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Stem cell-derived viral Ag-specific T lymphocytes suppress HBV replication in mice

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Cover Letter

COLLEGE OF MEDICINE

HEALTH SCIENCE CENTER

Department of Microbial Pathogenesis and Immunology

Jianxun (Jim) Song
Professor

June 03, 2019

Editors-in-Chief
JoVE

Dear Editors:

Enclosed please find our manuscript entitled "Stem cell-derived viral Ag-specific T lymphocytes suppress HBV replication in mice" by Xiong *et al.*, which we would like to submit for publication in ***JoVE***.

The study presented in this protocol aimed to demonstrate that viral antigen (Ag)-specific $CD8^+$ T cells (CTLs) from pluripotent stem cells (PSCs), i.e., PSC-CTLs, suppress HBV replication in a murine model. We believe that two novel methods of this study will make it interesting to general readers of ***JoVE***. First, we establish the generation of functional viral Ag-specific CTLs from the induced PSC (iPSCs), i.e., iPSC-CTLs. Second, we reveal that adoptive transfer of viral Ag-specific iPSC-CTLs greatly suppresses HBV replication in an animal model and prevents the HBV surface Ag expression on hepatocytes. These results indicate that stem cell-derived viral Ag-specific CTLs can markedly suppress the HBV replication.

We affirm that all of the authors concur with the submission of this manuscript, and that the methods of this manuscript have not been previously reported. Additionally, we have no conflicting financial interests, and would be happy to discuss with you about our results, if needed.

Yours sincerely,

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TITLE:

Stem Cell-Derived Viral Ag-Specific T Lymphocytes Suppress HBV Replication in Mice

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KEYWORDS:

stem cell, HBV, immunotherapy, adoptive cell transfer, viral replication, mouse

SUMMARY:

Presented here is a protocol for the effective suppression of hepatitis B virus (HBV) replication in mice by utilizing adoptive cell transfer (ACT) of stem cell-derived viral antigen (Ag)-specific T lymphocytes. This procedure may be adapted for potential ACT-based immunotherapy of HBV infection.

ABSTRACT:

Hepatitis B virus (HBV) infection is a global health issue. With over 350 million people affected worldwide, HBV infection remains the leading cause of liver cancer. This is a major concern, especially in developing countries. Failure of the immune system to mount an effective response against HBV leads to chronic infection. Although HBV vaccine is present and novel antiviral medicines are being created, eradication of virus-reservoir cells remains a major health topic. Described here is a method for the generation of viral antigen (Ag) -specific $CD8^+$ cytotoxic T lymphocytes (CTLs) derived from induced pluripotent stem cells (iPSCs) (i.e., iPSC-CTLs), which have the ability to suppress HBV infection. HBV replication is efficiently induced in mice through hydrodynamic injection of an HBV expression plasmid, pAAV/HBV1.2, into the liver. Then, HBV surface Ag-specific mouse iPSC-CTLs are adoptively transferred, which greatly suppresses HBV replication in the liver and blood as well as prevents HBV surface Ag expression in hepatocytes. This method demonstrates HBV replication in mice after hydrodynamic injection and that stem cell-derived viral Ag-specific CTLs can suppress HBV infection. This protocol provides a useful method for HBV immunotherapy.

INTRODUCTION:

Following acute infection, the adaptive immune system (i.e., humoral and cellular immunity) controls the bulk of acute HBV-related hepatitis. Still, several people in the HBV-endemic regions cannot eliminate the viruses and subsequently convert as chronic individuals. More than 25% of chronic patients (>250 million people) worldwide develop progressive liver disease, resulting in liver cirrhosis and/or hepatocellular carcinoma (HCC)[1]. As a result, eradication of insistently infected cells remains a general healthiness problem, even though there is an available vaccine[2] and numerous antiviral medicines are under development. Standard treatment for HBV infection includes IFN-α, nucleoside, and nucleotide analogues. These agents have direct antiviral activity and immune modulatory capacities. Nevertheless, seroconversion of HBe antigen $(Ag)^+$ carriers with anti-HBe antibody (Ab) and loss of serum HBV deoxyribonucleic acid (DNA) appear individually in approximately 20% of treated patients, and whole immunological control of the virus verified by the deprivation of the HBsAg is no more than 5%[3]. Moreover, the response to treatment is often not durable. Prophylactic vaccination with recombinant HBs Ag is highly effective in preventing infection, but therapeutic HBs Ag vaccination is not effective. Clearly, T cell-mediated immune responses play a critical role in controlling HBV infection and liver impairment; however, in chronic hepatitis patients, HBV-reactive T cells are often deleted, dysfunctional, or convert exhausted[4-6]. Consequently, in individuals with persistent HBV infection, no attempts to reinstate HBV-specific immunity (i.e., T cell-based immunity) by means of anti-viral remedy, immuno-modulatory cytokines, or curative immunization have achieved success.

Adoptive cell transfer (ACT) of HBV Ag-specific T cells is an efficient treatment directed to eventually eradicate remaining hepatocytes wih HBV[7,8]. ACT of HBV-specific CTLs into HBV-infected mice has been shown to cause transient, mild hepatitis, and a dramatic drop in HBV ribonucleic acid (RNA) transcripts in hepatocytes. In these studies, CTLs did not inhibit transcription of HBV genes but enhanced the degradation of HBV transcripts[9]. HBV-specific CTLs are important to prevent viral infection and mediate the clearance of HBV[10,11]. For ACT-based remedies, in vitro expansion of HBV-specific T cells with a high reactivity for in vivo resettlement has been suggested to be an ideal method[12-14]; nevertheless, the present approaches are restricted regarding their abilities to generate, separate, and grow appropriate quantities and qualities of HBV-specific T cells from patients for the potential therapies.

Although clinical trials present safety, practicability, and prospective therapeutic activity of cell-based treatments by means of engineered T cells that are specific to HBV virus-infected hepatocytes, there are worries about the unfavorable effects occurring from autoimmune responses because of cross-reactivity from mispairing T cell receptor (TCR)[15,16], off-target Ag recognition by non-specific TCR [17] and on-target off-toxicity by a chimeric Ag receptor (CAR)[18,19] with healthy tissues. Currently, the genetically modified T cells, which only have short-term persistence in vivo, are usually intermediate or later effector T cells. To date, pluripotent stem cells (PSCs) are the only source available to generate high numbers of naive single-type Ag-specific T cells[20-23]. Induced PSCs (iPSCs) are simply converted from a patient's somatic cells through the use of gene transduction of several transcription factors. As a result, the iPSCs have similar characteristics as those of embryonic stem cells (ESCs)[24]. Owing to the flexibility and possibility for the infinite ability to self-renew, in addition to tissue replacement, iPSC-based

treatments may be widely applied in regenerative medicine. Furthermore, the regiments underlying iPSCs may substantially improve current cell-based therapies.

The overall goal of this method is to generate a large amount of HBV-specific CTLs from iPSCs (i.e., iPSC-CTLs) for ACT-based immunotherapy. The advantages over alternative techniques are that HBV-specific iPSC-CTLs have a single-type TCR and naive phenotype, which results in more memory T cell development after the ACT. It is demonstrated that the ACT of HBV-specific iPSC-CTLs increases the migration of functional $CD8^+$ T cells in the liver and reduces HBV replication in both the livers and blood of infected mice. This method reveals a potential use of viral Ag-specific iPSC-CTLs for HBV immunotherapy and may be adapted to generate other viral Ag-specific iPSC-T cells for viral immunotherapy.

PROTOCOL:

All animal experiments are approved by The Texas A&M University Animal Care Committee (IACUC; #2018-0006) and are conducted in compliance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care. Mice are used during 6–9 weeks of age.

1. Generation of viral Ag-specific $CD8^+$ T cells from iPSCs (iPSC-$CD8^+$ T cells)

1.1 Creation of the retroviral constructs

NOTE: TCR α and β genes are linked with 2A self-cleaving sequence. The retroviral vector MSCV-IRES-DsRed (MiDR) is $DsRed^+$ [23].

1.1.1. Sub-clone $HBs_{183-191}$ (FLLTRILTI)-specific A2-restricted human-murine hybrid TCR (s183 TCR) genes (Vα34 and Vβ28) into the MiDR to create the s183 MiDR construct (**Figure 1A**)[25].

1.2 Retroviral transduction

NOTE: The Platinum-E (Plat-E) cells are used for packaging retroviruses (carrying s183 TCR genes), which will be used for retroviral transduction. The Plat-E cells are an effective retrovirus packaging cells underlying the 293T cells, which were developed by means of unique packaging constructs via the EF1α promoter to express retroviral structure protein, including gag, pol, and ecotropic env.

1.2.1 On a 100 mm culture dish, seed 3 x 10^6 Plat-E cells in 8 mL of DMEM culture medium containing 10% fetal calf serum (FCS) in an incubator at 37 °C with 5% CO_2, 1 day before transfection.

1.2.2 On day 0, transfect the s183 MiDR construct into the Plat-E cells using a DNA transfection reagent[25].

1.2.3 On day 1, seed 1 x 10^6 iPSCs (GFP^+) into a gelatin pre-coated culture plate.

1.2.4 On days 2–3, collect retroviruses-containing supernatant from Plat-E cell culture to transduce iPSCs with the s183 TCR in the presence of 1,6-dibromohexane solution[25].

1.2.5 On day 4, trypsinize the s183 TCR gene-transduced iPSCs, centrifuge at 400 *x g* for 5 min and seed 3 × 10^5 iPSCs in a 100 mm culture dish pre-coated with 3 x 10^6 irradiated SNL76/7 (irSNL76/7) feeder cells[25].

1.2.6 On day 5 or 6 of confluence, trypsinize the cells, centrifuge at 400 *x g* for 5 min and process for cell sorting. Gating on live cells, sort GFP and DsRED double-positive cells (the s183 TCR gene-transduced iPSCs) using a high-speed cell sorter. Similar to step 1.2.5, co-culture the sorted cells on irSNL76/7 feeder cells for future use[25].

1.3 Differentiation of HBV-specific iPSC-$CD8^+$ T cells

NOTE: The OP9-DL1/DL4 stromal cells overexpress both Notch ligands DL1 and DL4, and co-culturing iPSCs with the iPSCs can promote Notch signaling-mediated T cell differentiation[26].

1.3.1 Grow s183 TCR gene-transduced iPSCs (s183/iPSCs) in the OP9-DL1-DL4 cell monolayer in α-minimum essential medium (MEM) media containing 20% fetal bovine serum (FBS)[27]. Include murine Flt3 ligand (mFlt-3L; final concentration = 5 ng/mL) in the culture.

1.3.2 On day 0, seed 0.5–1.0 x 10^5 S183/iPSCs in a 10 cm culture dish previously grown with OP9-DL1-DL4 cells. Validate that the OP9-DL1-DL4 cells are in a condition of 80%–90% confluency.

1.3.3 On day 5, rinse the iPSCs with 10 mL of phosphate-buffered saline (PBS), aspirate off the PBS, add this to 4 mL of 0.25% trypsin, and incubate in a 37 °C incubator for 10 min. Afterward, add a supplementary 8 mL of iPSC media to end trypsin digestion. Accumulate all the digestive solutions containing the cells and centrifuge at 400 x *g* for 5 min at 15–30 °C.

1.3.4 Aspirate the supernatant and resuspend cells in 10 mL of iPSC media. Transfer the cell suspension to a new 10 cm Petri plate and incubate in an incubator for 30 min at 37 °C.

1.3.5 After 30 min, collect the iPSC media containing the floating cells. Pass the cell suspension through a 70 µm cell strainer and calculate the cell number.

1.3.6 Seed 5 x 10^5 cells in the culture dish previously grown OP9-DL1-DL4 cells with a condition of 80%–90% confluent as described in step 1.3.1.

NOTE: For T cell differentiation, each 2–3 days, the iPSC-derived cells need to re-seed with a fresh layer of the OP9-DL1-DL4 cells.

1.4 Evaluation

1.4.1 Morphological changes of differentiating iPSCs

1.4.1.1 Observe cells under a microscope on various days.

NOTE: By day 5, colonies have mesoderm-like characteristics, exhibiting a classic spindle-shape morphology resembling human dermal fibroblasts and sustained growth in vitro. By day 8, small round clusters of cells begin to appear.

1.4.2 Analysis of differentiating iPSCs by flow cytometry

1.4.2.1. On various days of co-culture, analyze iPSC-derived cells as described previously[25] (**Figure 1B,C**).

1.4.3. Functional analysis of differentiating iPSCs

1.4.3.1. On day 28 of co-culture, collect iPSC-$CD8^+$ T cells from cultures through harvesting the floating cells, trypsinize the leftover cells with 0.25% trypsin, re-suspend in 8 mL of iPSC media, centrifuge for 5 min at 400 *x g* at 15–30 °C, remove the media, then resuspend the cells in 10 mL of media.

1.4.3.2. Keep the re-suspended cells in a fresh 10 cm dish in a 37 °C incubator for 30 min and assemble the floating cells. Then rinse the cells one times with a cold PBS.

1.4.3.3. Incubate 3 x 10^6 T cell-depleted splenocytes ($CD4^-CD8^-$) from spleens of H-2 class I knockout, HLA-A2.1-transgenic (HHD) mice with 5 µM s183 peptide (FLLTRILTI) in 200 µL of media at 4 °C for 30 min.

1.4.3.4. Produce a mixture of iPSC-$CD8^+$ T cells with splenocytes pulsed with s183 peptide (T cells: splenocytes = 1:4; use 0.75 x 10^6 T cells). Incubate the mixture of cells at 37 °C in a CO_2 incubator for 40 h. During the last 7 h, add 4 µL of diluted brefeldin A into the culture (final concentration of 1,000x, which will be diluted in 1x culture media) to block transport processes during cell activation.

1.4.3.5. Stain the cells and perform flow cytometric analysis of intracellular IFN-γ as described previously (**Figure 2**).

2. Induction of HBV replication through hydrodynamic delivery of HBV plasmid

NOTE: pAAV/HBV1.2 construct was generated as described previously[9]. The HBV 1.2 complete DNA is incorporated in the vector pAAV.

2.1 Hydrodynamic deliveries of HBV plasmid through the tail vein

2.1.1 Heat HHD mice using a heat lamp for 5 min in the cage in order to dilate the tail vein.

2.1.2 Detain the animal by means of a restrainer, and clean mouse tails with 70% ethanol spray.

2.1.3 Delivery of plasmid into the liver

2.1.4 Measure the mouse body weight by using a measuring scale.

2.1.5 Dilute 10 µg of HBV plasmid in the 8% equivalent of body mass PBS (e.g., 1.6 mL for a 20 g mouse). Load diluted plasmid in a 3 mL syringe with a 26 G × ½" (0.45 × 12 mm) hypodermic needle.

2.1.6 Locate one of the two lateral tail veins in the middle third of the tail and position the needle into either lateral vein. Administer the injection containing HBV plasmid through the tail vein within 3 - 5 s.

2.2 Quantification of viremia from blood serum of infected mice

NOTE: HBV replication occurs from day 3–35 in the mouse serum. The DNA replication peaks on day 7 and reduces gradually. The HBV DNA is not cleared from the serum until day 35.

2.2.1 Collection of blood serum from infected mice

2.2.1.1 Collect approximately 0.1 mL of blood in a microcentrifuge tube from each mouse on 3, 5, 7, and 10 days post-infection by retro-orbital bleeding in a 1.5 mL microcentrifuge tube, then incubate at room temperature (RT) for 20 min.

2.2.1.2 Centrifuge the sample at 6,000 *x g* for 15 min at 4 °C and collect the serum supernatant after centrifugation.

2.2.2 Purify the DNA from the blood serum using a commercially available DNA extraction kit following the manufacturer's recommendations. Briefly, lyse the cells in a silica-based column, perform the washes, and extract DNA by adding 100% ethanol to the elution column and centrifuging at 6000 *x g* for 1 min. Elute the DNA in 50 µL of RNase-free water.

2.2.3 Use 100 ng of HBV DNA from the elution for real-time PCR analysis. Use the following primers and probes: forward 5' TAGGAGGCTGTAGGCATAAATTGG 3'; reverse 5' GCACAGCTTGGAGGCTTGT 3'; probe 5' TCACCTCTGCCTAATC 3'.

2.3. Use HBV genome containing plasmid (pAAV/HBV1.2) for standard curve and perform real-time PCR in a total volume of 10 µL (**Figure 3**).

2.3.1 Set up the PCR reaction in a total volume of 10 µL as shown in **Table 1**.

2.3.2 Set up the PCR program in the thermocycler as shown in **Table 2**. The programmed temperature transition rate is 20 °C/s for denaturation/annealing and 5 °C/s for extension. Measure the fluorescence at the end of the annealing phase for each cycle for real-time PCR monitoring.

3. Reduction of HBV replication by ACT of viral Ag-specific iPSC-CD8$^+$ T cells

3.1 Adoptive cell transfer (ACT)

3.1.1 Differentiate s183/iPSCs (1.3.7) upon the OP9-DL1-DL4 stromal cells in the presence of mFlt-3L and mIL-7 for 8 days as described in section 1.3.

3.1.2 On day 22, collect iPSC-CD8$^+$ T cells from the 10 cm plate with trypsin, then wash and resuspend each 10 cm plate in 10 mL of fresh media. Add the cells to a fresh 10 cm plate and return to the incubator for 30 min as done in section 1.3. After 30 min, collect floating cells.

3.1.3 Use a 70 µm nylon strainer to pass cells to eliminate cell clusters and count the cell number. Adapt the cells to a concentration of 1.5 x 10^7 cells/mL in cold PBS solution and use a 70 µm nylon strainer to pass cells to eliminate cell clusters again if needed. Keep cells on ice until the ACT.

3.1.4 Inject 200 µL cell suspension (3 x 10^6 cells) into 4–6 week-old HHD mice through the tail vein.

3.2 Induction of HBV replication

3.2.1. On day 14 after cell transfer, perform the hydrodynamic delivery of HBV plasmid through the tail vein as described in section 2.1.

3.3 Virus protein detection from infected liver

3.3.1 Sacrifice mice on days 3, 5, 7, 14, and 21 post-infection. For euthanasia, in each cage, use 1–2 L of carbon dioxide (CO_2) in the first stage. When the animal develops the loss of consciousness, gain the CO_2 flow rate around 4–5 L/min. Perform mouse euthanasia using CO_2 inhalation

3.3.2 Separate the liver by cutting the surface skin of the peritoneum utilizing scissors and forceps and slightly dragging the liver back to uncover the internal skin lining the peritoneal cavity. Collect and cut liver samples (length x width x height = 0.5 cm x 0.5 cm X 0.3 cm) of the infected mice to fit easily into the embedding cassette and block in 10% neutral buffer formalin for 4–24 h.

3.3.3 Decalcify the liver samplesusing 2.5 M formic acid; rinse in xylene for 3 min, rinse 2x in 100% ethanol, rinse 2x in 95% ethanol, rinse 2x in deionized (DI) water for 2 min, then decalcify the liver samples in 1 mM ethylenediaminetetraacetic acid (EDTA) correspondingly. Handle at a sub-boiling temperature (90 °C) for 20 min.

3.3.4 Cool the fixed liver tissues for 30 min. Rinse the tissues with 1x PBS for 4 min and embed the tissues in paraffin. Dehydrate the tissues in a series of increasing concentrations of ethanol to replace the water, and then immerse in xylene immersion. Embed the infiltrated tissues into wax blocks. Make evenly vertical and horizontal sectioning for staining. Prepare 4 µm sections with a sliding microtome.

3.3.5 Pass the sections through the deparaffinization and rehydration using xylene and ethanol, then perform immunofluorescent staining of the sections.

3.3.6 Stain the sections with 200 µL of HBV surface Ag-specific antibody (1:100 dilution in the blocking solution). Incubate the sections with the antibody for 2 h at RT in a 75%–100% humidified chamber, and wash 5x in 1x PBS for 5 min.

3.3.7 Use an anti-fade reagent containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining to counterstain the slides. Add the coverslip with about 300 µL of the diluted DAPI staining solution (300 nM in 1x PBS) and validate the entire coverslip covered. Keep the slides in the dark at 4 °C until inspection under a fluorescent microscope.

3.4 Inflammatory cell infiltration in infected mice

3.4.1 Sacrifice mice as described in step 3.3.1. Collect the liver and make sections as described in step 3.3.4.

3.4.2 Stain the section with hematoxylin and eosin (H&E) to evaluate infiltration of inflammatory cells into the liver.

3.4.3 Store slides in the dark at 4 °C until further analysis under a fluorescent microscope.

3.4.4 Visualize slides under a fluorescence microscope to detect the infiltration of inflammatory cells into the liver (**Figure 4**).

3.5 HBV DNA detection of infected liver

3.5.1 Isolation of viral DNA

3.5.2 Euthanize mice and collect the liver samples according to section 3.3.

3.5.3 Lyse the liver tissues in Nonindet P-40 (NP-40) lysis buffer (50 mM Tris-HCL, 1 mM EDTA, 1% NP-40) containing a protease inhibitor cocktail.

3.5.4 Briefly centrifuge at 16,000 *x g* to remove the nuclei and cell debris.

3.5.5 Incubate the cytoplasmic lysate with micrococcal nuclease (nuclease S7; 150 units/mL) and $CaCl_2$ (5 mM) at 37 °C for 90 min to degrade the nucleic acid outside nucleocapsids (NCs).

3.5.6 Inactivate the nuclease S7 by the addition of 10 mM EDTA.

3.5.7 Precipitate the nucleocapsids with polyethylene glycol (PEG), disrupt by 0.5% sodium dodecyl sulfate (SDS), and digest with 0.6 mg/mL proteinase K (PK) at 37 °C for 1 h.

3.5.8 Recover the viral nucleic acids by phenol chloroform extraction and ethanol precipitation.

3.5.9 Resolve the extracted viral DNA on a 1.2% agarose gel and detect by standard Southern blot analysis using a ^{32}P-labeled HBV DNA probe.

REPRESENTATIVE RESULTS:

As shown here, HBV viral Ag-specific iPSC-CD8$^+$ T cells are generated by an in vitro culture system. After ACT of these viral Ag-specific iPSC-CD8$^+$ T cells substantially suppress HBV replication in a murine model (**Supplemental File 1**). Mouse iPSC are transduced with the MIDR retroviral construct encoding a human-mouse hybrid HBV TCR gene ($HBs_{183-191}$-specific, s183), then the gene-transduced iPSCs are co-cultured with OP9-DL1/DL4 cells expressing Notch ligands (both DL1 and DL4) molecules in the presence of rFlt3L and rIL-7. On day 28 of in vitro co-culture, the iPSC-derived cells substantially express CD3 and Ag-specific TCR (T cell markers). Flow cytometric analysis of the CD3$^+$CD8$^+$ population shows that the HBV TCR transduction dramatically increases the generation of viral s183-specific CD8$^+$ T cells (**Figure 1**).

On day 28 of in vitro co-culture, the CD4$^-$CD8$^+$ single-positive (SP) iPSC-CD8$^+$ T cells are isolated and stimulated by T-depleted splenocytes pulsed with s183 peptide, and cytokine production is assessed. The iPSC-CTLs produce large amounts of IL-2 and IFN-ϒ as detected by intracellular staining or ELISA (**Figure 2**). HBV infection is induced in HLA-A2.1 transgenic (HHD) mice by hydrodynamic injection of 10 µg of pAAV/HBV1.2 DNA plasmid through the tail veins of mice. HBV replication is detected from day 3–21 in the serum of HHD mice. The DNA replication peaks on day 6 and reduces gradually using real-time PCR analysis (**Figure 3**).

Two weeks following the ACT, mice are challenged with a hydrodynamic injection of pAAV/HBV1.2 DNA plasmid. The viral titer is measured by real-time PCR from serum at various timepoints after the injection. The results demonstrate that viral replication is significantly reduced at all timepoints in the mice receiving HBV viral Ag-specific T cells compared to the mice receiving control cells (**Figure 4A**). HBV surface protein expression is also examined in the liver in the above setting of treatment. Mice are euthanized at various days after the HBV injection and the liver samples are isolated for histologic examination. Samples are stained for

HBV surface protein and examined under a fluorescence microscope. HBV surface protein is observed as substantially decreased in the mice receiving HBV viral Ag-specific pre-iPSC-CTLs, as compared with the mice receiving control cells (**Figure 4B)**. This experiment clearly demonstrates that viral Ag-specific iPSC-CD8$^+$ T cells have the ability to reduce HBV replication in the murine model.

FIGURE AND TABLE LEGENDS:

Figure 1: Generation of HBV viral Ag-specific iPSC-CD8$^+$ T cells. Mouse iPSCs are transduced with the following retroviral constructs: HBs$_{183\text{-}91}$ TCR (MiDR-s183 TCR) or OVA$_{257–264}$ TCR (MiDR-OVA TCR), and the transduced iPSCs are co-cultured with OP9-DL1/DL4 stromal cells for T lineage differentiation. (**A**) Schematic representation of the retroviral construct MiDR-s183 TCR expressing s183-specific TCR. Ψ = packaging signal; 2A = picornavirus self-cleaving 2A sequence; LTR = long terminal repeats. (**B**) Morphology of T cell differentiation on days 0, 7, 14, and 22. (**C**) Flow cytometric analysis for the iPSC-derived cells on day 28. CD3$^+$CD8$^+$ cells (left) are gated as indicated and analyzed for the expression of CD8 and TCRVβ28 (right). Data shown are representative of three individual experiments. The values represent mean ± SD (**p < 0.01; paired t-tests).

Figure 2: Functional analysis of the HBV viral Ag-specific iPSC-CD8$^+$ T cells. On day 28 of in vitro co-culture, the SP CD8$^+$s183 TCR pentamer$^+$ iPSC-T cells are sorted. The iPSC-T cells and CD8$^+$ T cells transduced with MiDR-s183 TCR are stimulated by T-depleted splenocytes (APCs) from HHD mice and pulsed with s183 peptide (FLLTRILTI). (**A**) Intracellular staining of IFN-ϒ after 7 h (gated on CD8$^+$ cells) (T/APCs = 1:4). (**B**) ELISA of IFN-γ after 40 h. Data shown are representative of three individual experiments. The values represent mean ± SD (n.s., p > 0.05; paired t-tests).

Figure 3: Induction of HBV replication in HHD mice by hydrodynamic injection. HHD mice are *i.v.* administrated with HBV plasmid via hydrodynamic tail vein injection. 10 μg of the plasmid is injected with 8% of total body mass PBS. On indicated time points after injection, the serum is isolated from the blood and DNA is extracted for real-time PCR. Data shown are representative of three individual experiments. The values represent mean ± SD. Data are representative of five mice per group of three independent experiments**.**

Figure 4: Reduction of HBV replication by ACT of viral Ag-specific iPSC-CD8$^+$ T cells. HHD mice are i.v. adoptively transferred with viral Ag-specific iPSC-CD8$^+$ T cell progenitors (on day 22 of the in vitro culture) and administrated with HBV plasmid at week 2 after the cell transfer. (**A**) Serum HBV copies. At the indicated timepoints after injection, serum is isolated from blood, and DNA is extracted for real-time PCR analysis. Data shown are representative of three individual experiments. The values represent mean ± SD. (**B**) Liver tissue histology. Mice are euthanized at day 8 post-HBV infection. Liver samples are isolated and stained for histologic examination. Upper panel shows the HBsAg protein expression in infected mice (IHC staining) and lower panel shows the inflammatory cell infiltration (HE-staining). Data are representative of five mice per group of three independent experiments.

Table 1: PCR reaction volume

Table 2: PCR program

DISCUSSION:

This protocol presents a method to generate the viral Ag-specific iPSC-CTLs for use as ACT to suppress HBV replication in a murine model. In chronic HBV infection, the viral genome forms a stable mini chromosome, the covalently closed circular DNA (cccDNA) that can persist throughout the lifespan of the hepatocyte. Targeting the clearance of the viral mini chromosome may result in a cure of chronic HBV infection. Current antiviral therapy targets the virus reverse transcriptase but rarely establishes immunological control over HBV replication driven by cccDNA. HBV-specific $CD8^+$ CTLs can mediate the killing of the infected hepatocytes and accelerate the clearance of cccDNA. Nevertheless, the HBV-specific CTLs are deleted, dysfunctional, or succumb to exhaustion in patients with chronic HBV infection. ACT with the HBV-specific CTLs is a favorable treatment for chronic HBV infection[28,29]. Naive or central memory T cell-derived T lymphocytes (i.e., highly reactive immune cells) are ideal effectors for ACT-based therapies owing to their great proliferation, lower tendency for death compared to terminally differentiated cells, and excellent ability to respond to homeostatic cytokines. However, such ACT has been often not feasible due to difficulties in obtaining sufficient numbers of CTLs from patients. It has been previously showed that reprogramming of Ag-specific CTLs or T_{regs} from iPSCs can be used for cell-based therapies[20,23,27,30]. This report demonstrates a method to generate viral Ag-specific iPSC-CTLs and use these cells for ACT-based immunotherapy in a murine model of HBV replication.

Although there are transgenic mouse models of HBV replication, these models are challenging because the central tolerance induced by the transgenic gene products causes mice to be immune tolerant to HBV Ags. Additionally, transgenic mice are not suitable for monitoring viral clearance as the integrated HBV genome persists in each mouse cell[31,32]. Also, despite that successful vaccines have been developed for preventing the infection, the treatment or immunotherapy after HBV infection has not been developed. Furthermore, the experimental approaches to HBV pathogenesis have been hampered because the host range of HBV infection is limited to men and chimpanzees, and in vitro culture system for the propagation of HBV is not sufficient. $CD8^+$ T cells are promising effector cells against various types of viral infection; however, T cell response against HBV is not abundant. Specificity, functioning, and lack of sufficient numbers to mount an immune response may be the cause. This report reveals the method of hydrodynamic injection that efficiently induces HBV replication in mice. The method allows delivery of a large amount of HBV plasmid directly into the liver. The model exhibits continuous viremia for more than 8 weeks with detection of HBV mRNA, protein, and DNA at different days after injection. This method for HBV replication in mice is useful for HBV immunotherapy.

In summary, HBV replication was successfully induced in mice through hydrodynamic injection procedure, and viral Ag-specific iPSC-CTLs were used as immunotherapy for HBV infections.

However, there are two limitations of the method: (1) more than three weeks of in vitro T cell differentiation may reduce the translational applications of the generated T cells for ACT; and (2) HBV hydrodynamic injection can efficiently induce HBV replication in the liver; however, it does not form the cccDNA, which is the main reason for the persistent HBV infection. Nevertheless, the method provides an alternative approach for HBV replication and treatment. A combination regimen using anti-HBV drugs and the ACT of viral Ag-specific iPSC-CTLs is likely to reduce HIV reservoirs, thereby resulting in a therapy for chronic HIV infection.

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DISCLOSURES:

The authors have nothing to disclose.

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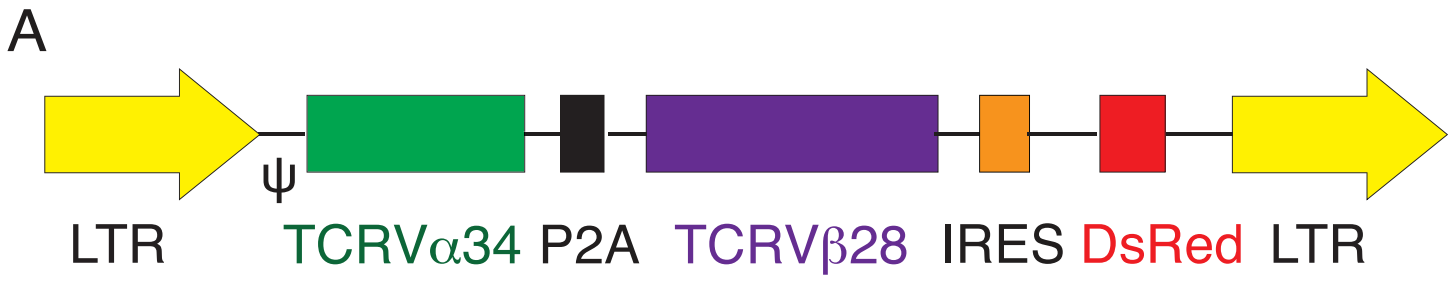
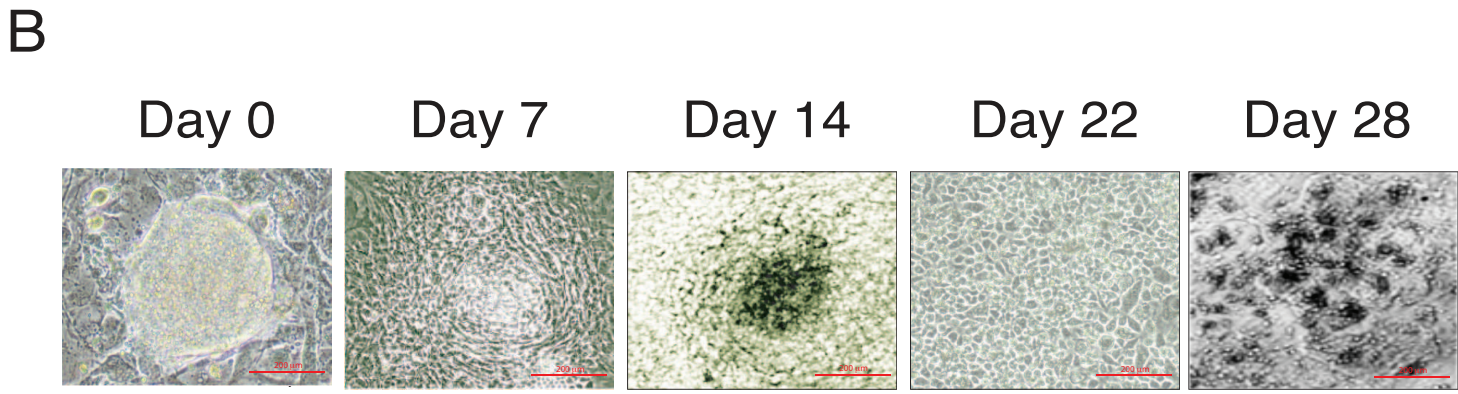
Figure 1





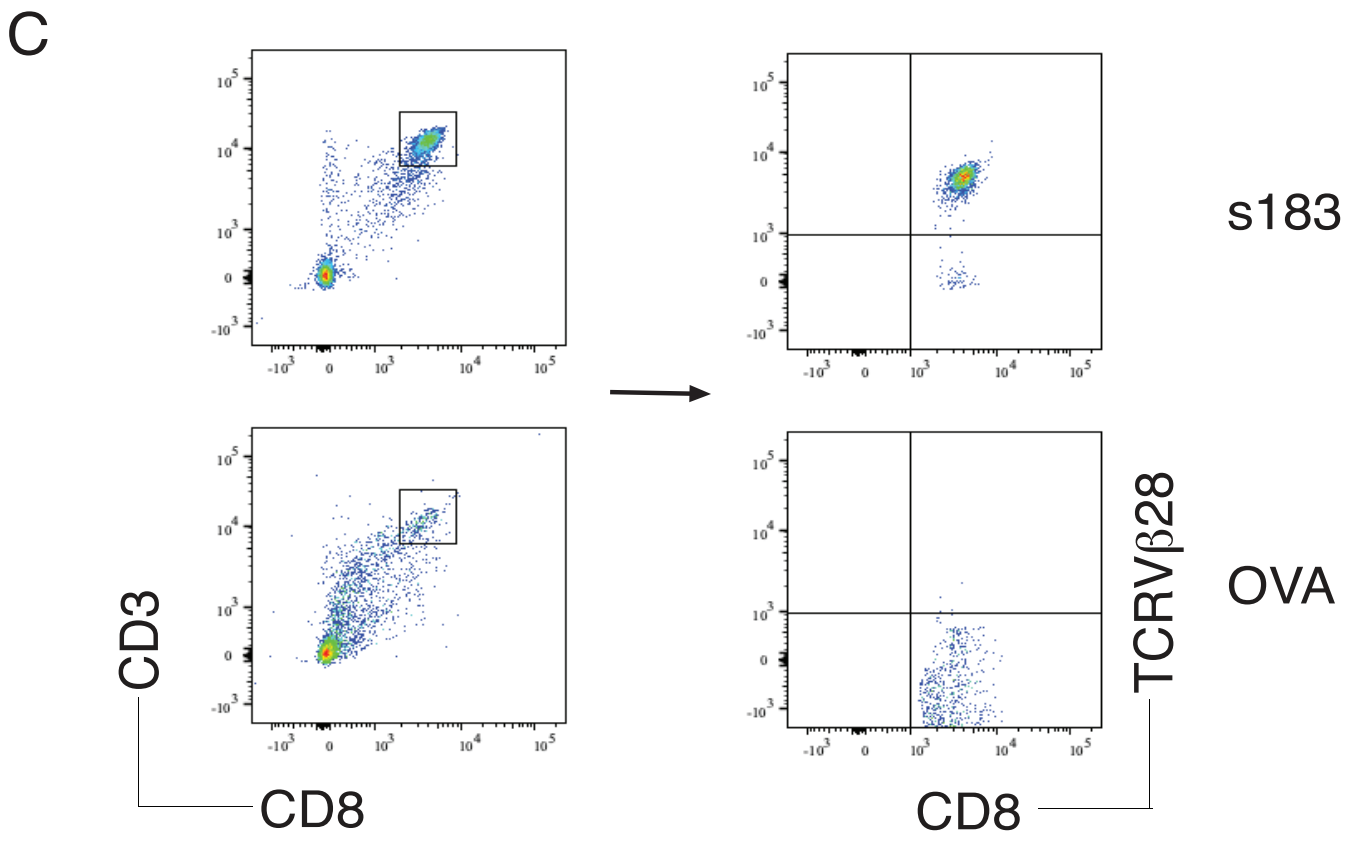


Figure 2

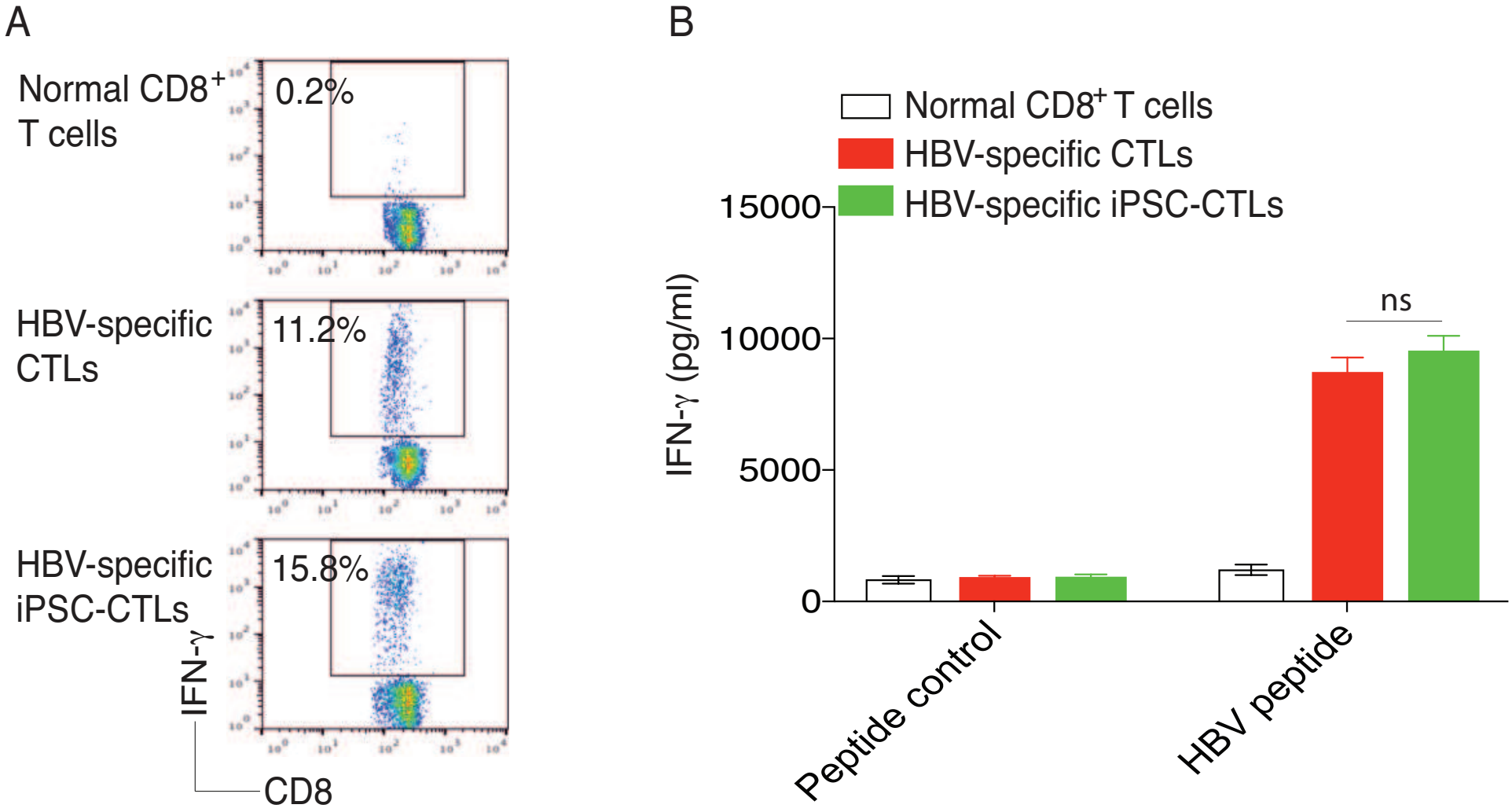


Figure 3

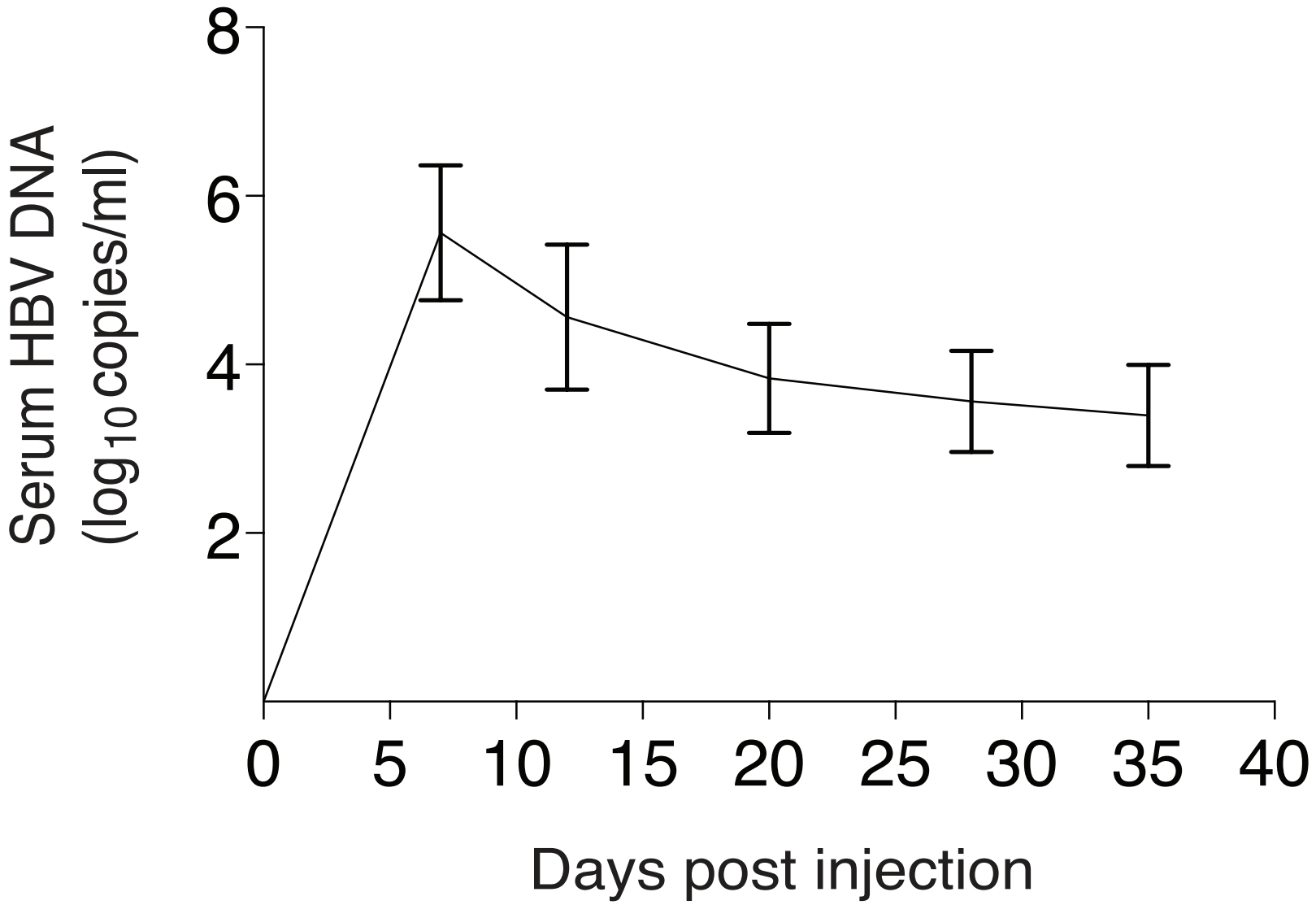


Figure 4

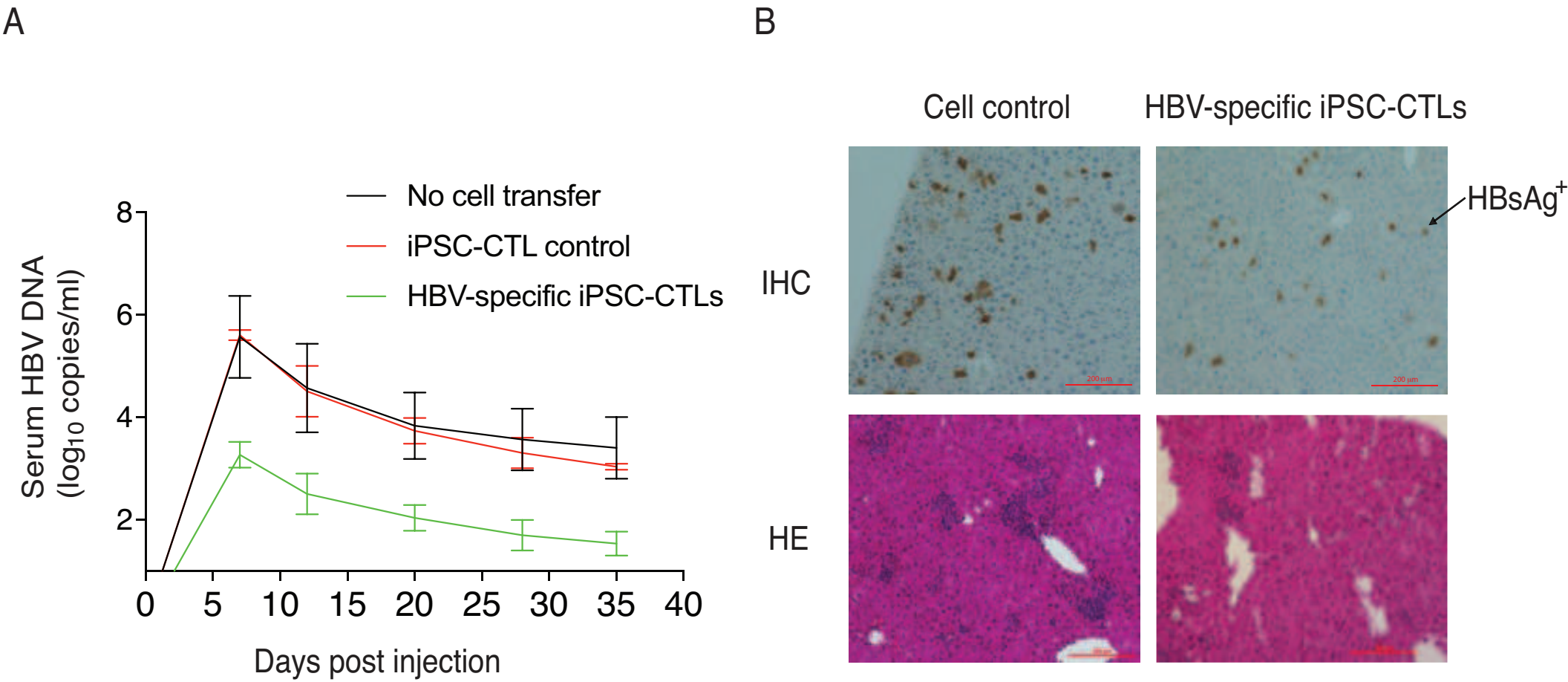


Table 1

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DNA template	2 µl
DNA master hybridization	1 µl
25 mM MgCl2	0.8 µl
0.3 µM the probe	3 µl
5 µM of each primer	3.2 µl
Total	10 µl

Table 2

Click here to access/download;Table;Table 2 (PCR reaction - program).xlsx

	Temperature	Time
Denaturation	95 °C	5 s
Annealing	53 °C	10 s
Extension	72 °C	20 s
	5 °C	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
HHD mice	Institut Pasteur, Paris, France		H-2 class I knockout, HLA-A2.1-transgenic (HHD) mice
iPS-MEF-Ng-20D-17	RIKEN Cell Bank	APS0001	
SNL76/7	ATCC	SCRC-1049	
OP9	ATCC	CRL-2749	
pAAV/HBV1.2 plasmid	Dr. Dr. Pei-Jer Chen (National Taiwan University Hospital, Taiwan)		HBV DNA construct
HBs183-91(s183) (FLLTRILTI)-specific TCR genes	Dr. Adam J Gehring (Toronto General Hospital Research Institute, Toronto, Canada)		FLLTRILTI-specific A2-restricted human-murine hybrid TCR genes (Vc

OVA257–264-specific TCR genes	Dr. Dario A. Vignali (University of Pittsburgh, PA)		SIINFEKL-specific H-2Kb-restricted TCR genes
Anti-CD3 (17A2) antibody	Biolegend	100236	
Anti-CD44 (IM7) antibody	BD Pharmingen	103012	
Anti-CD4 (GK1.5) antibody	Biolegend	100408	
Anti-CD8 (53-6.7) antibody	Biolegend	100732	
Anti-IFN-γ (XMG1.2) antibody	Biolegend	505810	
Anti-TNF-α (MP6-XT22) antibody	Biolegend	506306	
α-MEM	Invitrogen	A10490-01	
Anti-HBs antibody	Thermo Fisher	MA5-13059	
ACK Lysis buffer	Lonza	10-548E	
Brefeldin A	Sigma	B7651	
DMEM	Invitrogen	ABCD1234	
FBS	Hyclone	SH3007.01	
FACSAria Fusion cell sorter	BD	656700	
Gelatin	MilliporeSigma	G9391	
GeneJammer	Agilent	204130	

HLA-A201-HBs183-91-PE pentamer	Proimmune	F027-4A - 27	
HRP Anti-Mouse Secondary Antibody	Invitrogen	A27025	
mFlt-3L	Peprotech	250-31L	
mIL-7	Peprotech	217-17	
Nuclease S7	Roche	10107921001	
Paraformaldehyde	MilliporeSigma	P6148-500G	Caution: Allergenic, Carcenogenic, Toxic
Permeabilization buffer	Biolegend	421002	
Polybrene	MilliporeSigma	107689	
ProLong™ Gold Antifade Mountant with DAPI	Invitrogen	P36931	
QIAamp MinElute Virus Spin Kit	Qiagen	57704	

α34 and Vβ28)

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Jianxun (Jim) Song
Professor

June 5, 2019

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2. Please address specific comments marked in the manuscript.
Done.

3. There are still parts in the manuscript which matches with the previously published literature. Please reword the parts marked in green. Some of the highlighted steps which need rewording are marked with a comment. Please check.
Done.

4. I have adjusted the highlights to form a cohesive story. Please check.
Agreed.

5. Please confirm the filming location, College Station or Bryan Texas.
Bryan.

Yours sincerely,

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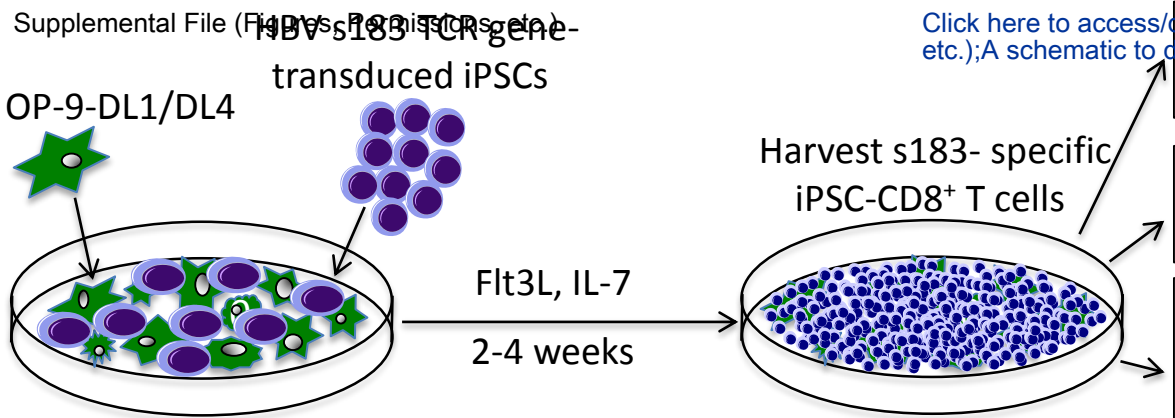

1: Generation of s183-specific iPSC-$CD8^+$ T cells

2: Functional analysis of the s183-specific iPSC-$CD8^+$ T cells

3: Cell-based therapies of HBV replication in mice

1. Induction of HBV replication in mice by hydrodynamic injection.
2. Reduction of HBV replication by ACT of s183-specific iPSC-$CD8^+$ T cells.