

RESEARCH PAPER



A dendritic cell receptor-targeted chimeric immunotherapeutic protein (C-HBV) for the treatment of chronic hepatitis B

Allan Ma^a, Bruce Motyka^b, Klaus Gutfreund^b, Yuenian Eric Shi^d, and Rajan George^a

^aAkshaya Bio Inc., Edmonton, Canada; ^bDepartment of Pediatrics, University of Alberta, Edmonton, Canada; ^cDepartment of Medicine, University of Alberta, Edmonton, Canada; ^dDepartment of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

ABSTRACT

In chronic Hepatitis B Virus (HBV) infections HBV-specific T cells are functionally impaired. Immunotherapy may restore HBV-specific T cell responses essential for sustained disease remission off-treatment and induction of a functional cure. Chimigen[®] Molecules are fusion proteins of antigen(s) with the Fc fragment of a xenotypic antibody designed to target specific receptors on dendritic cells (DCs). Here we describe the production and pre-clinical evaluation of Chimigen[®] HBV (C-HBV), containing HBV PreS1 and PreS2 peptide fragments, HBV core and murine Fc, produced in insect cells. C-HBV binding to immature DCs and internalization by endocytosis was FcγRII (CD32) and mannose receptor (CD206) dependent and led to increased MHC I and MHC II surface expression. Upon exposure of human T cells isolated from HBV un-infected healthy and chronically HBV-infected donors to C-HBV-pulsed mature DCs *ex vivo*, C-HBV induced vigorous T cell proliferation and enhanced expression of IFN-γ, TNF-α, perforin and granzyme B in both CD4⁺ and CD8⁺ T cell subsets. Re-stimulation of C-HBV-activated T cells from chronically infected donors with HBV PreS1/PreS2 and core overlapping peptides induced IFN-γ production in both CD4⁺ and CD8⁺ populations. C-HBV-activation of peripheral blood mononuclear cells (PBMCs) from chronically HBV-infected patients stimulated granzyme B production by CD4⁺CD25⁻ T responder (Tresp) cells, accompanied by an increase in Annexin V staining on CD4⁺CD25⁺ T regulatory (Treg) cell phenotype, consistent with apoptosis. The observed HBV-specific cellular responses induced by C-HBV *ex vivo* suggest that C-HBV is a promising immunotherapeutic candidate for the treatment of chronic HBV infections.

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Introduction

More than 2 billion people worldwide have been infected by the hepatitis B virus (HBV), and among this population, an estimated 257 million are chronically infected, thus hepatitis B remains a major health concern.¹ Many patients with chronic hepatitis B infection are at a high risk for developing liver cirrhosis and hepatocellular carcinoma. Worldwide, an estimated 887,000 people died due to complications resulting from chronic HBV infection in 2015.¹

Prophylactic vaccines containing the small HBV surface antigen (HBsAg) are effective in eliciting protective immunity against HBV infection,² but do not promote resolution of established chronic infections. HBsAg-based vaccines produce neutralizing antibodies which block HBV entry into uninfected hepatocytes. However, these are ineffective for established chronic/persistent HBV infections. There is considerable evidence from various studies that in the resolution of HBV infection, HBV-specific T cells are the major players and among them core-specific T cells are the most important.^{3–5} An effective HBV immunotherapy should be able to induce T cell activation which has been lacking, for various reasons. New antibodies produced may also play a role in assisting the cellular response to clear the virus and viral antigens. To date, treatment options for patients with

chronic hepatitis B consist mainly of pegylated interferon-α or nucleoside/nucleotide analogues such as entecavir and tenofovir. Current therapies achieve sustained off-treatment responses in a minority of highly selected patients, and a functional cure, hallmarked by a sustained clearance of circulating HBsAg and normalization of aminotransferase (ALT) levels following discontinuation of treatment remains most difficult to achieve.⁶ Hence, the emergence of drug-resistant strains of virus during long-term suppressive therapy with nucleoside/nucleotide analogues and cost remain as major concerns.⁷ Unlike hepatitis C virus infections,⁸ a virological cure for chronic HBV might not be possible due to the stability of covalently closed circular (ccc) HBV DNA and the lack of a cellular mechanism for its elimination.⁹

Functional impairment of HBV-specific immune response is a key feature of chronic HBV infection^{10,11} and has been attributed to many factors including T cell exhaustion¹² associated with overexpression of inhibitor receptors such as programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) on T cells.¹³ Fiscicaro et al.¹⁴ have suggested mitochondrial dysfunction as a cause of T cell exhaustion in HBV-infected individuals. Another proposed mechanism for the impaired T cell response is the increasing

number of regulatory T cells (Treg) during chronic infection which suppress or downregulate the induction and proliferation of effector T cells¹⁵ and dendritic cell dysfunction.¹⁶ Immunotherapeutic approaches which aim to induce/restore effective cellular and humoral responses against HBV antigens have been of considerable interest in recent years^{17,18} but have not been shown to be able to elicit clinically relevant responses to date.^{19,20} An ideal immunotherapy would “break” the immune tolerance status in chronic HBV-infected hosts to restore specific antiviral immunity and eliminate persistent infection by activating T cell responses which appear to be critical in achieving a functional cure.

Based on the observation that spontaneous resolution of HBV is accompanied by immune system re-constitution,²¹ various immunotherapeutic approaches to induce novel HBV-specific cellular and humoral responses have been attempted.^{22–24} These immunotherapy agents include DNA and viral vectors, proteins or peptide-based vaccines with various adjuvants. Many of these approaches so far have not been successful.

Chimigen® molecules are a novel class of immunotherapy agents that target viral antigens to antigen presenting cells, especially dendritic cell (DC) receptors, for efficient uptake, processing, and presentation via major histocompatibility complex class I (MHC I) and class II (MHC II) pathways. DCs play a central role in the activation of the immune responses.²⁵ Antigen uptake by DCs is mediated by receptors, such as C-type mannose²⁶ and Fcγ receptors.²⁷ A major advantage of targeting receptors on DCs is the facilitation of antigen delivery to appropriate compartments for the induction of an effective immune response without the use of adjuvant^{28,29} and with reduced antigen dose.³⁰

Chimigen® HBV (C-HBV) consists of an immune response domain (IRD) containing HBV PreS1 peptide fragment (119 amino acids), PreS2 peptide fragment (55 amino acids), the entire core antigen (185 amino acids), and a target binding domain (TBD) comprised of a linker fragment of eight amino acids and xenotypic Fc fragment from mouse IgG₁ (Figure 1). The order of the antigens was similar to the open reading frame of the HBV genome. The TBD mediates the binding to specific DC receptors such as Fcγ receptors.²⁷ The xenotypic nature of the murine Fc makes the molecule more immunogenic. In addition, the expression of the fusion protein in Sf9 insect cells which imparts primitive non-mammalian glycosylation without terminal sialic acid residues,^{31,32} increases the immunogenicity³³ of the molecule and also permits the targeting of mannose receptors (CD206) for antigen uptake, processing, and presentation.³⁴

The rationale for including HBV Core and PreS1/Pre2 antigens as components of C-HBV was based on prior studies showing the efficacy of these antigens in eliciting T cell immunity. The effect of circulating viral HBeAg and HBsAg as well as the lack of sufficient T cell responses to HBV core are two of several mechanisms suggested to explain the establishment of chronicity by HBV.³⁵ Resolution of chronic HBV achieved by adoptive transfer of bone marrow from HLA-identical donors with natural immunity to HBV correlated with core-specific T cell activation,³ and several strategies have been attempted to improve T cell responses to core.^{36–41} It has been suggested that PreS1/S2 peptides may also be relevant for eliciting immune responses.^{38,42,43} PreS protein was shown to be capable of stimulating preS-specific CD8+ and CD4 + T cell responses in BALB/c mice and HBV transgenic mice.

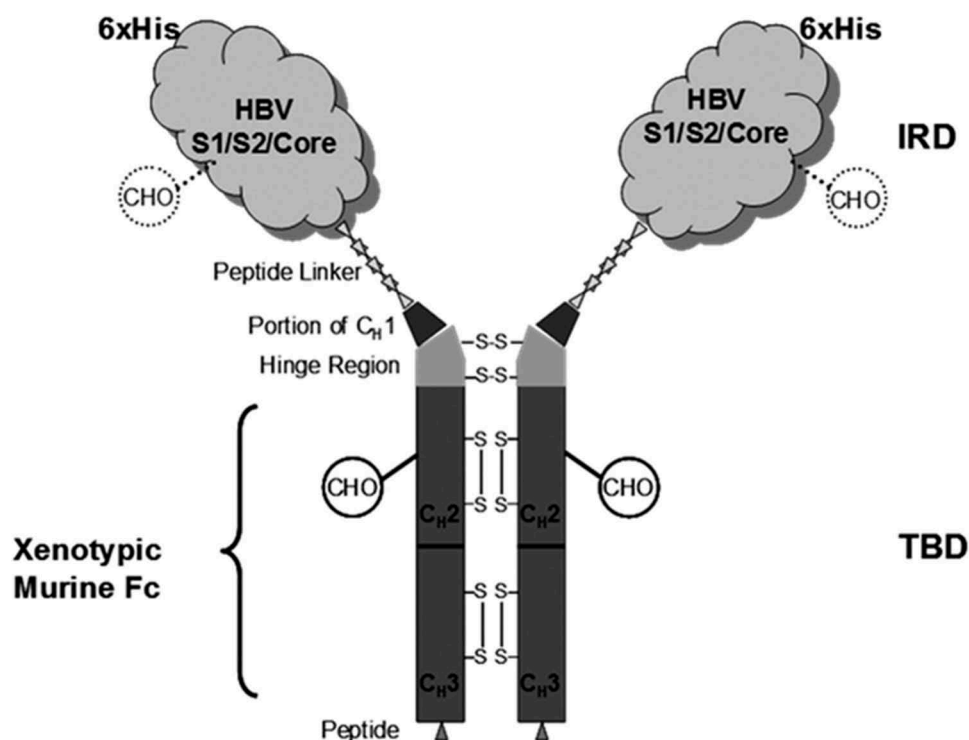


Figure 1. Schematic representation of C-HBV.

The fusion protein is comprised of antigen (HBV S1/S2 and core) and the Fc portion of a xenotypic (murine) monoclonal antibody. Immune Response Domain (IRD), Target Binding Domain (TBD), CHO-denotes glycosylation.

Furthermore, preS immunization provided protection against hydrodynamic transfection of HBV DNA in mice.⁴² These studies suggest that preS protein may also elicit cellular responses against HBV.

Here we describe the production of Chimigen® HBV (C-HBV) in insect cells and the evaluation of its ability to elicit HBV-specific T cell responses *ex vivo*, with cells derived from uninfected healthy and chronically HBV-infected donors.

Materials and methods

Cloning of C-HBV

DNA sequences for the HBV S1 fragment (119 amino acids), S2 fragment (55 amino acids) and entire core protein (185 amino acids) were obtained from Genbank DNA databanks (HBV 991, subtype adw, Accession Number X51970.1). The TBD sequence was derived from a murine IgG1 antibody heavy chain sequence (Genbank Accession Number D78344.1). A gene that encoded the three HBV proteins, an eight amino acid linker peptide and TBD in a single open reading frame was commercially synthesized and codon-optimized for enhanced production in insect cells by GenScript. The synthetic gene was cloned into a pUC57 plasmid and was designed to have a unique Sal I site at the 5' end and a unique Hind III site at the 3' end. The coding sequence of the TBD insert was modified to remove putative protease cleavage sites within the hinge region. The three mutations corresponded to R8A, K13A, and T18A (the numbering of position 1 of the TBD insert refers to the valine residue in the VDKK C_{H1} domain). For proper post-translational processing of the protein, the signal sequence from *Autographa californica* nuclear polyhedrosis virus (AcNPV) gp64 protein was cloned into pFastBac-HTa (Thermo Fisher Scientific, 10584-027). Two oligonucleotides that encode a unique 5' Ava II site and a 3' Rsr II site (5'GCATGGTCCATGGT AAGCGCTATTGTTTTATATGTGCTTTTGGCGGCGGCGG-CGCATTCTGCCTTTGCGGATCTGCAGGTACGGTCCGAT-GC-3' and 5'-GCATCGGACCGTACCTGCAGATCCGCAAAG GCAGAATGCGCCGCCGCCCAAAGCACATATAAAA-CAATAGCGCTTACCATGGACCATGC-3') were synthesized and annealed together. After digestion with Ava II (New England Biolab, R0153S), and Rsr II (New England Biolabs, R051S), the fragment was cloned into Rsr II digested pFastBac-HTa, which places the gp64 signal sequence just upstream of the 6xHis tag to generate pFastBacHTa-gp64. The S1/S2/Core/TBD insert in pUC57 was isolated by digestion with Sal I (New England Biolabs, R3138S) and Hind III (New England Biolabs, R0104S) and cloned into Sal I and Hind III digested pFastBacHTa-gp64.

Generation of baculovirus

Recombinant bacmids were generated using the Bac-To-Bac® cloning system (Thermo Fisher Scientific, 10359-016) in *E. coli* strain DH10Bac (Thermo Fisher Scientific, 10361-012). The gene for C-HBV cloned into pFastBacHTa-gp64 was transformed into *E. coli* strain DH10Bac. The recombinant bacmids were isolated and used for transfecting Sf9 insect cells to generate the recombinant baculoviruses that express the recombinant protein in insect cells. The baculovirus stock was

amplified to produce a high titer stock, and titer (pfu, plaque forming units per mL) was determined with the Baculovirus Titering Kit (Expression Systems, 97-101).

Production of recombinant proteins in wave bag bioreactors

Sf9 insect cells (Thermo Fisher Scientific, 11496015) were seeded at 1×10^6 cells/mL into 100 mL ESF 921 (Expression Systems, 96-001-01) media in a 500 mL flask. Cultures were incubated at 27.5°C with shaking at 130 rpm on an Innova Model 2100 Benchtop Platform Shaker (Eppendorf, M11940000) for 3-4 d (until the cell density reached $6-8 \times 10^6$ cells/mL). When the culture reached the desired cell density, an aliquot of the culture (1×10^6 cells/mL) was seeded into 1 L ESF 921 media in a 2 L flask. Cultures were incubated at 27.5°C with shaking at 130 rpm, in a bench-top shaker-incubator until the cell density reached $6-8 \times 10^6$ cells/mL (3-4 d).

A Wave Bioreactor System 2/10EH (GE Healthcare, 28-4115-00) and Cellbag 10L/O (GE Healthcare, CB0010L-01) was used for 5 L cultures with the ESF921 media. The seed culture (1 L), grown as described above, was used to inoculate 4 L of ESF 921 media. The rocking of the Wave Bag Bioreactor was set at 32 rpm, 5° rocking angle, atmospheric air flow at 0.30 Lpm (liters per minute) and temperature at 27.5°C. Rocking of the bag continued until the cell density reached $2-3 \times 10^6$ cells/mL.

For the production of C-HBV protein, the Sf9 cells were infected with an MOI of 2 pfu/mL. The bioreactor was allowed to rock at 32 rpm, 5° rocking angle, air flow (30% O₂) of 0.30 Lpm and at 27.5°C. The cells were harvested at 42 h following the infection by centrifugation at $1600 \times g$ for 10 min, at 4°C. Pellets of infected Sf9 cells were washed, frozen in liquid nitrogen and stored at -80°C.

Purification of C-HBV

C-HBV-containing N-terminal 6xHis tag was purified using Ni-affinity chromatography. Frozen-infected Sf9 cell pellets were re-suspended in 32 mL lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate, 0.5 M sodium chloride, pH 7.4) per 100 mL of frozen cell pellet. The lysate was sonicated using a Misonix 3000 Ultrasonic Liquid Processor (Misonix, S-3000) three times at 100 W for 30 s on ice. Tween-20 (1%) and imidazole (20 mM) were added to the lysate, the pH was adjusted to 7.4 and stirred at room temperature for 2 h. After stirring, the lysate was filtered through a 5 µm syringe top filter (Pall Corporation, 4650) and then a 0.45 µm syringe top filter (Pall Corporation, 4654).

The protein was purified using an AKTA Explorer 100 (GE Healthcare, 18111241). The solubilized filtered lysate was loaded onto a 5 mL HisTrap FF column (GE Healthcare, 17531901). The column was washed with 10 column volumes of 6 M guanidine HCl, 20 mM sodium phosphate, 0.5 M sodium chloride, 20 mM imidazole, 0.05% Tween 20, pH 7.4 buffer and subsequently with wash buffer 1 (6 M guanidine HCl, 20 mM sodium phosphate, 10 mM ethylenediamine, 20 mM imidazole, 0.05% Tween 20, pH 7.4) and wash buffer 2 (6 M guanidine HCl, 20 mM sodium

phosphate, 40 mM imidazole, 0.05% Tween 20, pH 7.4). Finally, the protein was eluted with the elution buffer (6 M guanidine HCl, 20 mM sodium phosphate, 150 mM imidazole, 0.05% Tween 20, pH 7.4) and collected in 1 mL fractions.

The fractions containing the purified protein were pooled and concentration of the protein was determined using Compat-Able protein assay preparation reagents (Thermo Fisher Scientific, 23215) and Micro BCA assay kit (Thermo Fisher Scientific, 23235). Next, 2-mercaptoethanol (Sigma-Aldrich, M3148) to 10 mM was added and incubated for 1 h at room temperature. The purified protein was transferred into a Slide-A-Lyzer cassette (Thermo Fisher Scientific, PI-66810) and dialyzed against Redox-shuffling buffer (0.5 M guanidine HCl, 0.5 M Tris, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.05% Tween 20, pH 7.4) overnight at 4°C followed by dialysis against 0.5 M Tris, 0.05% Tween 20, pH 7.4 for 12 h. The protein was finally dialyzed against a buffer containing 10 mM sodium phosphate, 0.15 M NaCl, 0.05% Tween 20, pH 7.4 for a minimum of 4 h.

The purified protein was sterilized by filtration through a 0.2 µm syringe-top filter (Pall Corporation, 4602). The concentration of the purified C-HBV protein was determined using the Micro BCA assay. Purified protein is stored at 4°C and is stable for over a period of at least 18 months under these conditions.

Biochemical evaluation of C-HBV

Purified C-HBV was characterized by SDS-PAGE. The protein sample was mixed with loading buffer containing the reducing agent DTT (2 mM) (Fermentas, R0891) and loaded onto a 7.5% TGX precast polyacrylamide gel (Bio-Rad, 456–1023). The gel was run at 100 V for 75 min. PageBlue (Thermo Fisher Scientific, PI 24620) was added; the gel was stained for 1 h and destained with several changes of ddH₂O.

The purified protein, following electrophoresis on 7.5% SDS-PAGE, was electroblotted onto Hybond ECL nitrocellulose membranes (GE healthcare, RPN303D) and used for Western detection using antibodies specific for components of the C-HBV protein. Antibody-binding was detected by chemiluminescence using the ECL kit (GE Healthcare, RPN2232). For C-HBV, four different antibodies were used: 6xHis horseradish peroxidase-conjugated mAb (Clontech, 631210), anti-mouse IgG (Fc specific) horseradish peroxidase-conjugated antibody (Sigma-Aldrich, A0168), anti-HBV core (Abcam, ab8638) and anti-PreS2 (Abcam, ab8635) mAbs.

For glycoprotein staining, the electroblotted Hybond ECL nitrocellulose membrane was incubated in Tris-buffered saline (TBS) solution (20 mM Tris, 150 mM NaCl, pH 7.5) containing 2% Tween 20 for 10 min at room temperature. After rinsing the membrane with TBS, the nitrocellulose membrane was incubated with ConA-HRP (Sigma-Aldrich, L6397) at a concentration of 1 µg/mL in TBS with 0.05% Tween 20, 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂ for 30 min at room temperature. The membrane was washed with TBS containing 0.05% Tween 20 and the positive bands were visualized by chemiluminescence using the ECL kit (GE Healthcare, RPN2232).

Immunological characterization of C-HBV using PBMCs isolated from healthy non-infected and chronically HBV-infected donors

Human PBMC

PBMCs were isolated from a leukapheresis preparation obtained from a healthy donor with the HLA-A2 haplotype (Biological Specialty Corporation, 213-15-04). Leukapheresis preparations were tested with the complete battery of FDA required infectious disease state tests and verified to be HBsAg negative, HBeAg antibody negative, and HBV DNA negative. PBMCs were obtained by Ficoll-Hypaque (Sigma-Aldrich, GE17-1440-02) density gradient centrifugation. PBMCs at 3×10^7 cells/cryovial (Thermo Scientific Nalgene, AY509X32) in freezing media (40% matched donor plasma, 50% AIM V (Thermo Fisher Scientific, 12055–091) and 10% DMSO), were stored in liquid nitrogen.

Chronic hepatitis B carriers that provided blood for isolation of PBMCs for this study were enrolled in study MS10 Pro00001044 approved by the Health Research Ethics Board of the University of Alberta. For this study, inactive hepatitis B carrier patients who had not received any anti-viral treatment were recruited from the outpatient clinics of the Liver Unit of the Division of Gastroenterology at the University of Alberta, Edmonton. The collection of peripheral blood (60 mL in seven ACD solution A tubes, Thermo Fisher Scientific, B364606) was performed using protocols approved by the Health Research Ethics Board at the University of Alberta. Upon patient consent, blood was obtained to screen for the expression of HLA-A2. After lysing red blood cells with ammonium chloride, blood cells were washed with D-PBS and stained with an AF 488-labeled anti-HLA-A2.01 antibody (Bio-Rad, MCA2090A488) or isotype control antibody. After staining, the cells were washed with D-PBS, fixed and acquired on a flow cytometer. HLA-A2 positive and HLA-A2 negative patients were included in the study. PBMCs and plasma were separated from whole blood by Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, 10771). Plasma was heat-inactivated and used for cell culture (2.5% in AIM V) and in the medium for cryopreservation (10% DMSO, 90% plasma) of PBMCs which are not used immediately in the *ex vivo* assays.

Isolation and differentiation of monocytes to iDC

Cryopreserved PBMCs were used to generate iDCs in a procedure modified from Whiteside et al.⁴⁴ PBMCs were cultured on 100 mm tissue culture dishes (BD Falcon, 353003) for 1 h at 37°C in AIM V media with 2.5% matched plasma (heat inactivated). Following culture, non-adherent cells were removed by washing with AIM V media. The adherent cells were then cultured for 24 h in AIM-V/2.5% matched serum containing IL-4 (R&D Systems, 204–01) and GM-CSF (R&D Systems, 215-GM-010) (1000 IU/mL of each). Following culture, the iDCs were harvested, washed once with AIM V media containing 2.5% matched serum, followed by two washes with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific, 14190–250) containing 0.1% (w/v) BSA

(PBSB). The phenotype of the isolated immature DCs was assessed by labeling for various cell surface markers including CD64, CD32, CD16, CD206, CD205, CD209, HLA-ABC, HLA-DR, CD14, CD11c, CD86, CD80, CD40, CD83, CD19 and CD3.⁴⁵

Binding of C-HBV protein to iDCs

Binding of C-HBV to iDCs prepared from uninfected donor was evaluated by flow cytometry. All steps were performed at 4°C. Cells were incubated for 60 min in PBSB containing various concentrations of protein or the corresponding dialysis buffer (2×10^5 cells/well in 96-well v-bottom plates) (Corning Costar, 3894) in a volume of 25 μ L. Protein binding was detected by incubation of the cells with rat anti-mouse IgG1-biotin (BD Biosciences, 553441) in PBSB for 20 min. The cells were washed with PBSB and then incubated with Streptavidin-PE-Cy5 (BD Biosciences, 554062) for 20 min. After washing with PBSB, cells were resuspended in PBSB containing 2% paraformaldehyde (PF) and the binding assessed by FACS. To determine the specificity of the receptors which bind C-HBV, iDCs were incubated with buffer control, isotype control mAb, anti-CD32 mAb, anti-CD206 mAb, or both anti-CD32 and anti-CD206 together for 1 h at 4°C before incubation with C-HBV for 1 h at 4°C. Bound C-HBV was detected with Penta-His AF 647 (Qiagen, 35370).

FACS acquisition and analysis

Cells were acquired with a FACSCalibur fitted with CellQuest Pro acquisition and analysis software (BD Biosciences, 342974). A gate was made on the viable cell population as determined by the forward scatter (FSC) and side scatter (SSC) profiles and $\geq 20,000$ events were acquired. The percentage of specific positive cells was calculated as $(\% \text{ positive cells test sample} - \% \text{ positive cells control}) / (100 - \% \text{ positive cells of control}) \times 100$. The relative mean fluorescent intensity (MFI) was determined as $\text{MFI of the test sample} - \text{MFI of the control sample}$.

Confocal fluorescence imaging

An inverted laser scanning confocal microscope (Zeiss, LSM 510) with an oil-immersion objective (NA = 1.3, 40 \times , Zeiss, 420762-9800) was used for imaging. Pacific blue, AF 488, 555, 647 were excited at 405, 488, 543 and 633 laser lines, respectively. During the image collection, the background level was set to deliver a non-zero count in each pixel and the gain was set to avoid saturating any of the pixels. This protocol would ensure that no information is lost at either end of the intensity scale hence the fluorescence intensity is quantitatively imaged as a function of position across the cell. In dual or triple labeling experiments, to minimize cross-over fluorescence, images were collected separately at each wavelength. One high-resolution image (Zoom 10-12, pixel resolution = 0.03-0.04 μ m) from an average of two scans was collected from each cell and 30 to 40 images from different cells were collected from different cells. All results were an average from double or triple individual experiments (total about 90 cells) to ensure the accurate determination

of mean of the cell population. Cells without labeling and cells labeled with secondary antibody only were imaged to obtain images representing the autofluorescence and nonspecific labeling, respectively. All imaging was conducted at room temperature. Images were processed using ICS and ICCS software from NIH Image (<http://rsb.info.nih.gov/nih-image/>) plugged in Image J program. Results were derived after background corrections which subtract the auto fluorescence and nonspecific labeling.

Analysis of C-HBV binding, internalization, and processing by confocal microscopy

The binding, internalization, and processing of the C-HBV by iDCs from uninfected donor was studied by confocal microscopy. iDCs were prepared as described above and transferred to chambered slides (MatTek, CCS-8) and incubated for an additional day. This is the optimal time for DC morphology and cell surface receptor expression. For the binding assay, all steps were performed at 4°C. First, iDCs were cooled on ice for 5 min and incubated with 3% BSA in DPBS to prevent nonspecific binding before the addition of C-HBV. Cells were incubated for 30 min in PBSB with C-HBV and rinsed three times with DPBS/0.25% BSA. Protein binding was detected by incubation of the cells with F(ab')₂ fragment of goat anti-mouse AF 488 (Thermo Fisher Scientific, A-11017) for 30 min at room temperature. Cells were fixed with 4% PF for 30 min at room temperature. Cells were mounted with SlowFade Gold antifade reagent (Thermo Fisher Scientific, S36936) and coverslips were sealed on before confocal imaging.

To confirm that CD32 is involved in C-HBV binding to iDCs, anti-CD32 antibody was used to block the binding. iDCs were rinsed with RPMI 1640 (Thermo Fisher Scientific, 11875093) and incubated with rabbit anti-human CD32 (Bio X Cell, BE0224-R100MG, 80 μ g/mL) for 1 h at 4°C. After washing, DCs were incubated with C-HBV (2 μ g/mL) at 4°C for 30 min. Cells were fixed with 4% PF for 30 min at room temperature. Binding was detected by confocal fluorescence microscopy with a donkey anti-rabbit AF 488 (Thermo Fisher Scientific, A-21206, 400 μ g/mL) followed by goat anti-mouse AF 555 (Thermo Fisher Scientific, A28180, 6 μ g/mL).

Internalization of C-HBV by iDCs was studied either by directly incubating the cells in media containing C-HBV at 37°C, 7% CO₂ or by first labeling the surface receptors at 4°C in PBSB, washing away the unbound protein, and then studying the uptake of receptor-bound C-HBV over time (0, 10, 30, and 60 min) and at different concentrations (1, 2, 4, 10, 20, 100, 400 μ g/mL) at 37°C, 7% CO₂ in RPMI/2.5% matched plasma. Internalization was stopped by soaking the cells with cold PBS. Cells were fixed with 4% PF at room temperature for 30 min, permeabilized with 0.5% Triton X-100, 10% FBS, 3% BSA in PBS for 10 min and C-HBV detected with goat anti-mouse IgG1 AF 555 (Thermo Fisher Scientific, A-21127, 10 μ g/mL) by confocal microscopy.

Co-localization with transferrin

In order to study receptor-mediated endocytosis of C-HBV, C-HBV loaded DCs were incubated with goat

anti-Fc IgG-AF 555 (Thermo Fisher Scientific, A-21127) for 3 h at 37°C/7% CO₂, washed, and incubated with AF 488 transferrin conjugate (Thermo Fisher Scientific, T13342) for 1 h at 37°C/7% CO₂ or incubated with both goat anti-Fc Fab IgG-AF 555 and AF 488 transferrin conjugate for 3 hr at 37°C/7% CO₂. After washing, cells were fixed with 4% PF for 30 min at room temperature, mounted and analyzed by confocal microscopy.

Co-localization with MHC I and II

The expression of MHC I & II molecules and their co-localization with C-HBV were examined using confocal microscopy. iDCs were incubated in AIM V medium with donor-matched serum and cytokines (IL-4, GM-CSF) for 3 d. Following this, they were incubated in RPMI 1640 media alone (control), with LPS (100 ng/mL, Sigma-Aldrich, L4391), or with C-HBV (2 µg/mL) at 37°C for 4, 8 or 24 h, the cells were washed and fixed using 4% PF at 4°C for 30 min. Cycloheximide (5 µg/mL, Sigma-Aldrich, C4859), used to inhibit protein synthesis, was added 30 min prior to initiation of experiment and remained present in the media during incubation. The cells were permeabilized using 0.5% Triton X-100 in FBS at room temperature for 10 min. Following this, the cells were labeled with mouse anti-human HLA-DR (MHC II) Pacific Blue (BioLegend, 327016, 10 µg/mL), mouse anti-human HLA-ABC (MHC I) AF 647 (BioLegend, 311416, 10 µg/mL), mouse anti-human calnexin (ER marker) FITC (Novus Biologicals, NBO2-36570 F, 5 µg/mL), LAMP (late endosome/lysosome marker) AF 488 (BioLegend, 121607, 10 µg/mL), or goat anti-mouse IgG1 (C-HBV) AF 555 (Thermo Fisher Scientific, A-21127, 10 µg/mL).

Antigen Presentation Assays (APAs) and generation of antigen-loaded mature DCs

The immune responses of naïve T cells from uninfected donor to antigen presented by DCs were assessed using APAs. The general protocol for the APA is schematically shown in Figure 2. For chronic HBV-infected donor samples, due to the limitation of volume of samples, antigen was added to whole PBMCs.

Immature DCs were generated as described above and incubated for 8 h with antigen or buffer (control). The cells were then cultured for 16 h with poly (I:C) (Sigma-Aldrich, P0913, 20 µg/mL). The extent of maturation of the DCs was assessed by phenotype analysis. Most of the DCs were mature as they expressed high levels of the DC maturation markers CD83, CD86, and CD80.⁴⁶ The matured antigen-loaded DCs (mDCs) were washed and co-cultured with T cells.

The iDCs from chronic HBV carrier donors were seeded into 96 well v-bottom plates at 3×10^4 cells/well and cultured for another 24 h (without cytokines). Dialysis buffer, C-HBV, and its components or tetanus toxoid (TT) were added to the cells for 6–8 h. Cells were then incubated with poly (I:C) (20 µg/mL) for 24 h before co-culture with PBMCs.

Isolation of human PBMC-derived T cells

T cells were isolated from PBMCs of uninfected donor by negative selection using Dynal Biotech T cell negative selection kit (Thermo Fisher Scientific, 11344D) as per manufacturer's procedure. Exceptions were that matched plasma was used instead of BSA and FBS. CD3+ T cells comprised greater than 98% of the isolated population. The T cells were used either labeled with carboxyfluorescein N-succinimidyl ester

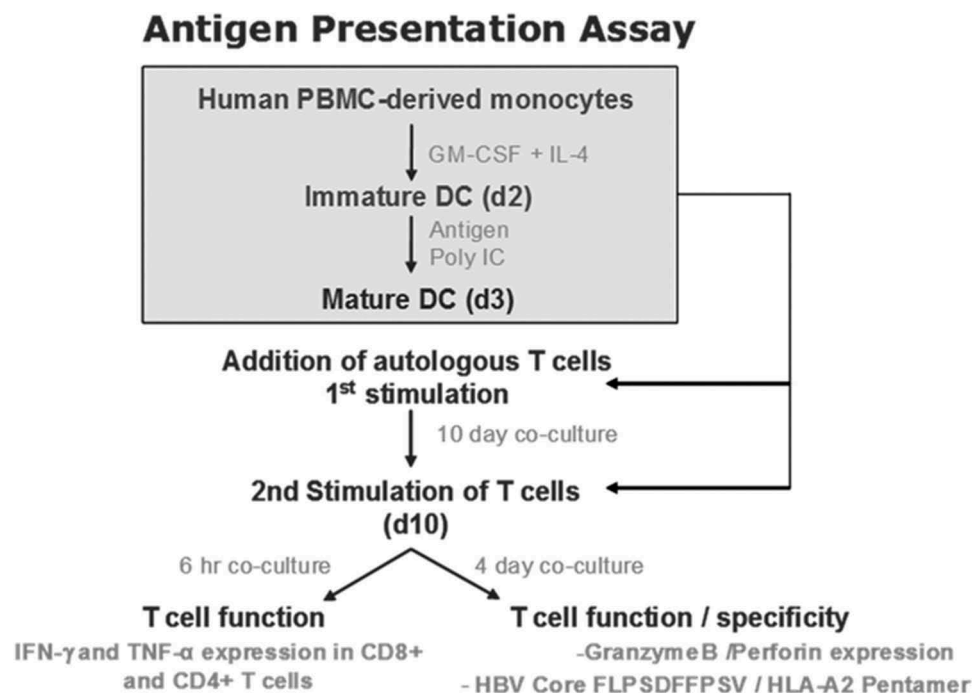


Figure 2. Antigen Presentation Assay (APA) schematic. Protocol used for PBMC-derived DC/T cell APA

(CFSE) (see below) or left unlabeled and added directly to cell cultures with antigen-loaded mature DCs.

T cell proliferation assay

Freshly isolated or previously frozen T cells from uninfected donors or PBMCs from chronic HBV carrier donors (in a 15 mL Tube, Corning, C352196) were washed once with D-PBS/5% autologous plasma (AP). The cells were resuspended at 4×10^6 cells/mL in D-PBS/5% autologous plasma (AP) and 110 μ L of a 50 μ M dilution of CFSE (Thermo Fisher Scientific, 65-0850-84) in D-PBS was added per 1 mL of cells. The cells were mixed and incubated at room temperature for 15 min. After incubation, the tube of cells was topped up to 15 mL with D-PBS/5% AP and the cells were pelleted by centrifugation. The cells were washed twice with 15 mL D-PBS/5% AP. The CFSE-labeled T cells or PBMCs were then resuspended at 4×10^6 cells/mL with AIM V/2.5% AP for co-culture with antigen-loaded mDCs. T cells from the uninfected donor were incubated with antigen-loaded mDCs at ratios of $1-2 \times 10^5$ T cells to $2-4 \times 10^4$ mDCs per well of a 96 well v-bottom plate in AIM V/2.5% matched serum. T cells were cultured for 7 d. PBMCs from chronic HBV carrier donors were seeded into 96 well plates at 4×10^5 cells/well, incubated for 24 h at 37°C and the PBMCs were stimulated with mDCs loaded with dialysis buffer, C-HBV, its components or TT (tetanus toxoid, EMD Chemicals Inc, 582231). Seven days after stimulation, the CFSE-labeled T cells and PBMCs were washed with D-PBS/2% FBS and stained with fluorescently labeled anti-human-CD3-PE (BD Biosciences, 552127), anti-CD4-APC (BD Biosciences, 555349) and anti-CD8-PerCP (eBioscience, 46-0087-42) antibodies for 20 min on ice. The cells were then washed twice with D-PBS/2% FBS, fixed and acquired using a FACSCalibur flow cytometer. Pentamer binding to activated T cells were analyzed 7 d following the two stimulation APA protocol (Figure 2).

Detection of intracellular IFN- γ and TNF- α

T cells from the uninfected donor were incubated with antigen loaded mDCs at a ratio of $1-2 \times 10^5$ T cells to $2-4 \times 10^4$ mDCs for 10 d and then re-stimulated with freshly prepared antigen-loaded mDCs. T cell function was assessed after 6 h of second stimulation. For the determination of intracellular cytokine production, brefeldin A (BD Biosciences, 347688) at 1 μ g/mL was added to prevent cytokine release. The production of IFN- γ and TNF- α was quantified using a standard intracellular cytokine labeling protocol (BD Biosciences). In brief, this consisted of labeling the cells with specific fluorochrome-conjugated mAbs for the detection of CD3 (anti-CD3-APC, BD Biosciences, 555335) and CD8 (anti-CD8-PE-Cy5, BD Biosciences, 555368), followed by fixing and permeabilization. Following this, the cell samples were incubated with anti-IFN- γ -PE (BD Biosciences, 562016) and anti-TNF- α -FITC (BD Biosciences, 554512). Between 20,000 and 100,000 cells were acquired per sample using a BD FACSCalibur. The assay protocol is presented in Figure 2.

PBMCs from chronic HBV-infected donors were seeded into 96 well plates at 4×10^5 cells/well and were co-cultured

with antigen-loaded PBMCs for 10 d. PBMCs were co-cultured again (second stimulation) with the mDCs for 30 min at 37°C, at which time 1 μ g/mL Golgi Plug (BD Biosciences, 555029) was added, and incubated for an additional 6 h. After 6 h, the cells were washed with D-PBS/2% FBS and stained with antibodies to cell surface markers, anti-CD8 PerCP (BD Biosciences, 347314) and anti-CD3 APC (BD Biosciences, 555335), for 20 min on ice. The cells were washed twice with D-PBS/2% FBS, fixed, permeabilized and stained with anti-TNF- α FITC (BD Biosciences, 554512) and anti-IFN- γ PE (BD Biosciences, 554791) for 30 min on ice. The cells were washed twice with Cytotfix/Cytoperm buffer (BD Biosciences, 554714), resuspended in D-PBS/2% FBS/2% PF and acquired using FACSCalibur flow cytometer.

Detection of intracellular GrB and Pfn

The production of GrB and Pfn was quantified using a standard intracellular cytokine labeling protocol (BD Biosciences). In brief, after the second stimulation of T cells from uninfected donor or PBMCs from chronic HBV-infected donors, were labeled with specific fluorochrome-conjugated mAbs for detection CD3 (anti-CD3-APC, BD Biosciences, 555335) and CD8 (anti-CD8-PE-Cy5, BD Biosciences, 555368), followed by fixing and permeabilization. Cells were harvested 4 d after the second stimulation and incubated with anti-GrB-FITC (BD Biosciences, 560211) and anti-Pfn-PE (BD Biosciences, 563763). About 20,000–40,000 cells were acquired per sample using a BD FACSCalibur. The assay protocol is presented in Figure 2.

Pentamer analysis

Pentamer binding⁴⁷ was used to quantify the specificity of CD8 + T cells by using a standard pentamer and intracellular staining protocol (ProImmune and BD Biosciences). T cells from uninfected donor were labeled with anti-CD8-PE-Cy5 (BD Biosciences, 555368), anti-CD3-APC (BD Biosciences, 555335), and the unconjugated HLAA*0201 Pentamer HBV Core FLPSDFFP (ProImmune, Peptide code 023). The unconjugated Pentamers were detected with a PE-conjugated Fluorotag (ProImmune, K2A-D). Approximately 50,000 cells were acquired using the FACSCalibur. The assay protocol is presented in Figure 2.

IFN- γ and TNF- α intracellular staining following stimulation with S1, S2, and Core overlapping peptides

PBMCs from chronic HBV carrier donors stimulated for 10 d *ex vivo* with C-HBV were re-stimulated with overlapping peptides (Mimotopes) of S1, S2 and Core antigens (15 amino acid peptides, 5 amino acid overlap) to induce cytokine production. The peptides were derived from the sequences of PreS1/S2 fragments and core in C-HBV. Peptides were resuspended in 50% acetonitrile and diluted to 1 μ M in 0.25% acetonitrile before adding to culture. The PBMCs were incubated for 30 min at 37°C, at which time 1 μ g/mL Golgi Plug was added for an additional 6 h of incubation. After 6 h, the cells were washed with D-PBS/2% FBS and stained with

antibodies to cell surface markers, anti-CD8-PerCP (BD Biosciences, 321314) and anti-CD3-APC (BD Biosciences, 555335) for 20 min on ice. The cells were washed twice with D-PBS/2% FBS, fixed, permeabilized and stained with anti-TNF- α -FITC (BD Biosciences, 554512) and anti-IFN- γ -PE (BD Biosciences, 554791) for 30 min on ice. The cells were washed twice with perm/wash buffer (BD Biosciences, 554723), resuspended in D-PBS/2% FBS/2% PF and acquired using FACSCalibur flow cytometer.

Analysis of regulatory T cells and apoptosis

The percentage of CD4⁺ CD25⁺ regulatory T cells (Treg) or CD4⁺ CD25⁻ responder T cells (Tresp) producing GrB in PBMCs from chronic HBV-infected donors was assessed by standard phenotype analysis and intracellular staining protocol. For examining the GrB expression in Tresp, the PBMCs were stimulated with C-HBV for 7 d and then labeled with anti-human CD4 – PerCP (BD Biosciences, 347324), anti-human CD25-APC (BD Biosciences, 560987) antibodies. Following cell surface staining, the cells were fixed, permeabilized, and then incubated with anti-human GrB-FITC (BD Biosciences, 560211). Between 30,000 and 40,000 cells were acquired using FACSCalibur flow cytometer. For monitoring apoptosis of Treg cells, the PBMCs were stimulated with C-HBV for 7 d and then labeled with anti-human CD4-PerCP, anti-human CD25-APC antibodies and Annexin V-FITC (BD Biosciences, 556419). The cells were fixed after cell surface staining. Approximately 12,000 cells were acquired using FACSCalibur flow cytometer.

Evaluation of cytolytic activity by DC-based cytotoxic assay using Real-time cell electronic sensing (RT-CES) system

The RT-CES system (xCELLigence, Roche) has been used for assessing a variety of cytotoxicity assays,^{48–52} including natural killer (NK) cell-mediated cytotoxic activity.⁵³ Briefly, target cells cultured in E-plates are mounted onto a device station placed inside a CO₂ incubator. Under the control of RT-CES software, the sensor analyzer automatically monitors cell index (CI), a parameter which represents cell status according to changes in electrode impedance in the wells. Since the CI value is proportional to the culture area covered by attached cells, cell damage and death result in detachment of cells from the wells leading to decrease in CI value. The data of real-time CI were collected and plotted according to experimental analysis. CI at each point is defined as:

$$CI = \max_{i=1 \dots N} \left[\frac{(R_{\text{cell}}(f_i))}{(R_b(f_i))} \right]$$

where N is the number of the frequency points at which the impedance is measured, and $R_b(f_i)$ and $R_{\text{cell}}(f_i)$ are the frequency-dependent electrode impedance with or without cells present in the well, respectively. To minimize random error among individual wells, a normalized CI at each point is also calculated by dividing the CI at the time point by the CI at a reference time point. A normalized CI is 1 at the reference time point.

In order to assess C-HBV-induced CTL-mediated cytolytic activity, a DC-based RT-CES cytotoxicity assay was developed. In this assay, antigen-loaded mature DCs derived from human PBMCs of healthy donors or from chronic HBV carriers were used as the target cells. Isolated T cells from uninfected donors or whole PBMCs from chronic HBV-infected donor, activated using the antigen presentation by DCs, were used as effector cells. T cells or PBMCs were co-cultured with C-HBV (or the respective control antigens)-loaded mDCs for 10 d. The target DCs generated from autologous PBMCs were plated into a microelectronic sensor plate (E-plate; $0.6\text{--}1 \times 10^5$ /well) and loaded with protein and its controls followed by maturation with poly (I:C). The E-plate was then put on RT-CES system for monitoring DC growth. On day 10, expanded T cells were harvested from the plate and transferred to corresponding wells in target DCs E-plate ($6\text{--}8 \times 10^5$ /well). Changes in CI were dynamically monitored using the RT-CES system. The monitoring was continued for 72 h after the co-culture of T cell/PBMC-target DC. At the end of the 72 h co-culture, the cells were harvested for intracellular staining of GrB production in CD8⁺ and CD4⁺ T cells and were evaluated using multi-color flow cytometry.

Results

Production and characterization of C-HBV protein

The C-HBV fusion protein containing N-terminal 6xHis tag (Figure 1) was produced in Sf9 insect cells using the baculovirus expression system and purified under denaturing conditions by Ni-chelation affinity chromatography. Following separation by SDS-PAGE under reducing conditions, purified C-HBV appeared as a major band with a molecular weight of ~78 kDa (Figure 3A). Along with the major monomer band, some aggregation products, possibly including the dimer of the protein, was observed. Minor bands of smaller molecular weight proteins, possibly degradation productions, were also seen. Detailed structural studies on the protein have not been carried out. C-HBV protein was characterized by immunoblot using monoclonal antibodies (mAb) recognizing the 6xHis tag, HBV PreS2, HBV Core and murine IgG1 Fc (Figure 3B). Concanavalin A (ConA), a lectin with affinity for terminal α -D-mannosyl residues on glycoproteins, was used to determine if purified C-HBV was glycosylated. Purified protein reacted with ConA (Figure 3C) consistent with glycosylation of the C-HBV fusion protein when produced in Sf9 insect cells.

Receptor-mediated binding and internalization of C-HBV by immature DCs

Chimigen[®] proteins are designed to target Fc receptors on DCs by virtue of the Fc fragment present in the fusion proteins and the glycosylation of the protein would allow binding to C-type lectin receptors on immature DCs (iDCs). To verify the binding of C-HBV to DCs, iDCs were assessed by flow cytometry following incubation with C-HBV (Figure 4). C-HBV binding to iDCs increased in a concentration-dependent manner. The specific binding to Fc γ RII and CD206 receptors was determined using blocking mAbs to CD32 and CD206. Compared to buffer

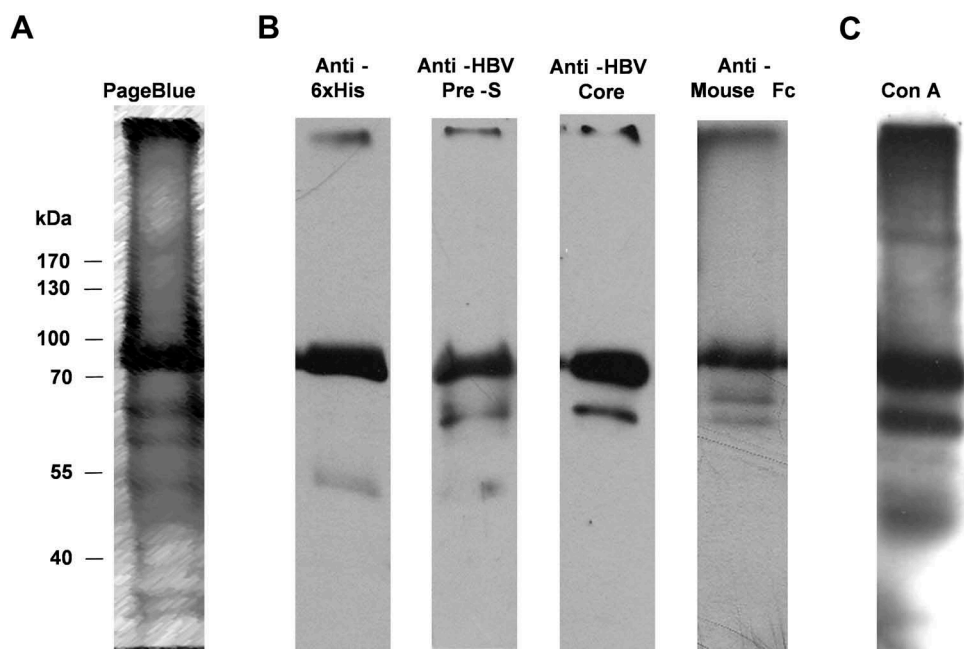


Figure 3. Biochemical characterization of purified C-HBV.

C-HBV was purified using Ni-IDA (HisTrap FF) affinity chromatography and characterized by SDS-PAGE (A), Western blots (B) and glycostaining (C). 7.5% SDS PAGE gel was stained with PageBlue. Standard molecular weight markers are shown. Western blots using antibodies specific to 6xHis, IRD domain (anti-HBV Pre-S2 and anti-HBV Core) and TBD (anti-Fc). Glycosylation of C-HBV was detected with ConA-HRP stain.

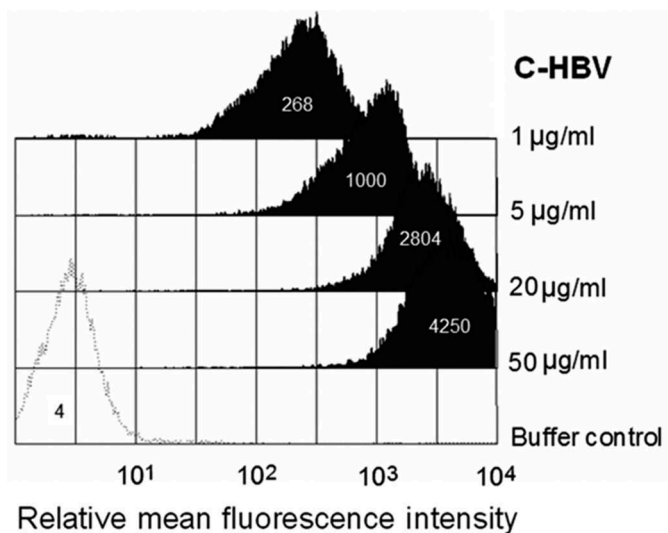


Figure 4. Binding of C-HBV to iDCs-FACS analysis.

PBMC-derived iDCs from HBV un-infected donors were incubated for 1 h at 4°C with 1–50 µg/ml of C-HBV. Bound protein was detected by flow cytometry following labeling with biotinylated anti-mouse IgG mAb and SA-PE-Cy5.

control, both anti-CD32 and anti-CD206 inhibited binding by approximately 65% and 46%, respectively (Figure 5A,B). Addition of both anti-CD32 and anti-CD206 together, further inhibited C-HBV binding to iDCs ~72% (Figure 5B). The specificity of receptor-binding was further evaluated using confocal microscopy. Anti-CD32 antibody was used to block the binding to CD32 before exposure to C-HBV protein. Blocking the CD32 receptor inhibited binding of the fusion protein to the iDC surface, suggesting that the CD32 receptor is involved in binding of the protein (Figure 5C). Therefore, CD32 and CD206 appear

to be major receptors involved in the binding and uptake of C-HBV.

Confocal microscopy also was used to study the binding of C-HBV to iDCs and the role of receptor-mediated internalization, intracellular processing and antigen presentation by DCs. The iDCs incubated with C-HBV at 4°C showed an intense staining on their surface compared with buffer only controls indicating that C-HBV bound to the cell surface (Figure 6). Internalization of C-HBV by iDCs was evaluated by directly incubating iDCs in media containing C-HBV at 37°C (Figure 6). Internalization was also studied by initially binding the C-HBV to iDCs at 4°C, removing the unbound protein by washing, and monitoring the internalization of C-HBV by tracking the uptake of the receptor-bound protein at 37°C over time (0, 10, 30, and 60 min). C-HBV localized in punctate staining pattern, often in the vicinity of the cell nuclei, suggesting C-HBV was internalized (Figure 7A,B), and binding and internalization increased in a concentration-dependent manner (Figure 7B,C). The role of receptor-mediated endocytosis in the uptake of C-HBV was studied by its co-localization with transferrin. Immature DCs were pulsed with C-HBV at 4°C, washed, and incubated for 15 min at 37°C. The cells were fixed, permeabilized and labeled with antibodies to detect C-HBV as well as the transferrin receptor. The transferrin receptor is taken up by receptor-mediated endocytosis and then recycled back to the plasma membrane from early endosomes.^{54,55} At 4°C, C-HBV bound to the cell surface while the transferrin receptor was present predominantly within cells (Figure 8). At 37°C, endosomes were observed to form, some of which contained both the C-HBV protein and transferrin receptors. The co-localization of both molecules suggests that C-HBV may be taken up by receptor-mediated endocytosis (Figure 8). Furthermore, expression of

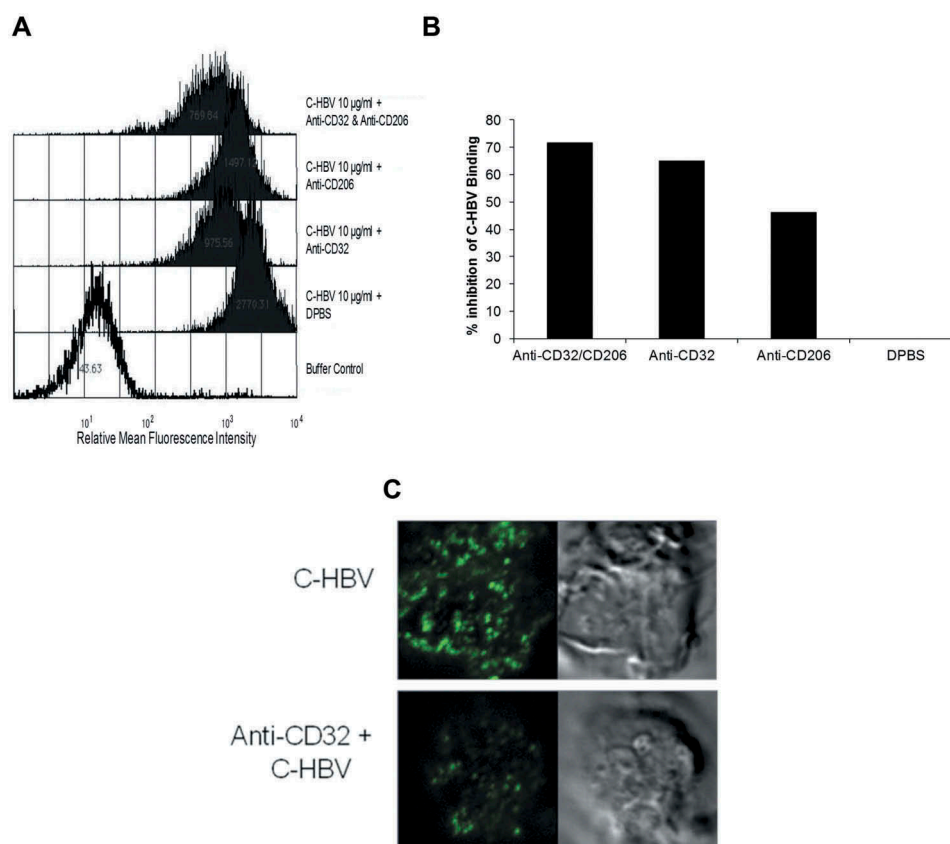


Figure 5. Inhibition of C-HBV binding to iDCs by antibodies to CD32 and CD206.

PBMC-derived iDCs from HBV un-infected donors were incubated with buffer (control), isotype mAb (control), anti-CD32 mAb, anti-CD206 mAb, or both together for 1 h before incubation with C-HBV at 4°C. Bound C-HBV was detected by FACS using Penta-His AF 647. For confocal microscopic evaluation of inhibition of C-HBV binding to iDC receptors, iDCs were incubated at 4°C with rabbit anti-CD32. Subsequently, the cells were incubated with C-HBV at 4°C. Binding was detected by confocal fluorescence microscopy with donkey anti-rabbit AF 488 followed by goat anti-mouse AF 555. Panel A, FACS scatter. Panel B, calculated percent inhibition. Panel C, confocal images of CD32 inhibition of C-HBV binding.

MHC I and MHC II was evaluated by confocal microscopy. Following stimulation with either LPS (positive control) or C-HBV for 24 h, there was a substantial increase in the intensity of MHC I and MHC II on the surface of DCs compared to untreated cells. The co-localization of C-HBV with MHC I and MHC II is shown in Figure 9. Treatment with the protein synthesis inhibitor cycloheximide inhibited the expression of the MHC I and II which indicated that iDCs synthesized new MHC molecules that migrated to the cell surface after stimulation (Figure 9).

T cell responses to C-HBV in healthy uninfected donor cells, ex vivo

The antigen presentation assay (APA) quantifies functional T cell immune responses and the ability of antigen-loaded mature DCs (mDC) to expand antigen-specific T cell clones. Peripheral blood mononuclear cell (PBMC)-derived monocytes from healthy (HBsAg, HBV DNA, and HbAb-negative) donors were differentiated to iDCs, loaded with antigen and further differentiated into mDCs using Poly (I:C), and these antigen-loaded mDCs were cultured together with autologous naïve T cells. For the analysis of T cell function and specificity, proliferation and production of IFN- γ , TNF- α , perforin (Pfn) and granzyme B (GrB) was assessed following re-stimulation with

antigen-loaded mDCs, and peptide-specific responses were assessed using MHC I pentamers.

Both CD4+ and CD8+ CFSE-labeled T cells responded to C-HBV with a higher percentage of proliferation than observed with either dialysis buffer or any of the individual components (HBV S1/S2, HBV core protein or mTBD) (Figure 10A,B). Generally, CD4 + T cells displayed a higher percentage of proliferation than CD8 + T cells (Figure 10A,B). These results show that antigen presentation of C-HBV by DCs to naïve T cells result in their activation and proliferation.

T cell activation was further evaluated by measuring the production of the Th1 cytokines IFN- γ and TNF- α following C-HBV antigen presentation by DCs, as these cytokines were shown to be involved in the elimination of cccDNA without cytotoxicity.^{56,57} Compared to buffer and controls (HBV S1/S2, HBV core protein or mTBD), stimulation with C-HBV induced a substantially higher percentage of IFN- γ producing CD4+ and CD8 + T cells (Figure 10C,D). Similarly, C-HBV induced a marked increase in the percentage of CD4+ and CD8 + T cells producing TNF- α (Figure 10E,F). This further confirmed the ability of C-HBV to activate both CD8+ and CD4 + T cells. The emphasis of the present study was on the cellular response as measured by Th1 cytokines in terms of their relevance to chronic hepatitis B, as originally suggested by Chisari and coworkers⁵⁸ and recently shown by Xia *et al.*⁵⁶ Nevertheless, analysis of Th2

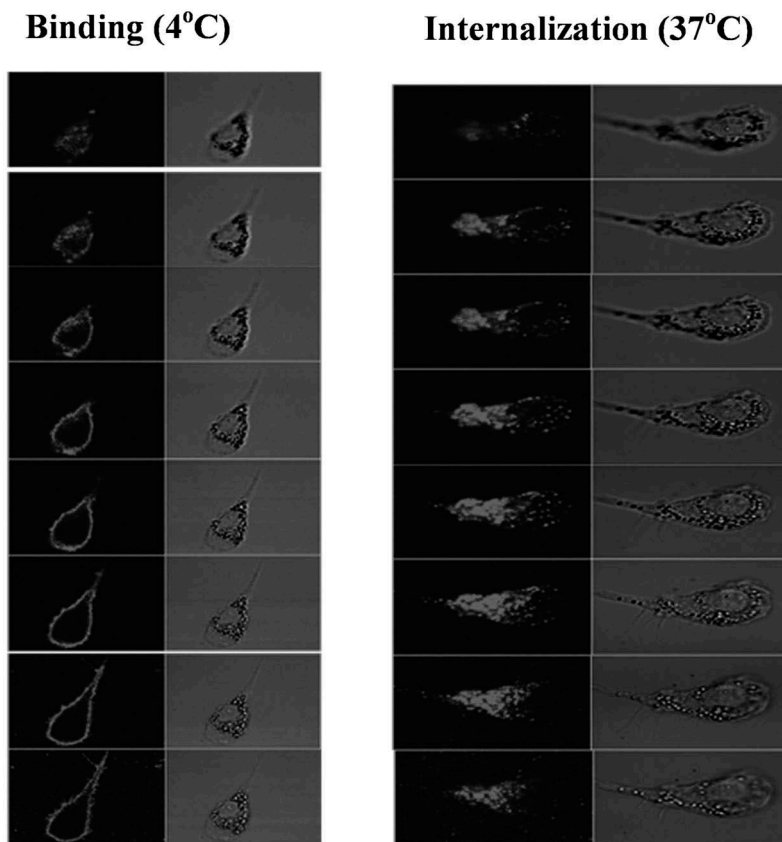


Figure 6. Binding and internalization of C-HBV by iDCs—confocal microscopy.

Immature DCs were incubated in PBSB with C-HBV at 4°C for binding and 37°C for internalization. C-HBV binding was detected by incubation of the cells with F(ab')₂ fragment of goat anti-mouse AF 488. Internalization was stopped by soaking the cells with cold PBS. Cells were fixed and C-HBV detected with anti-mouse IgG Fab AF 555. The z-stack (interval = 1 μm) is presented.

cytokines might have revealed further aspects of the immune response, even if such data do not fit the hypothesis that Th1 response is more relevant to the clearance of chronic hepatitis B. Both Th1 and Th2 responses to C-HBV were evaluated in sheep,⁵⁹ by measuring lymphocyte proliferation and IFN-γ secretion, as well as the production of HBV antigen-specific antibodies. The results in sheep show a mixed Th1/Th2 response, but nature of the involvement of Th2 cytokines could not be predicted, as this data is not available.

The expression of GrB and Pfn in T cells was also quantified as a means to assess the capability of C-HBV to induce a cytotoxic T cell response. There was marked induction of both GrB and Pfn in CD8 + T cells following stimulation with C-HBV and tetanus toxoid (TT, positive control) compared to HBV S1/S2, HBV core or mTBD (Figure 10G,H). Compared to TT and component antigens in the fusion protein, C-HBV induced a higher percentage of CD8 + T cells expressing GrB and Pfn. There was a significant increase in the population of HBV core peptide-specific pentamer-positive CD8 + T cells from C-HBV-stimulated cultures (Figure 11). The results from the *ex vivo* APA using cells from healthy HBV un-infected donors show that the C-HBV fusion protein is internalized and processed by DCs promoting proliferation and activation of both CD8+ and CD4 + T cells and differentiation to effector T cells. The data showing internalization of C-HBV, co-localization with MHC

I and II, together with our T cell functional data, suggest that the DCs would have processed the protein for presentation in MHC I and II. The identification of core peptide-specific cells by pentamer staining suggests the processing of C-HBV. Furthermore, the recall responses to the peptide pools indirectly suggest that the processed peptides from HBV antigens were instrumental in defining the specificity of the activated T cells.

T cell responses to C-HBV in PBMCs isolated from chronic HBV carriers

Having shown the nature of the action of C-HBV in cells from HBV un-infected donors, the effect of C-HBV in PBMCs derived from chronically HBV-infected individuals was tested. Chronic HBV infection results in T cell exhaustion.¹² An effective immunotherapy for chronic HBV would ideally restore HBV-specific T cell responses. The ability of C-HBV to induce T cell responses was evaluated using the APA with PBMCs-derived DCs and T cells from chronic HBV carriers. T cells isolated from chronically HBV-infected individual donors (n = 9) stimulated *ex vivo* with C-HBV-pulsed DCs induced proliferation of both CD4+ and CD8+ CFSE-labeled T cells compared to the respective controls (dialysis buffer, HBV S1/S2, HBV core protein or mTBD) (Figure 12A,B). With cells isolated from healthy HBV-seronegative donors,

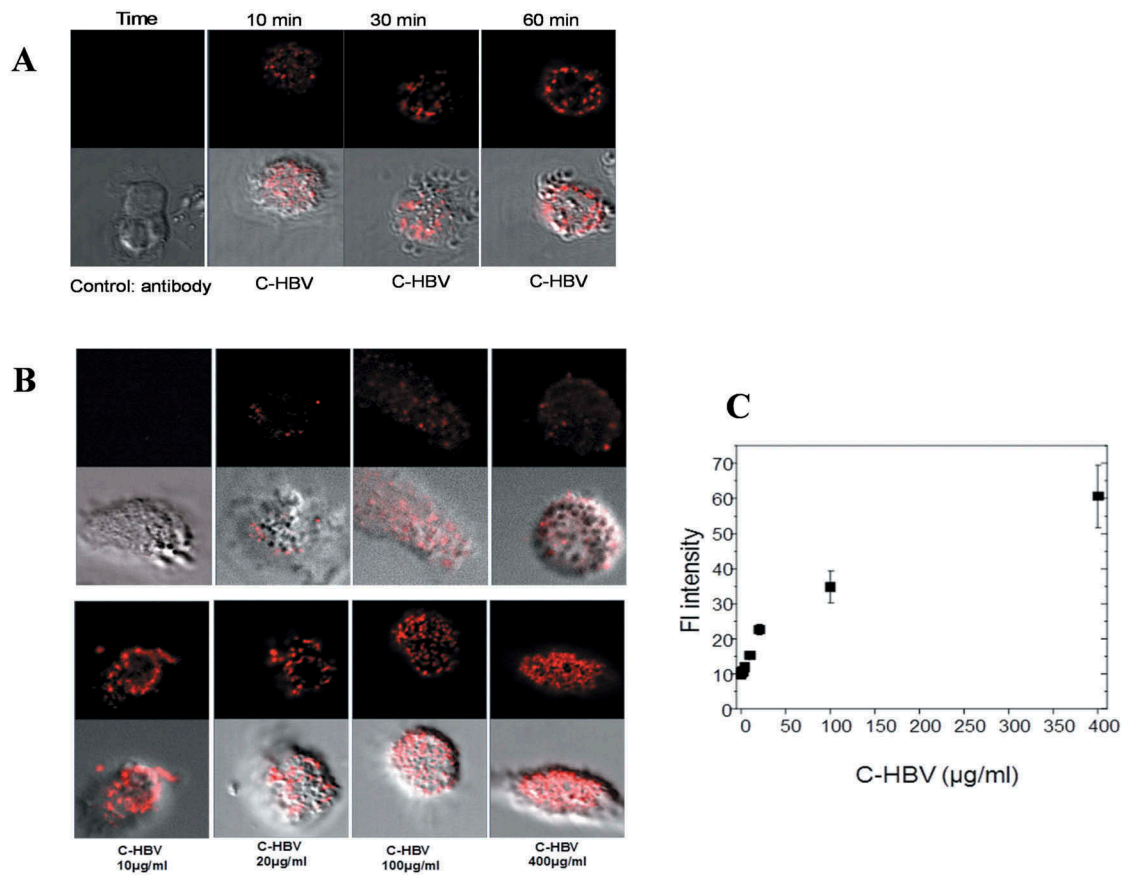


Figure 7. Time and concentration-dependent internalization of C-HBV by iDCs-confocal microscopy.

Immature DCs were incubated with C-HBV at 4°C. Unbound C-HBV was washed off and incubation was continued at 37°C. After 10, 30, 60 min of incubation, the internalization was stopped by soaking the cells with cold PBS. At the end of the last time point (60 min), cells were fixed and permeabilized and labeled with anti-mouse IgG1 AF 555 for confocal microscopy. Immature DCs were incubated with increasing concentrations of C-HBV (1, 2, 4, 10, 20, 100, 400 µg/mL) at 4°C. Unbound C-HBV was washed off and incubation was continued at 37°C. After 60 min of incubation, the internalization was stopped by soaking the cells with cold PBS, cells were fixed and permeabilized and labeled with anti-mouse IgG1 AF 555 for confocal microscopy. Panel A. Time-dependent internalization, Panel B. C-HBV concentration-dependence Panel C. Binding as a function of concentration.

CD4 + T cells displayed a higher percentage of proliferation than CD8 + T cells (Figure 10A,B). Similarly, using the APA *ex vivo*, after two stimulations of PBMCs, C-HBV induced a higher percentage of IFN- γ ⁺ (Figure 12C,D) and TNF- α ⁺ (Figure 12E,F) producing CD4⁺ and CD8 + T cells than either the buffer control, or any of the components of C-HBV (HBV S1/S2, HBV core protein or mTBD). The secretion of IFN- γ by PBMCs from chronically HBV-infected donors also showed similar pattern (data not presented). These results demonstrate that C-HBV is able to induce T cell Th1 cytokine production in PBMCs from chronically HBV-infected individuals.

C-HBV peptide epitope-specificity of T cell responses

The specificity of the CD8+/CD4 + T cells generated in the *ex vivo* assays were assessed following the re-stimulation of C-HBV-primed PBMCs with overlapping peptide pools containing HBV S1/S2 fragments or core sequences. The pools containing peptides (15 amino acid, 5 amino acid overlap, 9–14 peptides per pool), were used for each of the HBV antigens (S1/S2/Core). PBMCs from chronic HBV carriers (n = 6) were cultured in the presence of C-HBV, S1/S2, core antigen or buffer and recall responses were then assessed following

incubation of cells with the overlapping peptide pools from HBV S1/S2 fragments and core antigen by determining the percentage of IFN- γ producing CD8+ and CD4 + T cells. The PBMCs which were treated with C-HBV showed recall responses to many of the peptide pools (Figure 13A,B). In general, responses were more pronounced with peptides derived from HBV core, although S1/S2 peptides showed a recall response. These results suggest that C-HBV is able to induce HBV-specific T cell response, directed against multiple epitopes, especially within HBV core, shown to be important for the resolution of chronic HBV infection.³

Induction of functional responder T cells (T resp) by C-HBV

Regulatory T cells (Treg) have been shown to contribute to T cell dysfunction and HBV persistence by suppressing virus-specific T cell responses in chronically HBV-infected patients.¹⁵ Activated CD4 responder T cells (Tresp, CD4+ CD25-) were shown to produce GrB and actively remove effector regulatory T cells (Treg, CD4+ CD25+ FoxP3+) by apoptosis.⁶⁰ PBMCs isolated from two representative donors (Figure 14A, donor #14) and (Figure 14B, donor #23) were stimulated *ex vivo* with C-HBV and the respective controls. There was

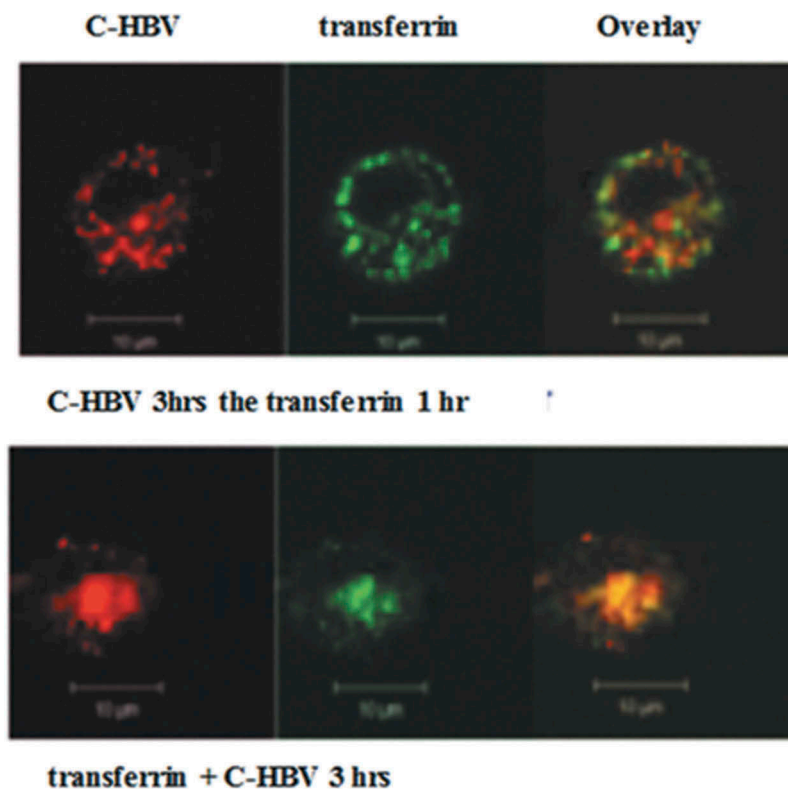


Figure 8. Co-localization of internalized C-HBV and transferrin-confocal microscopy.

In order to study receptor mediated endocytosis of C-HBV, C-HBV loaded DCs were incubated with anti-Fc Fab IgG-AF 555 for 3 h at 37°C/7% CO₂, washed, and incubated with AF 488 transferrin conjugate for 1 h at 37°C/7% CO₂ or incubated with both anti-Fc Fab IgG-AF 555 and AF 488 transferrin conjugate for 3 h at 37°C/7% CO₂. After washing, cells were fixed with 4% paraformaldehyde (PF) for 30 min at room temperature, mounted and analyzed by confocal microscopy.

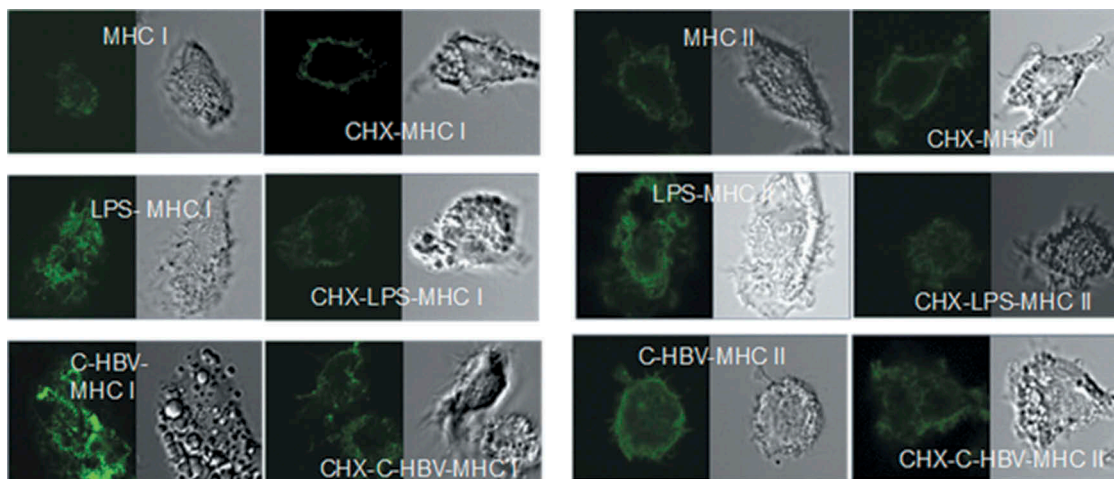


Figure 9. Co-localization of C-HBV and MHC class I & II-confocal microscopy.

The expression of MHC I & II molecules and their co-localization with C-HBV was examined using confocal microscopy. Immature DCs were incubated in AIM V media with donor-matched serum and cytokines (IL-4, GM-CSF) for 3 d and then incubated in RPMI media alone (control), with LPS (100 ng/mL), or with C-HBV (2 μg/mL) at 37°C for 4, 8 or 24 h. Cycloheximide (5 μg/mL) was added 30 min prior to initiation of experiment and remained present in the media during incubation. The cells were permeabilized, labeled with mouse anti-human HLA-DR (MHC II) Pacific Blue, mouse anti-human HLA-ABC (MHC I) AF 647, mouse anti-human calnexin (ER marker) FITC, LAMP (late endosome/lysosome) AF 488 or goat anti-mouse IgG1 (C-HBV) AF 555 and visualized by confocal microscopy.

a significant increase in the GrB production seen in the C-HBV treated group (Figure 14A,B). These results demonstrate that C-HBV is able to induce GrB production in Tresp cells in PBMCs from chronically HBV-infected individuals. Staining for Annexin V⁺ cells was used to monitor the apoptosis of Treg cells by activated Tresp cells. There was an increase in

Annexin V stained cells in CD4⁺ CD25⁺ cells (Treg phenotype) from C-HBV-stimulated PBMCs compared to the respective controls in chronic carrier PBMCs (Figure 14C). These results suggest that the apoptosis of Tregs may be mediated by C-HBV-activated Tresp cells and may possibly play a role in breaking tolerance to chronic HBV infection.

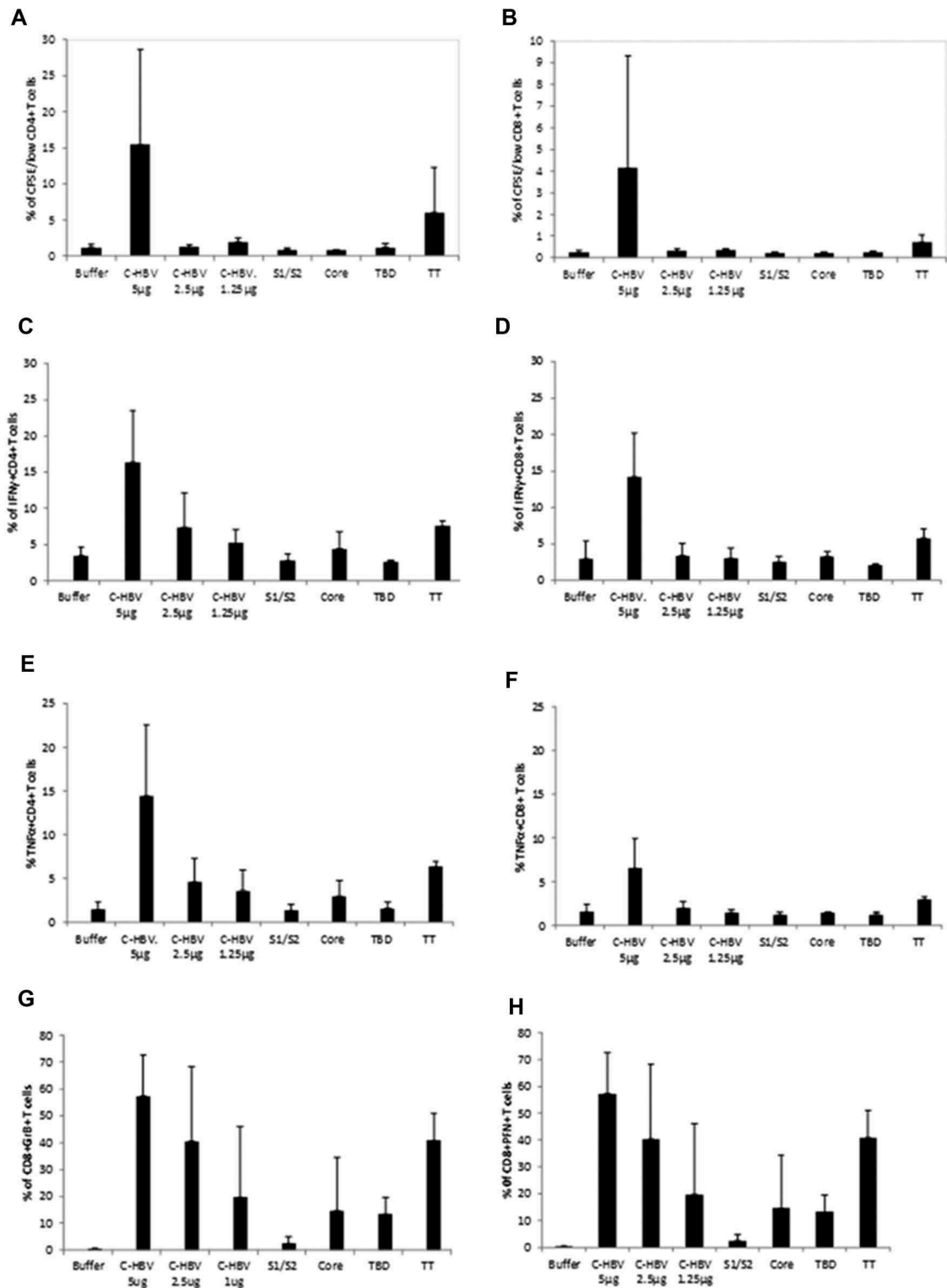


Figure 10. C-HBV- induced T cell proliferation, production of IFN- γ , TNF- α , GrB and Pfn in T cells.

CFSE-labeled T cells from an uninfected donor were incubated with antigen-loaded mDCs for 10 d. Cells were harvested and the degree of proliferation was assessed by loss of CFSE staining using flow cytometry. The individual components of C-HBV were used as controls (HBV S1/S2, HBV Core, TBD). Tetanus toxoid (TT) was used as a positive control for T cell activation. Each bar represents results from the average of triplicate wells within group (Panel A and B). T cells from an uninfected healthy donor were incubated with C-HBV-loaded mDCs for 10 d and then re-stimulated with C-HBV-loaded mDCs for 6 h. The percentage of T cells (CD4+ and CD8+) producing IFN- γ (Panel C and D) or TNF- α (Panel E and F) was determined by intracellular cytokine staining followed by flow cytometry. Each bar represents results from the average of triplicate wells within group. CD8+ T cells from an uninfected healthy donor were incubated with C-HBV-loaded mDCs for 10 d and then re-stimulated with C-HBV-loaded mDCs for 4 d. The percentage of T cells producing GrB (Panel G) or Pfn (Panel H) was determined by intracellular cytokine staining followed by flow cytometry.

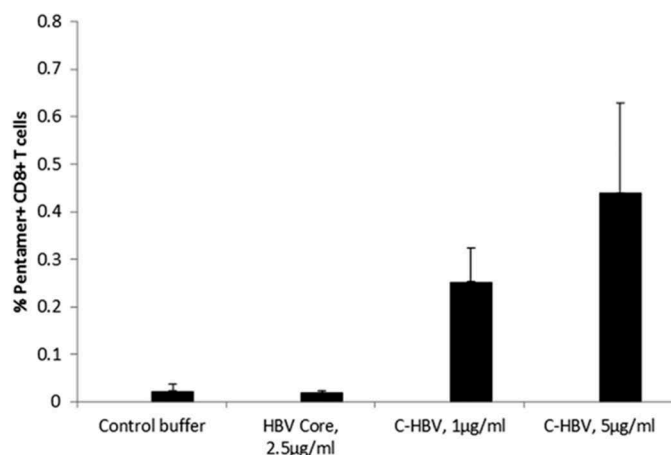


Figure 11. Generation of HBV core peptide pentamer-specific T cells.

CD8 + T cells from an uninfected healthy donor were stimulated twice with DCs loaded with C-HBV, HBV core or buffer control. After 7 d of incubation at 37°C, T cells were harvested and incubated with the HBV core pentamer. Subsequently, cells were labeled with a PE-Fluorotag and CD8-specific mAb. The percentage of pentamer-labeled CD8 + T cells was determined by FACS.

CTL-mediated cytolytic activity induced by C-HBV

Cell lysis under various conditions,^{48–52,61} including natural killer (NK) cell-mediated cytolytic activity⁵³ can be assessed using a real-time cell electronic sensing (RT-CES) system (xCELLigence, Roche). A DC-based RT-CES cytolytic assay was developed to study the effects of C-HBV on the induction of T cell-mediated cell killing using cells derived from PBMCs isolated from an HBV un-infected healthy donor (control) and HBV chronic carriers (n = 6). In this assay, PBMC-derived mDCs, loaded with different concentrations of C-HBV or buffer, were used as target cells, and T cells isolated from an uninfected donor or whole PBMCs from chronic HBV carriers were used as effector cells. Lysis of the target cell monolayer was monitored as described in the methods section. Cell indices from an un-infected donor (Figure 15A) and a representative HBV-carrier are presented (Figure 15D). The cell killing was similar in the other HBV-carrier samples (data not shown). The expression of GrB among the T cell population in un-infected (Figure 15B,C) and in HBV carrier (Figure 15E,F) is shown. The decrease in cellular index as a function of time, both in the un-infected and the HBV-infected donor cells, and the expression of GrB, suggests that C-HBV is able to promote the induction of cytotoxic T cell responses.

Discussion

Chronic hepatitis B remains a major healthcare concern worldwide, and in spite of the availability of very effective prophylactic vaccines over 250 million people are estimated to remain chronically infected.¹ Currently available antiviral agents can efficiently control virus replication but the induction of durable off-treatment responses remains unattainable in the vast majority of chronic hepatitis B carriers. Many novel therapies that target the HBV life cycle and host immune response are currently in various stages of clinical development.^{19,62} Strategies to develop agents for functional

cure of hepatitis B include the use of novel adjuvants and routes of administration,⁶³ HBV antigens produced *in situ* using vector plasmids,^{64,65} various RNAi technologies,⁶⁶ Toll-like receptor (TLR) effectors,⁶⁷ mAbs⁶⁸ and inhibitors of core capsid assembly.⁶⁹

The mechanisms HBV uses to avoid immune surveillance are not well understood.⁷⁰ A high viral load and the resulting increase in the concentration of viral antigens may make the immune system tolerant to the virus. Predictors of responsiveness to interferon therapy include a low baseline viral load, high ALT levels, younger age and HBV genotype amongst others.⁷¹ In pivotal clinical trials of *de novo* combination therapy of pegylated interferon with lamivudine,^{72–74} adefovir,⁷⁵ or entecavir⁷⁶ did not improve sustained virological response rates compared to monotherapy but recently interferon combined with tenofovir has been shown to improve post-treatment HBsAg clearance rates during longer-term follow-up,^{77,78} and in long-term nucleoside/nucleotide suppressed patients a switch to interferon or interferon “add on” therapy may enhance response rates in selected patients.^{71,79} This suggests that interferon-mediated immune clearance may be more effective in clearing the virus when the viral load has been reduced for extended periods.

Many mechanisms may contribute to the development of HBV tolerance including an immunosuppressive role mediated by regulatory T cells (Tregs) which exert suppressive function on immune responses that may cause persistent HBV infection⁸⁰ and the suppressive function of Tregs can be enhanced by virus infection.⁸¹ A number of studies have shown that Tregs in PBMCs isolated from HBV-infected patients inhibited HBV-specific immune response.^{82–84} These results suggest that the modulation of Treg might be a potential therapeutic strategy for the treatment of chronically infected patients. The lack of proper presentation of the appropriate viral antigen to the host immune system may also be a contributing factor. It has been reported that the functional defects of antigen-presenting cells, such as DCs, may cause the impaired T cell response during HBV infection.^{22,85}

Although treatment regimens for chronic hepatitis B have markedly improved outcomes during the last decade, the need for immune system activation to effectively induce sustained off-treatment disease remission and clearance of the hepatitis B surface antigen (“functional cure”) still remains a largely unachieved target.^{86,87} The host immune system plays a dominant role in the elimination of HBV infection. In acute HBV infections in adults, more than 90% of the infections can be cleared by the host immune system, whereas in infants with the immune system not fully functional the clearance rate is less than 10%.^{11,88,89} During clinical trials of lamivudine, it was noted that patients with elevated liver enzymes, a measure of immune system stimulation, cleared the HBV infection at a rate greater than patients with normal liver enzymes.⁹⁰ This observation emphasizes the need for the host immune system to be activated against viral antigens which are present in the chronic carrier which the immune system regards as “self” rather than “foreign”. Since impaired immune responses (immune tolerance/ignorance) prevail in chronic HBV carriers, any immunotherapy approach must be able to re-educate the host immune system to recognize the

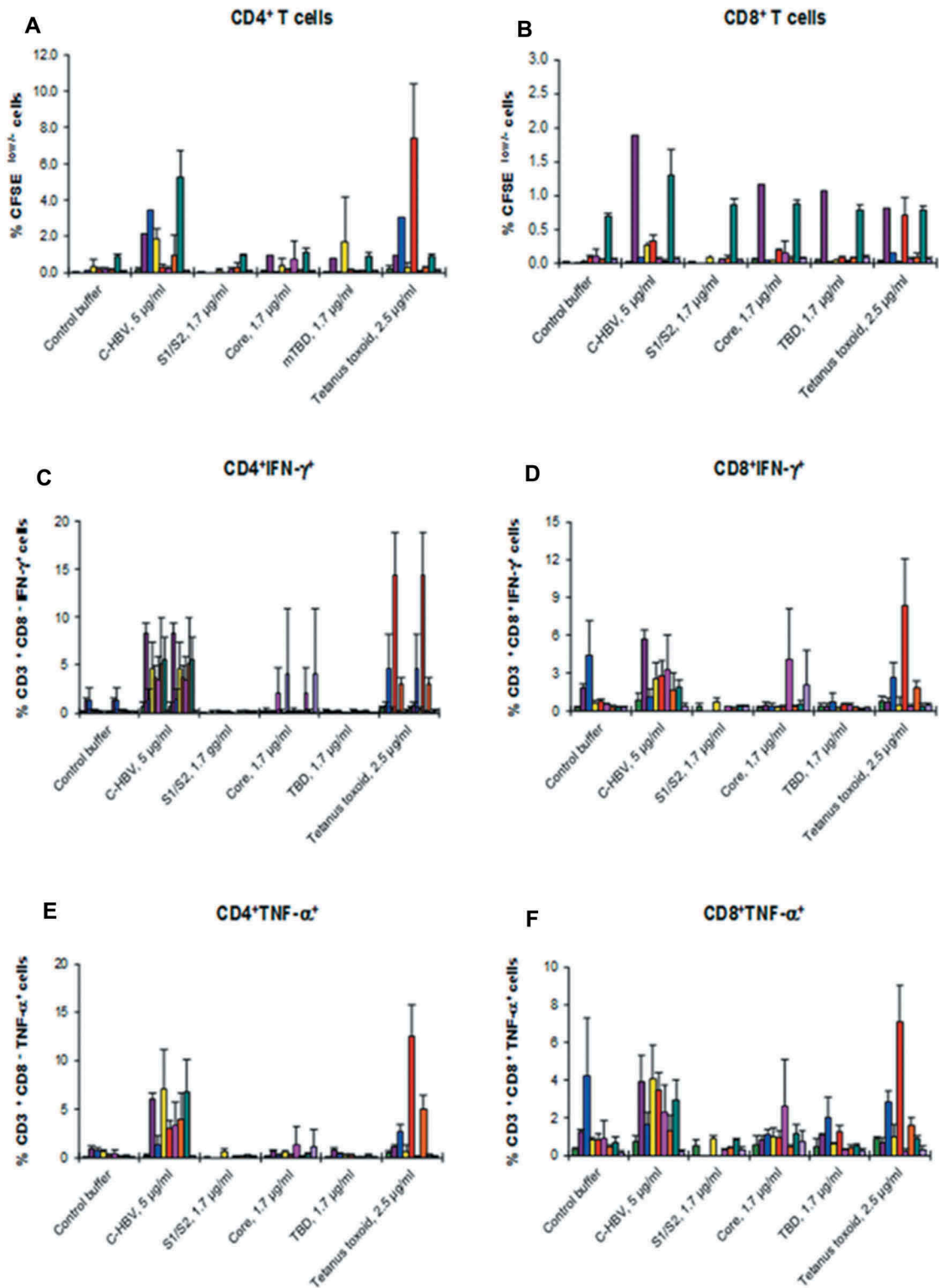


Figure 12. Induction of T cell proliferation and production of IFN- γ and TNF- α positive T cells by C-HBV in PBMCs from chronically HBV-infected donors. CFSE-labeled PBMCs from chronic HBV-infected patients ($n = 9$) were incubated with C-HBV or components for 7 d. Cells were harvested and the percentage of CFSE^{low/-} CD4⁺ (Panel A) and CD8⁺ (Panel B) T cells were determined by flow cytometry. Each colored bar represents a different donor. PBMCs were incubated with C-HBV for 10 d and then re-stimulated with mature C-HBV-loaded DCs for 6 h. The percentage of CD4⁺ (Panel C) or CD8⁺ (Panel D) T cells producing IFN- γ was determined by intracellular cytokine staining and flow cytometry. The percentage of CD4⁺ (Panel E) or CD8⁺ (Panel F) T cells producing TNF- α was determined by intracellular cytokine staining and flow cytometry. Data was derived from the average of triplicate wells. Each colored bar represents a different donor.

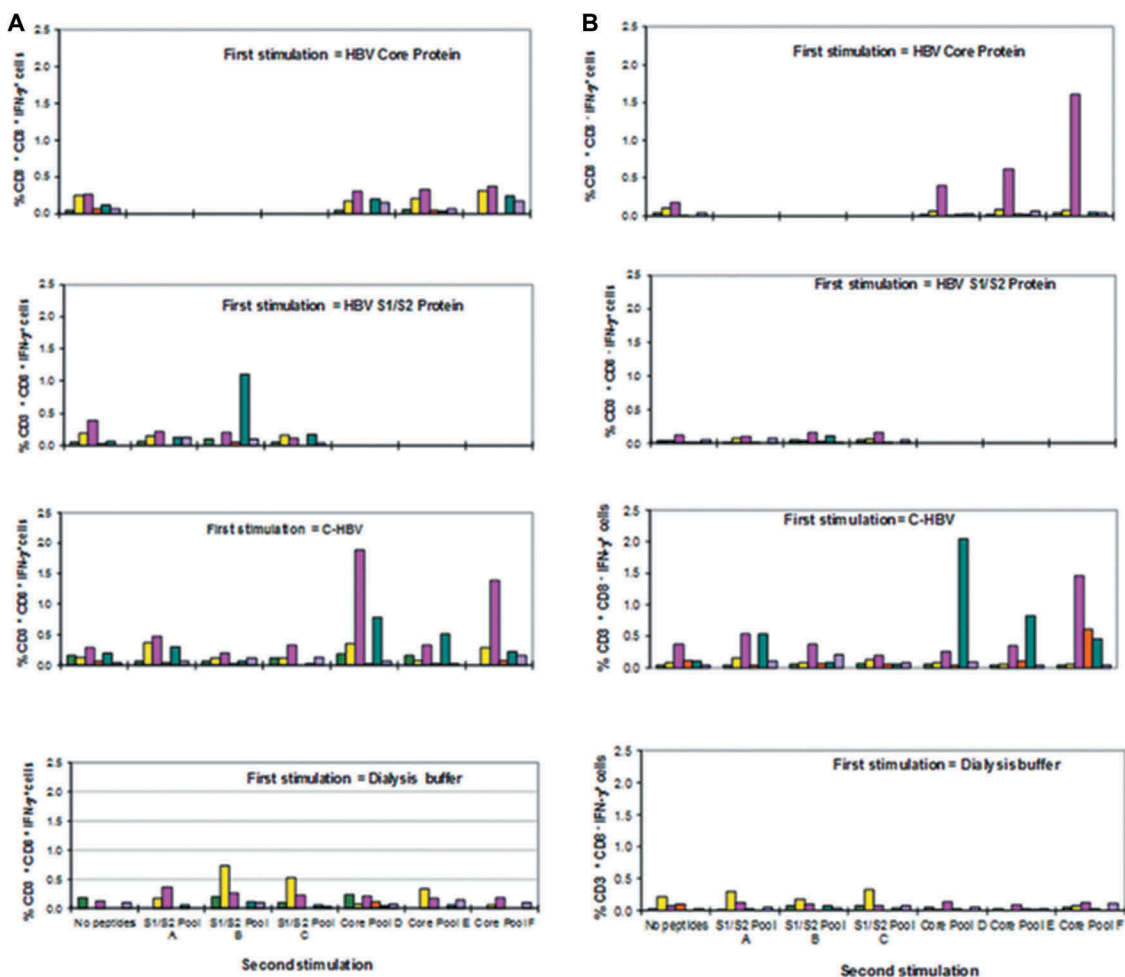


Figure 13. HBV peptide-specific T cell recall responses in chronic HBV carrier PBMC-derived T cells activated by C-HBV.

PBMCs from chronically infected donors were incubated first with either buffer (control), HBV core antigen, S1/S2 protein or C-HBV for 10 d. Pools of overlapping peptides (15 amino acids, 5 amino acid overlap) from S1/S2 and Core were added to the PBMC cultures and the cultures were incubated for 6 h. The percentage of CD8⁺ (Panel A) and CD4⁺ (Panel B) T cells producing IFN- γ was determined by intracellular cytokine staining and flow cytometry. Data was derived from the average of triplicate wells. Each colored bar represents a different donor.

virus and the viral antigens as “foreign” (to break tolerance/ignorance) so that newly activated CTL can clear infected cells and the humoral response can eliminate the circulating antigens.¹⁷

The correlation between immune responses and the spontaneous clearance of chronic HBV infection supports the renewed interest in developing HBV immunotherapy agents.²¹ These include antibodies, protein, and peptides with adjuvants, DNA and viral-vectored molecules which have been used in combination with currently available antiviral agents to decrease viremia. So far, none of these approaches have produced the expected results, and various explanations have been provided to explain the lack of efficacy of these molecules.^{21,91}

A number of therapeutic product candidates have been listed in a recent publication by Boni et al.²¹ The most advanced product candidate ABX 203 had advanced to Phase III clinical trial. ABX 203 is a mixture of 2 Virus Like Particles (VLPs) from HBsAg and HBcAg. The HBsAg is purified from *Pichia pastoris*, whereas the HBcAg is purified from *E. coli*. The vaccine is nasally and subcutaneously

administered.⁶³ This trial was recently terminated based on a futility analysis. Heat-inactivated whole yeast particles (Tarmogens) expressing HBV X, surface and core antigens⁹² was used in a Phase II clinical trial. As there was no significant decrease in serum HbsAg, the development was terminated due to lack of clinical efficacy.⁹³ One of the observations was that, although there was CD8 + T cell activation, there was neither CD4 + T cell activation nor antibody production. It is possible that the immune reaction to the large number of yeast particles overwhelmed the host immune system, with minimal response to the expressed HBV proteins. Yeast Immune Complex (YIC), an “Antigen-antibody (HBsAg-HBIG) immunogenic complex” with Alum as adjuvant, did not show clinical efficacy in a Phase III clinical trial.⁹⁴ The observation that “placebo” group showed superior response than treated group, implied lack of efficacy. Among the vectored vaccines, TG1050 has completed Phase Ib trial.⁹⁵ TG1050 is an adenovirus serotype 5 vectored vaccine encoding truncated HBV core, a modified HBV DNA polymerase and two HBV envelope domains. IFN- γ producing HBV-specific T cells were produced, but only minor decreases in

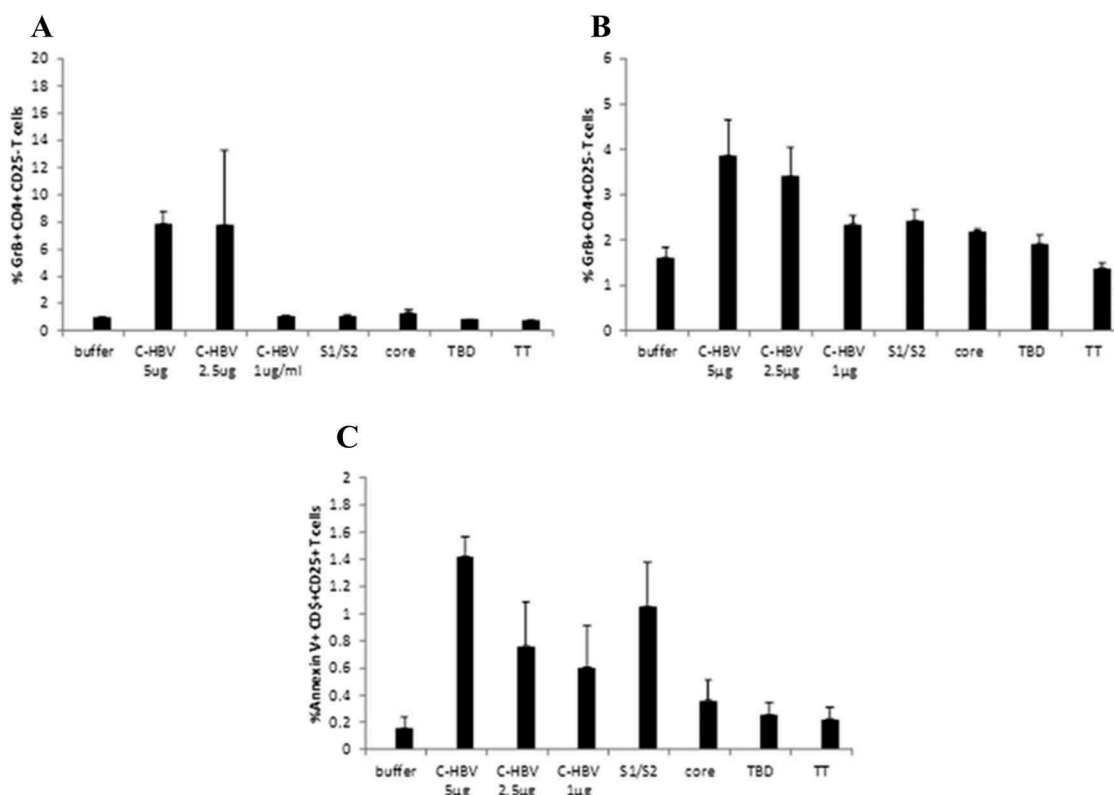


Figure 14. Production of GrB positive T responder cells by C-HBV in PBMCs from chronic HBV carriers. PBMCs from donor CHBV#14 (Panel A) and #23 (Panel B) were stimulated *ex vivo* with either dialysis buffer, C-HBV or components for 7 d. The percentage GrB+ CD4+ CD25+ T responder cells (Tresp) was determined by intracellular cytokine staining and flow cytometry. A batch of PBMCs from donor #23 was stained for Annexin V. The percentage of CD4+ CD25+ Annexin V+ (Treg cell phenotype) was determined by flow cytometry (Panel C). Each bar represents results from average of triplicate wells from the same group.

HBsAg was observed. Further clinical trials using a larger number of patients would provide a better understanding of the efficacy. The major issue in using adenoviral vector is the preexisting immunity against the vector, which can abrogate any clinical efficacy.^{96–98}

It has been reported that adjuvant-based vaccines can induce specific T cell responses to cancer cells, but most of the antigen-specific T-cells were sequestered, dysfunctional and deleted at the vaccination site due to the persisting “depot effect” of the adjuvanted vaccine⁹⁹ which is a possible explanation for the lack of efficacy of some of the listed HBV therapeutic candidates.

The success in restoring effective antigen-specific immune control of the viral infection may depend on various strategies including those that target antigen processing and presentation by the APCs for effective generation of CTLs, down-regulation of Tregs and induction of effective humoral responses.

The technology described herein employs the use of Fc fusion proteins to target DC receptors for antigen delivery and activation of immune responses. This platform technology combines the benefits of both recombinant (neo)-antigens and the xenotypic antibody in inducing a broad and antigen-specific immune response. Targeted delivery of antigens to DCs has been a challenge, although several studies have shown that antigen-targeting to DCs can be employed to enhance T cell priming and induction of effector T cell and humoral responses.^{28,100} The fusion protein (C-HBV) is

produced in Sf9 insect cells which have less complex N-glycosylation (terminal mannose, no sialic acid) than eukaryotic cells,³² switching the nature of the protein while maintaining the primary amino acid sequence intact. The glycosylation targets lectin receptors on DCs and the primitive nature of the glycosylation makes the whole molecule highly immunogenic in comparison to the mammalian or yeast-derived HBV antigens. Chimigen® Technology is designed such that the protein specifically targets DC receptors so that it is properly processed and presented by MHC I and II to generate multi-antigen, multi-epitope-specific T and B cell immune responses. Chimigen® Technology is a unique approach to vaccine and immunotherapy development which is expected to avoid many of the drawbacks seen with previous attempts.

Various antibody-based therapies partially achieve this goal by binding to the respective antigen and the antigen-antibody complex entering the DCs via receptor-mediated uptake.¹⁰¹ For example, Trastuzumab (Herceptin), a humanized IgG1 k mAb that binds to the extracellular domain of epidermal growth factor 2 (HER2), apart from inhibition of HER2 downstream signaling and antibody-dependent cell-mediated cytotoxicity, has recently been shown to enhance FcR-mediated uptake and cross presentation by HER2-derived peptides by DCs, thereby enhancing the generation of peptide-specific CTL.¹⁰² There are a large number of clinical trials currently in progress using mAb for various cancer and infectious diseases applications.^{103–105} Antibody-based

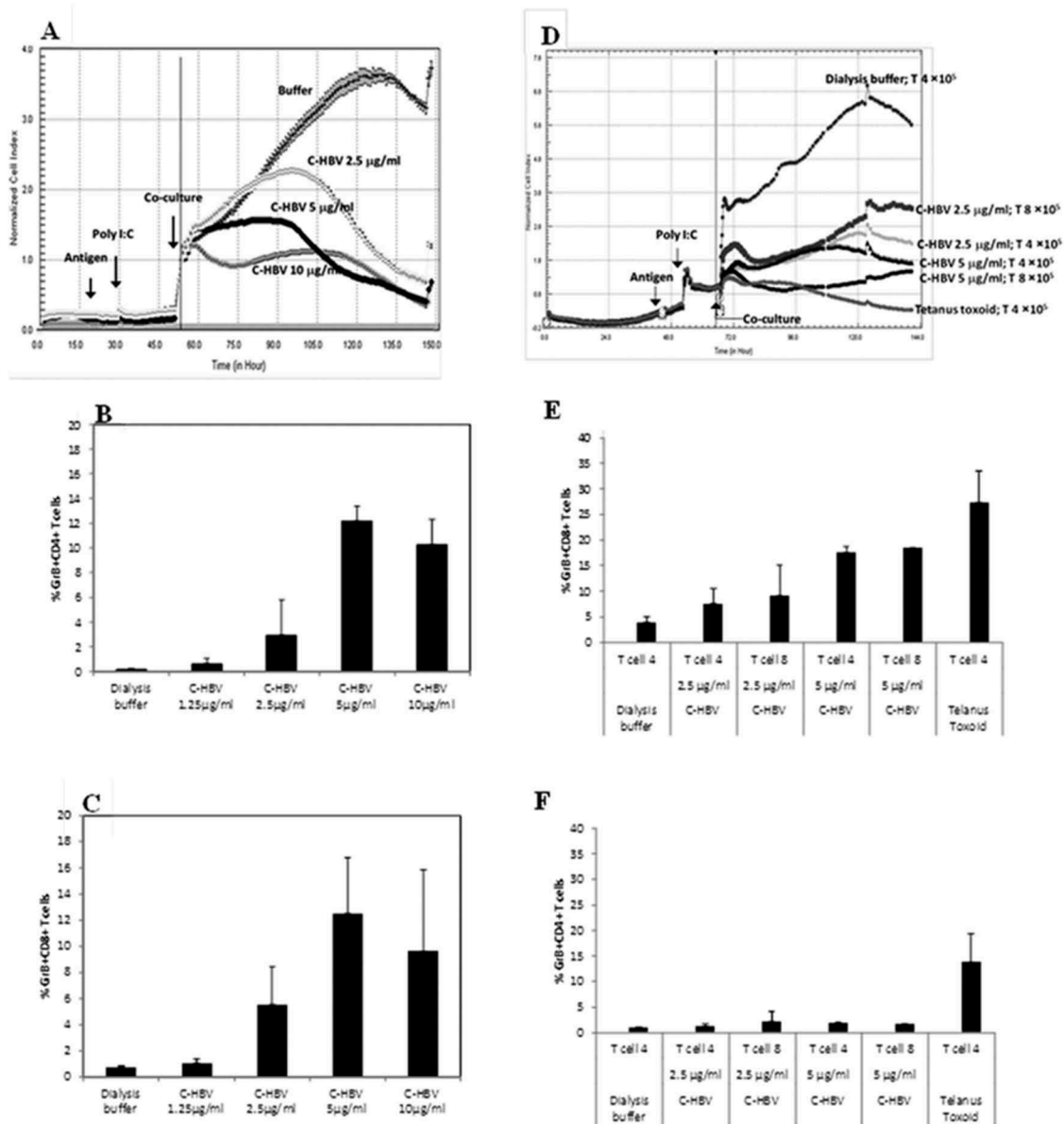


Figure 15. RT-CES dynamic monitoring of apoptosis by C-HBV-activated T cells.

Immature DCs (1×10^5 /well) were seeded and cultured in the wells of E-plates. After 20 h incubation at 37°C , iDCs were treated with different concentrations of C-HBV or dialysis buffer followed by maturation with poly (I:C), to allow the establishment of a monolayer of cells and the baseline cell index (CI) was recorded. At the indicated time point, C-HBV-activated non-purified (PBMCs) T cells (un-infected donor, Panel A) or C-HBV-activated T cells (HBV-infected donor, Panel D) were added into the antigen-loaded mDC culture monolayers. Changes in CI were monitored every 1 h using the RT-CES system. GrB production in CD4+ (Panel B) and CD8+ (Panel C) T cells from un-infected donor are shown. GrB production in HBV carrier donor CD4+ (Panel E) and CD8+ (Panel F) T cells are presented.

therapies require the antigen to be complexed *in situ* for efficient delivery to DCs. With levels of circulating antigens varying among individuals the efficiency of uptake by the DCs also depends on the nature of the complex. Aggregates of the complex could be engulfed by scavenger cells and macrophages resulting in a lack of appropriate antigen presentation.¹⁰⁶ Antigen-antibody complexes can bind to DC receptors which result in appropriate antigen uptake, processing and productive presentation, whereas aggregates may be taken up via micropinocytosis for degradation in lysosomes resulting in poor antigen presentation.¹⁰⁶ Another problem is that if the antibody is in excess of antigen, T cell responses can be inhibited.¹⁰⁷ In antibody-based therapies, the administered antibody concentration has to be adjusted to each

patient to optimally complex antibody with circulating auto-antigen.¹⁰⁸ In the case of Chimigen® Technology, this variable is avoided since the stoichiometry of antigen/antibody is maintained in the protein molecule. An added advantage is that this does not have to rely on circulating antigen for presentation.

C-HBV is a protein immunotherapy with no adjuvant and therefore, the depot effect is avoided.⁹⁹ Avoiding a depot effect of adjuvant would thus be a definite advantage. Unlike a protein administered with adjuvant, C-HBV would not be expected to remain at the site of injection but instead, be readily taken up by DCs as observed in a large animal model (sheep) following C-HBV injection.⁵⁹

The generation of HBV-specific T cells (IFN- γ , TNF- α , GrB, Pfn +ve) is the most relevant for the predicted anti-HBV immunotherapeutic effect. Further, the *ex vivo* assays demonstrated that not only do T cells proliferate, but they also differentiate into effector cells secreting antiviral cytokines and mediators of cytolytic activity.

C-HBV contained N-terminal 6xHis tag to facilitate the purification using Ni-chelate affinity chromatography. The protein showed no toxic effect either in *ex vivo* studies using human PBMCs, or *in vivo* studies using sheep.⁵⁹ Furthermore, the antibody responses observed against the 6xHis were of much lower magnitude than any of the HBV antigens. The presence of the tag could be an issue if used in clinical trials but there are products with His tag that have reached Phase 2, including one for HBV,^{92,93} with no safety issue identified and none has reached Phase 3 so far.

One of the limitations of the current study is the lack of Th2 cytokine data. The present study focused on the induction of Th1 immune responses by C-HBV, measured as the production of Th1 cytokines IFN- γ and TNF- α . The rationale was to assess for markers of cellular immunity and these were the most relevant.^{56,58} Analysis of Th2 cytokines might have provided additional information on the immune responses, even if such data would not fit the current hypothesis that Th1 responses are more meaningful in chronic hepatitis B treatment. Although the present study shows compelling results *ex vivo* in human cells for an effective immunotherapy for chronic hepatitis B, one other limitation of the study is that the clinical efficacy of this approach remains to be proven. The next step is to test C-HBV in clinical trials in humans.

Conclusion

The experimental results presented in this report demonstrate that C-HBV binds to specific receptors CD32 and CD206 on DCs, and subsequently is internalized and processed for presentation on both MHC I and II. Presentation of C-HBV to T cells from HBV un-infected healthy donors or chronic HBV carriers resulted in the proliferation of CD4+ and CD8 + T cells and the production of IFN- γ , TNF- α , GrB and Pfn, consistent with CTL function. The data presented herein provides “proof of concept” of the Chimigen® Technology platform for DC-receptor targeted delivery of antigen(s) and subsequent induction of effective CD4+ and CD8 + T cell responses. The induction of CD4 + T cells from chronic HBV carrier PBMCs along with the production of T responder cells and the targeted removal of cells with a T regulatory phenotype provides preliminary supportive evidence for a role of C-HBV to break immune tolerance to HBV.

Abbreviations

| | |
|-------|---|
| APA | Antigen presentation assay |
| AF | Alexa Fluor |
| APC | Allophycocyanin |
| C-HBV | Chimigen® HBV |
| CFSE | Carboxyfluorescein N-succinimidyl ester |
| Cy | Cyanine |

| | |
|---------------|-------------------------------------|
| DC | Dendritic cells |
| FACS | Fluorescence activated cell sorting |
| FITC | Fluorescein isothiocyanate |
| HBV | Hepatitis B virus |
| IFN- γ | Interferon gamma |
| GrB | Granzyme B |
| Pfn | Perforin |
| PE | Phycoerythrin |
| PBMC | Peripheral blood mononuclear cell |
| PerCP | Peridinin-chlorophyll-protein |
| TNF- α | Tumor necrosis factor alpha |

Disclosure of potential conflict of interest

The authors, RG, AM, BM, YES and KG declare they have no conflict of interest.

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ORCID

Bruce Motyka  <https://orcid.org/0000-0002-8448-2777>
 Klaus Gutfreund  <https://orcid.org/0000-0002-2944-7143>
 Rajan George  <https://orcid.org/0000-0002-5633-9754>

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