects of unstimulated CM on HepG2-NTCP cells were minor compared to media alone (Fig. 3C). When TLR7 and TLR8-stimulated CM were compared, TLR8-stimulated CM enhanced antigen presentation to a greater extent than TLR7, resulting in a 2-fold and 20-fold increase in T cell activation, respectively. Importantly, enhanced HBV-specific CD8 T cell activation was dependent on the infection status of HepG2-NTCP cells (Fig. 3C).

TLR CM enhance CD8 T cell activation through up-regulation of antigen processing machinery.

To define phenotypic changes which may contribute to T cell activation after CM treatment, we analyzed expression of regulators of T cell activation and proteasome components. After 24-hour treatment with TLR7 and 8 agonists, we observed that direct TLR stimulation did not alter the expression of MHC I in HepG2-NTCP, regardless of infection status (Fig. 4A). However, exposure of HepG2-NTCP to TLR-stimulated CM enhanced the expression of MHC I, independent of infection status (Fig. 4B). Further characterization of HepG2-NTCP demonstrated that TLR7 and TLR8 agonists alone did not alter the expression of CD80, CD86, and PD-L1 in HepG2-NTCP cells (Supplementary Fig. 2A). When the effects of TLR7 and TLR8-stimulated CM were investigated, neither treatment

altered CD80 and CD86 expression, but both upregulated PD-L1 on HepG2-NTCP cells by 7.4 and 9.4-fold, respectively (supplementary Fig. 2B). When the roles for individual inflammatory cytokines were defined, we found that the treatment of HepG2-NTCP cells with recombinant TNF-α, IFN-α, and IFN-γ could enhance the presentation of HLA-A2 on HepG2-NTCP, but only IFN-γ induced expression of PD-L1 (supplementary Fig. 2C, D).

Previous work has suggested that HBs183-191 is an immunoproteasome restricted, HLA-A201 restricted, epitope. As activation of HBsAg-specific CD8 T cells appeared dependent on inflammation, we chose to further define the mechanisms regulating TLR-stimulated CM altered HBV antigen presentation. Therefore, we investigated the effect of CM on inflammation dependent antigen processing, in particular the immunoproteasome, due to the central role in processing antigens for presentation on MHC-I. When HepG2-NTCP were treated with TLR agonists alone, or CM, we observed minor effects on expression of constitutive proteasome components *PSMB5, 6,* & *7* (Fig. 4C). Similar to co-receptor data, direct TLR stimulation did not induce immunoproteasome components *PSMB8*, *9*, & *10*. While treatment of HepG2-NTCP with unstimulated CM induced modest upregulations in immunoproteasome components, TLR-stimulated CM drove significant upregulations in the expression of the immunoproteasome (Fig. 4C). To verify the immunoproteasome restriction of HBs193-191, siRNAs against the immunoproteasome (Supplementary table 2) were used to knockdown *PSMB*8, 9, and 10. Four days post siRNA treatment, HepG2-NTCP were treated with TLR-stimulated CM and then co-cultured with HBcAg and HBsAg-specific CD8 T cells. While immunoproteasome knockdown did not alter HBcAg-specific CD8 T cell responses, we found that knockdown of the immunoproteasome significantly reduced HBsAg-specific CD8 T cell recognition following TLR CM treatment (Fig. 4D).

To define which cytokines in CM alter CD8 T cell recognition of HBV-infected HepG2-NTCP cells, we utilized blocking antibodies against interferon alpha receptor 2 (IFNAR2), TNF-α, and IFN-γ due to

known roles in inducing the immunoproteasome (13,18). HBsAg-specific CD8 T cells were utilized because they showed the greatest change in response to TLR CM. We found that IFNAR2 blockade alone, prior to exposure of infected HepG2-NTCP to TLR7-CM, suppressed HBsAg-specific CD8 T cell activation by 41%. Blockade of TNF-α and IFN-γ in TLR7 CM did not suppress HBsAg-specific CD8 T cell activation when used individually or in combination (Fig 4E). The combination of IFNAR2 blockade with blocking antibodies failed to fully suppress HBsAg-specific CD8 T cell recognition, suggesting that other components in the CM may contribute to enhanced HBsAg-specific CD8 T cell activation. When TLR8-stimulated CM were treated with IFN-γ blocking antibodies, IFN-γ blockade alone suppressed HBsAg recognition by 70%. Neither TNF-α or IFNAR2 blockade contributed to HBsAg-specific CD8 T cell activation when used individually or in combination with other blocking antibodies (Fig. 4E). Together, these findings suggest that TLR7 and TLR8 agonists compounds enhance CD8 T cell recognition through different mechanisms, with TLR7 stimulation enhancing CD8 T cell recognition through type 1 IFN, while TLR8 stimulation is IFN-γ dependent.

HBV-specific siRNAs suppress HBV-specific CD8 T cell recognition

HBV-specific siRNAs have drawn clinical interest due to their ability to suppress the expression of viral antigens. However, the influence of HBV-specific siRNA treatment on HBV-specific CD8 T cell recognition has yet to be defined. To study HBV-specific siRNA treatment on HepG2-NTCP, two HBV-specific siRNAs were synthesized as described (3). When HBV-specific siRNAs were administered individually or separately, we saw that a singular treatment of infected HepG2-NTCP cells with HBV-specific siRNAs inhibited the expression of HBcAg by 34% within 2 days and 62% by 10 days post treatment (Fig. 5A, B). HBV-specific siRNAs strongly suppressed HBV replication, with a 96% reduction in HBV replication by 10 days post transfection (Fig. 5C). As anticipated for siRNAs targeting all HBV transcripts, treatment with HBV-specific siRNAs suppressed HBsAg synthesis by 73% within 2 days and reached 94% suppression by 10 days post treatment (Fig. 5D). Because the recognition of HBs183-191 without inflammation is weak, we chose to investigate how siRNA treatment affects HBcAg-specific CD8 T cell recognition of HepG2-NTCP cells. When HBcAg-specific

CD8 T cells were co-cultured with siRNA treated infected HepG2-NTCP, we found by 2 days post treatment, CD8 T cell recognition was suppressed by 69% (Fig. 5E). Further, the suppression of HBV-specific CD8 T cell responses increased by 10 days post siRNA treatment, reaching 82% reduction (Fig. 5E). Interestingly, while HBcAg-specific CD8 T cell recognition was suppressed by 69% by day 2 post transfection, HBcAg expression was only suppressed by 34%. When siRNA treatment was combined with TLR CM, we found that the enhanced antigen presentation induced by TLR CM in figure 3 was eliminated after siRNA-mediated HBV antigen reduction (Fig. 5F). These data demonstrate efficient suppression of HBV antigen production with oligo-based drugs significantly reduces visibility of infected hepatocytes to HBV-specific CD8 T cells and that enhancements to antigen presentation pathways can be negated through suppression of antigen expression.

Discussion:

Novel drug classes may alter CD8 T cell recognition of infected hepatocytes by altering antigen presentation or reductions in antigen expression. In the current study, we define how two classes of experimental therapeutics for the treatment of CHB alter the ability of HBV-specific CD8 T cells to recognize HBV-infected cells. We demonstrated that the underlying mechanism for TLR7 and TLR8 compounds is dependent on immune activation, with little to no direct effect on HBV-infected cells, in agreement with previous findings (9). We demonstrated that TLR7 and TLR8 CM were dependent on type 1 IFN and IFN-γ, respectively. Despite IFN-γ being present in both TLR7 and TLR8, we theorize that TLR7 CM was not sensitive to IFN-γ neutralization due to low concentrations of IFN-γ following dilution of CM for neutralization. Further, we found that TLR7 CM, but not TLR8 CM, was sensitive to IFNAR2 blockade. We demonstrated that the administration of HBV-specific siRNAs, which inhibit viral replication and antigen expression, strongly suppressed HBV-specific CD8 T cell recognition. Similarly, we demonstrated that HBV specific siRNA-mediated antigen reduction could negate the ability of TLR CM to enhance antigen presentation to HBV-specific CD8 T cells. Finally, we have conclusively demonstrated that the HBs183-191 epitope in the HLA-A201 background is

immunoproteasome restricted. Understanding how CD8 T cell recognition is altered by these drug classes will help inform logical combination strategies for Hepatitis B treatments.

Previous data have demonstrated that hepatoma cell lines and primary human hepatocytes are poor presenters of HBV antigens and only drive weak activation of HBV-specific CD8 T cells (16). Despite this, the consequences of chronic antigen presentation during chronic HBV infection are apparent, driving progressive exhaustion of HBV-specific CD8 T cell responses. However, despite CD8 T cell exhaustion in chronic HBV, the prevalence of HBV-specific CD8 T cells correlates with reduced viral and antigen loads, indicating exhausted cells retain some antiviral functionality (15). For these reasons, it was important to consider how immunomodulation or antigen reduction will impact CD8 T cells to understand the limitations of these approaches.

TLR7 and TLR8 agonists were developed to target immune cells and induce the production of cytokines with known antiviral effects, type I IFN and IFN-γ, respectively. TLR agonists have completed early clinical trials, demonstrating only modest antiviral effects against HBV, suggesting that cytokine production alone is insufficient to reduce HBV replication (12). However, innate immunomodulators will alter the intrahepatic environment through production of inflammatory cytokines. IL-12 and IL-18, produced after TLR stimulation support T cells and induce IFN-γ from NK, γδ T, and mucosal associated invariant T cells (10,19). IFN-α can protect T cells from NK cell mediated lysis (20). Therefore, it can be argued that innate immunotherapies will work best in combination with T cell targeted therapies rather than direct acting antivirals.

As we have demonstrated, the combination of innate immunomodulators with RNA interference (RNAi) against HBV is not synergistic, as RNAi reduced antigen expression, impairing presentation, negating the enhanced antigen presentation conferred upon hepatocytes by interferons. In contrast, therapeutic vaccination is more likely to benefit from combination with innate

immunomodulators. Vaccine induced HBV-specific CD8 T cells could be pulled to the liver through production of chemokines, display enhanced antiviral function supported by IL-12, and efficiently recognize infected hepatocytes due to increased antigen presentation. This response may be further boosted through expression of the immunoproteasome in hepatocytes, which broadens HBV epitope presentation. Our data, in agreement with previous work, definitively demonstrate that the HBs183-191 HLA-A201 restricted epitope is immunoproteasome dependent for efficient presentation (9,21). Therefore, it may be important to investigate if therapeutic vaccination can be utilized to induce T cell responses against potentially less exhausted immunoproteasome-restricted epitopes.

Our data also suggest that checkpoint inhibitors may be a rational drug combination with immunomodulation. TLR8 induces IL-12, which can restore exhausted CD8 T cell function (22). However, interferons produced following TLR activation increased PD-L1 expression. Therefore, combination of innate immunomodulator with anti-PD-1 drugs may enhance endogenous CD8 T cell activation while simultaneously removing inhibitory signals on hepatocytes. This combination could not be studied in our model as the HBV-specific CD8 T cells used do not display an exhausted phenotype.

RNA interference strategies for the treatment of chronic hepatitis B have demonstrated promising pre-clinical and early clinical data in their ability to drive lasting reductions in HBV antigens. However, RNAi affects viral transcripts, and like nucleoside analogues, targets HBV downstream of cccDNA. There is no published evidence to indicate that RNAi reduces cccDNA. Therefore, involvement of the immune response may be required to clear infected hepatocytes. It remains unlikely that antigen reduction alone drives restoration of HBV-specific T cell immunity. Recent data in CHB patients has demonstrated exhaustion markers, including TOX, is found in patients with resolved infection (23). Additionally, the murine lymphocytic choriomeningitis virus model

demonstrates that exhausted CD8 T cells commit to exhausted lineages and do not recover when removed from chronic infection (24). Further, elimination of HBsAg alone is insufficient to improve HBV-specific CD8 T cell immunity (25) However, preclinical models indicate that reduction of HBV antigens can permit effective boosting of HBV-specific T cells following therapeutic vaccination (26). Therefore, combining the reduction of HBV antigens by RNAi with therapeutic vaccines is an attractive strategy. This combination may take advantage of reduced exhaustive antigen presentation by hepatocytes allowing priming or boosting T cells in the periphery. An important consideration with this approach is the timing of viral rebound following RNAi cessation and vaccination. How long should antigen be suppressed before vaccination and how durable will vaccine-induced T cells be when HBV antigen expression is continually supressed? Will a gradual HBV rebound post-RNAi allow for controlled clearance of infected hepatocytes or re-impose an exhaustion profile on HBV-specific T cells? Furthermore, considering that RNAi suppresses the recognition of infected hepatocytes by CD8 T cells, it is unlikely that checkpoint inhibitors will yield antiviral benefit when used in combination.

It is important to note that data generated in this paper cannot fully recreate *in vivo* conditions within the liver. We used a hepatoma-derived cell line manipulated to allow for HBV infection and investigated responses in the absence of complex intercellular interactions of the liver environment. However, we feel the system is relevant to address our questions. HBV replicates naturally in HepG2-NTCP. HepG2-NTCP cells respond to interferons and express HLA-A0201, allowing us to test the processing and presentation of immunodominant HBV epitopes with HBV-specific transduced CD8 T cells, which recognize endogenously processed antigen. The CD8 T cells utilized do not fully replicate the phenotype of exhausted HBV-specific CD8 T cells typical of CHB, which may be impacted by alterations in antigen presentation to a greater extent, particularly when antigen presentation is reduced by RNAi. Therefore, while *in vitro*, this system allowed distillation of effects of experimental therapeutics with the immune response down to fundamental interactions between CD8 T cells and infected hepatocytes. Given that drugs and drug classes performed as expected, we

anticipate that our data is a robust predictor of what would be observed *in vivo*. We would also anticipate that these observations will extend to other targets that induce interferons (RIG-I/STING) or reduce HBV antigen production (antisense oligonucleotides). Further, this study utilized a first-generation siRNA that does not target HBV transcripts from integrated sequences. Therefore, later generation siRNAs may display an even greater suppressive effect through more efficient knockdown of HBV than the siRNAs used in this study.

Given that these drug classes are in phase 2 clinical trials as monotherapy or in combination with nucleotide analogues, the timing of our study is highly relevant. Our study provides important data on how to combine novel direct acting antiviral and immunotherapies for chronic hepatitis B to maximize T cell immunity. The striking decline in antigen presentation by RNAi has the potential to make infected cells invisible to CD8 T cells. This could present a significant obstacle for combination with immunotherapies and require careful immunological follow-up post-RNAi treatment.

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Figure legends:

Figure 1: Characterization of the HepG2-NTCP *in vitro* infection model for HBV. (A) HepG2-NTCP cells were infected with HBV and the titer of HBV in the cell culture supernatant over time was monitored through qPCR and graphed as Genome Equivalent (GE) per ml. (B) HBcAg expression HepG2-NTCP cells infected with 1000 MOI. HBcAg staining (bottom), overlayed and pseudo-coloured with DAPI (top; dapi = blue, HBcAg = red), 100x magnification. (C) HBV-specific CD8 T cell recognition of HepG2-NTCP infected +/- an MOI of 100. Longitudinal HBcAg-specific (left) and HBsAg-specific CD8 T cell responses (right). (D) Increasing MOI of infection corresponds with increasing HBV-specific CD8 T cell recognition of infected HepG2-NTCP. Positive controls for (C) and (D) were HepG2-NTCP pulsed with 1µM HBc18-27 or HBs183-191. Representative of 3 independent experiments. **p<0.01, ****p<0.001.

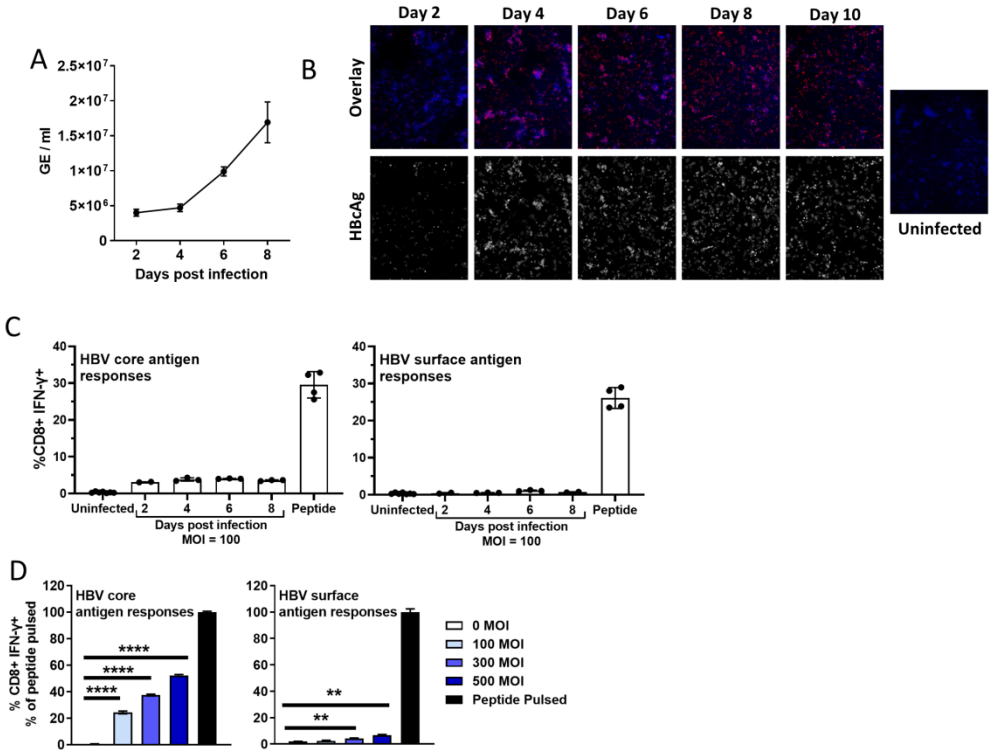
Figure 2: Nucleos(t)ide analogues do not suppress CD8 T cell recognition of infected cells. (A) HepG2-NTCP cells were treated +/- tenofovir disoproxil fumarate (TDF; 10 µM) for up to 9 days. untreated (top) and TDF treated (bottom), 100x magnification, overlayed DAPI (blue) and HBcAg (red). (B) TDF treatment suppresses HBV replication in HepG2-NTCP. (C) TDF treatment did not suppress HBcAg-specific CD8 T cell recognition of infected cells. TDF was replenished every 48 hours. *p<0.05, ****p<0.001.

Figure 3: Immunomodulatory compounds alter CD8 T cell recognition indirectly through immune activation. (A) Cytokine composition of CM as measured by cytometric bead array (N = 8 HD CM). (B) Direct TLR stimulation of HepG2-NTCP cells does not alter CD8 T cell recognition of HBcAg (left) or HBsAg (right). Representative of 3 experiments. (C) TLR-stimulated CM enhance CD8 T cell recognition of HBcAg (left) and HBsAg (right) in infected HepG2-NTCP (N = 5 HD CM). *p<0.05, **p<0.01, ***p<0.005.

Figure 4: Determining mechanisms for enhanced CD8 T cell recognition of infected cells. (A) Direct TLR stimulation of HepG2-NTCP does not alter MHC-1 presentation. (B) TLR-stimulated CM upregulate the presentation of MHC-1 on HepG2-NTCP. (A) and (B) are representative of 3 experiments. (C) Quantification of the constitutive- and immunoproteasome in TLR and CM treated HepG2-NTCP (N = 3 HD CM). statistics are relative to unstimulated CM. Knockdown of *PSMB* 8, 9, & 10 4 days post transfection suppresses HBsAg-specific CD8 T cell recognition, but not HBcAg-specific CD8 T cell recognition following TLR CM treatment. (E) Cytokine blockade demonstrates that TLR7 CM enhanced HBsAg-specific CD8 T cell activation through type 1 IFN, while TLR8 CM was IFN-γ dependent. *p<0.05, **p<0.01, ****p<0.001.

Figure 5: HBV-specific siRNA suppresses CD8 T cell recognition of infected cells. (A) HepG2-NTCP were transfected with HBV-specific siRNA. Expression of HBcAg, 200x magnification, overlayed DAPI (blue) and HBcAg (red), was monitored over time and quantified (B) using ImageJ on three fields of view. (C) Treatment of HepG2-NTCP with HBV-specific siRNA suppressed HBV replication and (D) HBsAg production. Normalized to vehicle treated, infected cells. (E) siRNA treatment of HepG2-NTCP cells suppresses HBcAg-specific CD8 T cell recognition of infected cells. Normalized to vehicle treated, infected cells. (F) HBV specific siRNA can suppress HBcAg and HBsAg-specific CD8 T cell recognition following TLR CM treatment. Normalized to peptide pulsed positive control. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

Figure 1



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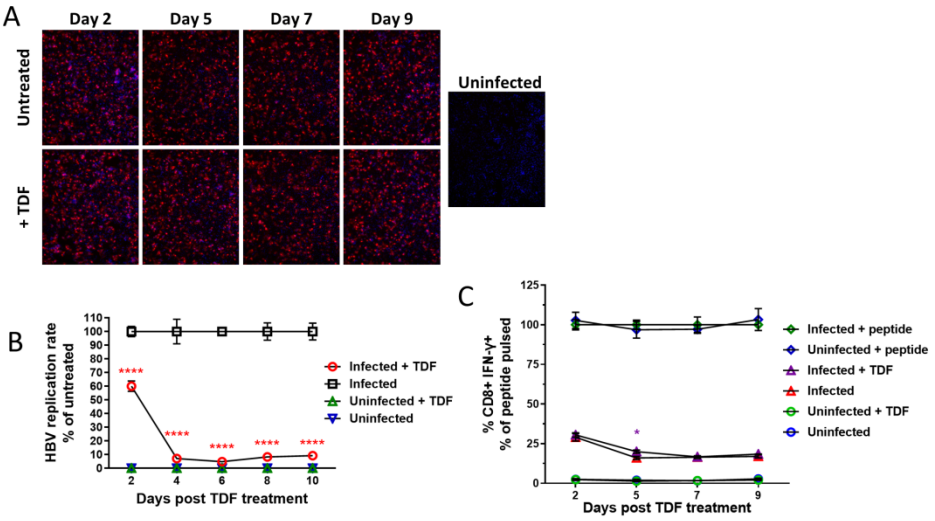

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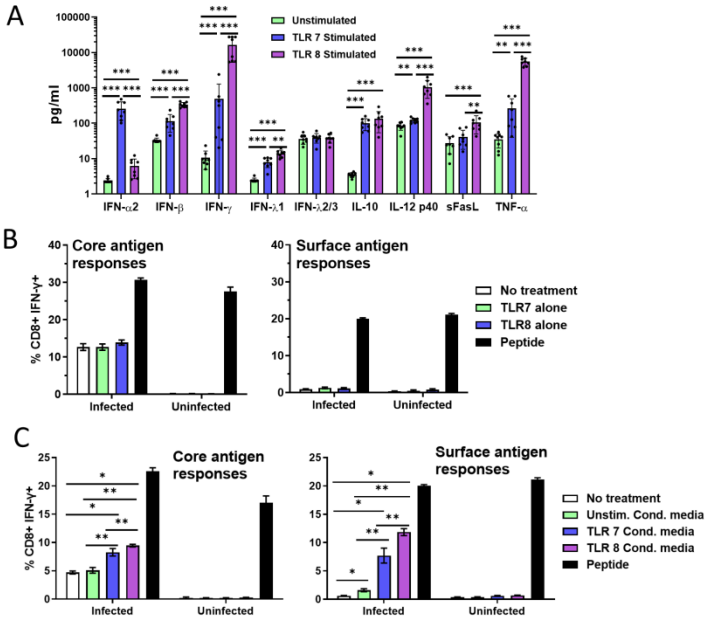

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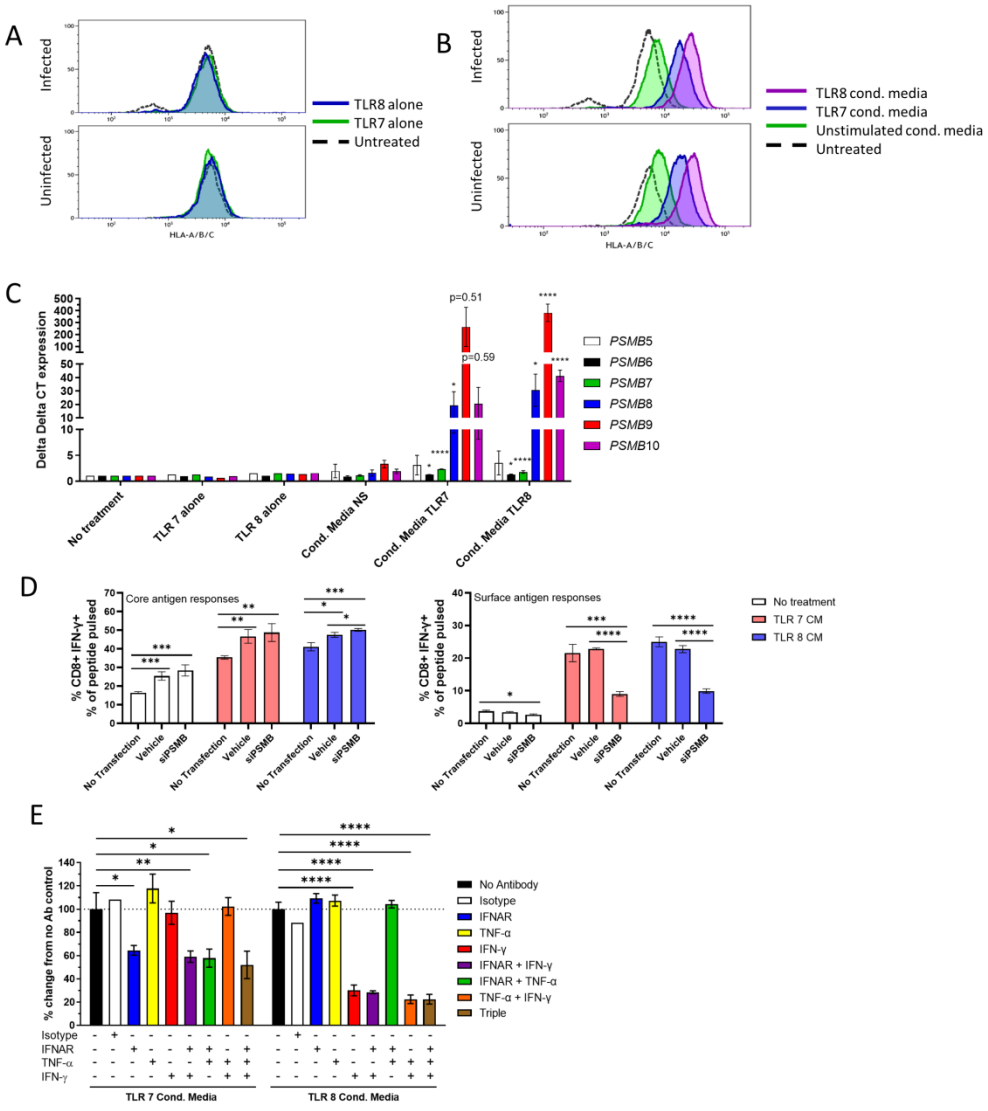

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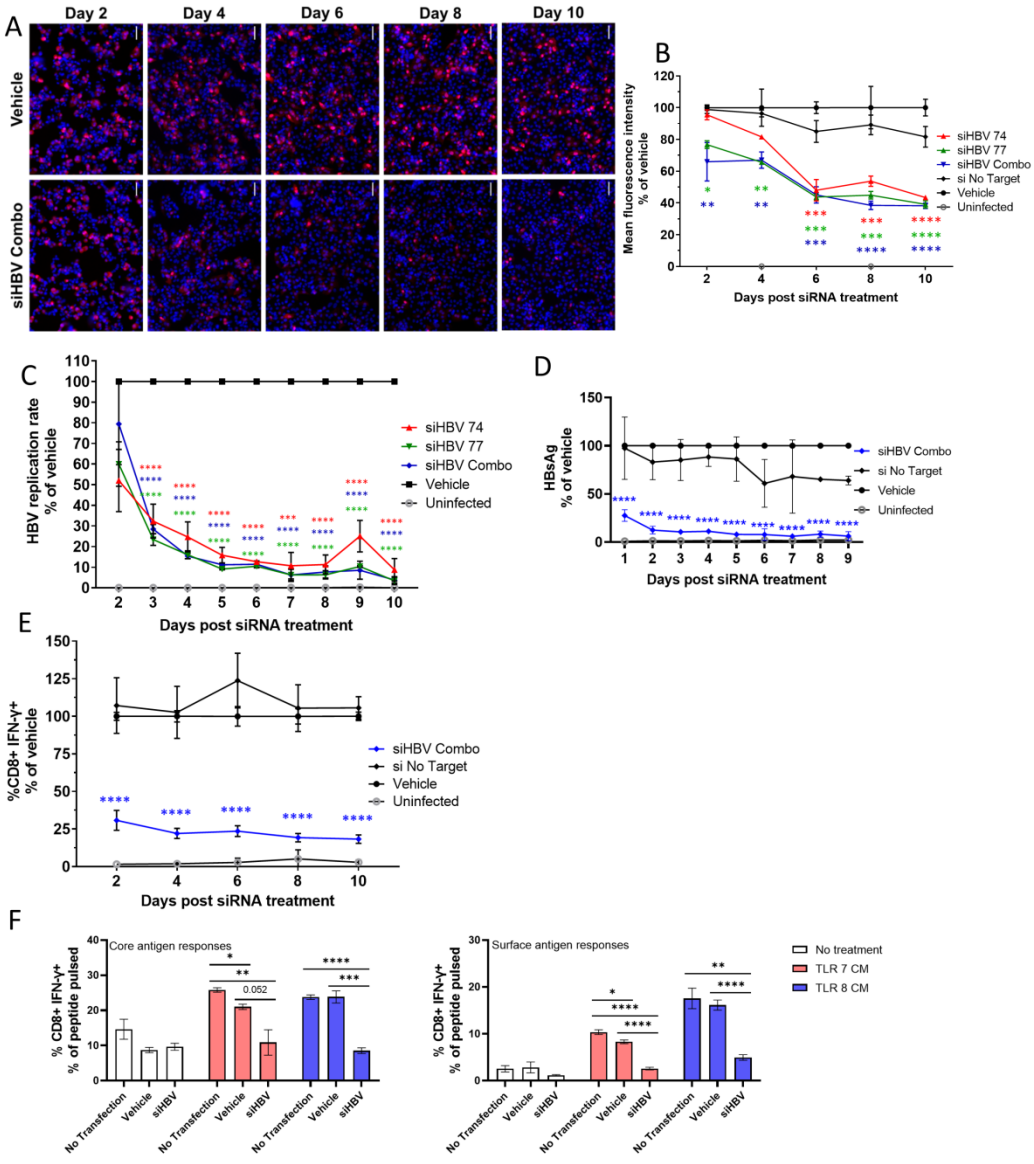
Figure 5



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