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TITLE:

Analysis of HBV-specific CD4 T-cell Responses and Identification of HLA-DR-restricted CD4 T-cell Epitopes Based on a Peptide Matrix

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Hepatitis B virus, CD4, T-cell response, epitope

SUMMARY:

Based on a hepatitis B virus (HBV)-derived peptide matrix, HBV-specific CD4 T-cell responses could be evaluated in parallel with identification of HBV-specific CD4 T-cell epitopes.

ABSTRACT:

CD4 T cells play important roles in the pathogenesis of chronic hepatitis B. As a versatile cell population, CD4 T cells have been classified as distinct functional subsets based on the cytokines they secreted: for example, IFN-γ for CD4 T helper 1 cells, IL-4 and IL-13 for CD4 T helper 2 cells, IL-21 for CD4 T follicular helper cells, and IL-17 for CD4 T helper 17 cells. Analysis of hepatitis B virus (HBV)-specific CD4 T cells based on cytokine secretion after HBV-derived peptides stimulation could provide information not only about the magnitude of HBV-specific CD4 T-cell response but also about the functional subsets of HBV-specific CD4 T cells. Novel approaches, such as transcriptomics and metabolomics analysis, could provide more detailed functional information about HBV-specific CD4 T cells. These approaches usually require isolation of viable HBV-specific CD4 T cells based on peptide-major histocompatibility complex-II multimers, while currently the information about

HBV-specific CD4 T-cell epitopes is limited. Based on an HBV-derived peptide matrix, a method has been developed to evaluate HBV-specific CD4 T-cell responses and identify HBV-specific CD4 T-cell epitopes simultaneously using peripheral blood mononuclear cells samples from chronic HBV infection patients.

INTRODUCTION:

Currently, there are 3 main approaches to analyze antigen-specific T cells. The first approach is based on the interaction between the T-cell receptor and the peptide (epitope). Antigen-specific T cells could be directly stained with peptide-major histocompatibility complex (MHC) multimers. The advantage of this method is that it could obtain viable antigen-specific T cells, suitable for downstream transcriptomics/metabolomics analysis. A limitation of this method is that it could not provide information about the whole T-cell response to a specific antigen, as it requires validated epitope peptides while the number of identified epitopes for a specific antigen is limited for now. Compared to hepatitis B virus (HBV)-specific CD8 T-cell epitopes, fewer HBV-specific CD4 T-cell epitopes have been identified[1,2], which made this method less applicable for analysis of HBV-specific CD4 T cells currently.

The second approach is based on the upregulation of a series of activation-induced markers after antigen peptide stimulation[3]. The commonly used markers include CD69, CD25, OX40, CD40L, PD-L1, 4-1BB[4]. This method has now been used to analyze antigen-specific T-cell responses in vaccinated individuals[5,6], Human Immunodeficiency Virus infection patients[7], and Severe Acute Respiratory Syndrome Coronavirus 2 infection patients[8,9]. Unlike the peptide-MHC multimers based assay, this method is not restricted by validated epitopes and could obtain viable cells for downstream analysis. A limitation of this method is that it could not provide information about the cytokine profile of antigen-specific T cells. Also, the expression of these activation-induced markers by some activated antigen-non-specific cells might contribute to the background signals in analysis, which could be a problem especially when the target antigen-specific T cells are rare. Currently, there is limited application of this method on HBV-specific CD4 T cells[4]. Whether this method could be utilized to analyze HBV-specific CD4 T cells in a reliable way needs further investigation.

The third approach is based on the cytokine secretion after antigen peptide stimulation. Like activation-induced marker-based analysis, this method is not restricted by validated epitopes. This method could directly reveal the cytokine profile of antigen-specific T cells. The sensitivity of this method is lower than the activation-induced marker-based method as it relies on the cytokine secretion of antigen-specific T cells and the number of cytokines tested is usually limited. Currently, this method is widely used in analysis of HBV-specific T cells. As cytokine secreting HBV-specific T cells could hardly be detected by direct ex vivo peptide

stimulation[10,11], the cytokine profile of HBV-specific T cells is usually analyzed after 10-day in vitro peptide stimulated expansion[12-16]. Arrangement of peptide pools in a matrix form has been utilized to facilitate identification of antigen-specific epitopes[17,18]. With the combination of peptide matrix and cytokine secretion analysis, a method has been developed to evaluate HBV-specific CD4 T-cell responses and identify HBV-specific CD4 T-cell epitopes simultaneously[16]. In this protocol, the details of this method are described. HBV core antigen is chosen as an example of demonstration in this protocol.

PROTOCOL:

Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Southwest Hospital's human research committee.

1. Design of the HBV-derived peptide matrix

1.1. Download amino acid sequences of the HBV core antigen from NCBI databases (GenBank: AFY98989.1).

1.2. Purchase HBV core antigen derived peptides (a panel of 35 15-mer peptides overlapping by 10 residues, purity > 90%, 4 mg/peptide) from a peptide synthesis service provider.

1.3. Set up a square 6×6 peptide matrix with each position in the matrix containing only one 1 peptide. There are 12 peptide pools: 6 row peptide pools and 6 column peptide pools, 5-6 peptides in each pool[16]. The row peptide pools and the column peptide pools in the matrix represent 2 separate formations of HBV core antigen.

1.4. For 3/4 of the purchased peptides, mix peptides in the same row/column of the matrix into 12 separate peptide pools by dissolving them together in dimethyl sulfoxide (DMSO) (2 μg/μL for each peptide). Store at -80 °C for analysis of HBV-specific CD4 T-cell responses.

1.5. Dissolve the rest of the peptides separately (10 μg/μL) and store at -80 °C for epitope identification.

2. Isolation of peripheral blood mononuclear cells (PBMCs)

2.1. Sample 5 mL of venous blood from chronic HBV infection patients.

NOTE: The blood volume should be roughly estimated according to the number of

peptide pools plus 1 background control and 1 positive control. Analysis of 1 peptide pool needs 3 x 10^5 PBMCs. On average, 1 x 10^6 PBMCs could be obtained from 1 mL of blood.

2.2. Isolate PBMCs from blood using Ficoll density gradient centrifugation (800 × g, 20 min) and cryopreserve isolated PBMCs in liquid nitrogen for later use.

2.3. Use a Pasteur pipette to collect granulocytes between the clear Ficoll layer and the red blood cell layer. Extract genomic DNA from granulocytes using a genomic DNA purification kit according to the manufacturer's protocol.

2.4. Send the DNA sample to genotyping service providers to determine the HLA-DRB1 genotype.

3. In Vitro Expansion of PBMCs Using a HBV Peptide Matrix

3.1. Thaw PBMCs.

3.1.1. Warm RPMI 1640 supplemented with 1:10,000 benzonase (25 U/mL) to 37 °C in a water bath.

NOTE: Benzonase helps to limit cell clumping during thawing. Each sample will require 20 mL of RPMI 1640 with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of media (37 °C) with 1:10,000 Benzonase (25 U/mL). Thaw no more than 5 samples at a time.

3.1.2. Remove samples from liquid nitrogen and quickly thaw frozen vials in a water bath (37 °C).

3.1.3. Transfer the thawed cell suspension to a 15 mL centrifuge tube. Add 1 mL of Benzonase RPMI 1640 (37 °C) dropwise to the tube. Slowly add 6 mL of Benzonase RPMI 1640 (37 °C) to the centrifuge tube, rinse cryovial with another 2 mL of Benzonase RPMI 1640 (37 °C) to retrieve all cells. Continue with the rest of the samples as quickly as possible.

NOTE: Slow dilution of cryopreserved samples is the key to maintain the viability of thawed cells.

3.1.4. Centrifuge (400 × g, 10 min), remove the supernatant, and loosen the pellet by tapping the tube.

3.1.5. Gently resuspend the pellet in 1 mL of warm Benzonase RPMI 1640. Mix gently,

and filter cells through a 70 µm cell strainer if needed (i.e., if any visible clump exists).

3.1.6. Aliquot a 10 µL suspension and dilute in Dulbecco's phosphate-buffered saline (DPBS), add Trypan blue (0.04%), load onto a hemocytometer, wait for 1 min, and count the number of viable cells (clear cells).

3.1.7. Add 9 mL of Benzonase RPMI 1640 (37 °C) to the tube, centrifuge (400 × g, 10 min), remove the supernatant, and loosen the pellet by tapping the tube.

3.2. Resuspend PBMCs in RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% human AB serum (complete culture medium). Adjust cell density to 1.5 x 10^6 cells/mL. Plate PBMCs in 96-well plates (flat bottom) at a density of 3 x 10^5 cells/well.

3.3. Add HBV derived peptide pools (2 µg/mL for each single peptide) to each well. For wells of background control and positive control, add the same amount of solvent (DMSO, 1 µL/mL). Add 10 U/mL IL-2 and 10 ng/mL IL-7. Incubate at 37 °C and 5% CO_2.

3.4. At day 3, supplement culture medium with 50 U/mL of IL-2 and 10 ng/mL of IL-7.

NOTE: During day 1 to day 3, no obvious T-cell proliferation will be observed. The total cell number will usually decrease by 1/3 to 1/2, due to the death of non-T cells such as B cells, NK cells, NKT cells, and monocytes.

3.5. At day 7, replace half of the culture medium with fresh medium containing peptides (4 µg/mL), IL-2 (100 U/mL), and IL-7 (20 ng/mL).

NOTE: To avoid disturbing the cells at the bottom, pipette about 90 µL of culture medium carefully from the top of the medium. During day 3 to day 7, robust T-cell proliferation will be observed, and proliferating T-cells usually aggregate to form clusters.

3.6. At day 10, gently pipette cell culture in each well 7-9 times to disaggregate cell clusters, count the number of viable cells, and transfer 2×10^5 cells in each well to a 96 well plate (round bottom) for HBV-specific CD4 T-cell response analysis.

3.7. Continue culturing the rest of cells for epitope identification at day 12, adjust the volume of culture medium to 100 µL (discarding excessive medium), and supplement culture with 100 µL of fresh complete culture medium containing

peptides (4 μg/mL), IL-2 (100 U/mL), and IL-7 (20 ng/mL).

NOTE: During day 7 to day 10, T-cells continue proliferating vigorously. Replace the culture medium as in step 3.5 if the medium turns yellow. In general, the cell number will exceed 6×10^5 at day 10. Each well usually shows similar cell number, count cell number in 3 wells and use the average value as an estimate of cell number for all the wells.

4. Analysis of HBV-specific CD4 T-Cell responses by intracellular flow cytometry

4.1. Stimulating PBMCs with peptide pools

4.1.1. For the cells transferred to the 96 well plate (round bottom), wash 3 times in a plate (550 × g, 3 min). Use 200 μL of medium for each wash (RPMI 1640 for the first 2 washes, complete culture medium for the last wash). Discard the supernatants.

NOTE: Removal of residual cytokines in the culture by repeated washing could effectively decrease the background in intracellular flow cytometry analysis.

4.1.2. For each well of cells stimulated with a specific peptide pool, add 200 μL of complete culture medium supplemented with the same peptide pools (2 μg/mL for each single peptide). For the well of background control, add complete culture medium supplemented with 1 μL/mL of DMSO. For the well of positive control, add complete culture medium supplemented with 1 μL/mL of DMSO, 150 ng/mL of phorbol 12-myristate 13-acetate (PMA), and 1 μmol/L of ionomycin.

NOTE: High dose of DMSO will block the cytokine secretion of T cells (most significant for TNF-α). Dose of DMSO higher than 5 μL/mL is not recommended. Generally, the dose of DMSO in our experiment does not exceed 1 μL/mL.

4.1.3. Incubate at 37 °C and 5% CO_2 for 6 h.

4.1.4. After 1 h of incubation, add Monensin (1.37 μg/mL) to the culture.

4.2. Flow cytometry

4.2.1. After 6 h of incubation. Remove supernatant after centrifugation (550 × g, 3 min), wash cells once with 200 μL of DPBS (550 × g, 3 min), stain surface markers (CD3, CD4, and CD8) and viability marker (using Fixable Viability Dye) in a 4 °C refrigerator for 30 min.

4.2.2. Wash once with 200 μL of DPBS (550 × g, 3 min). Fixate and permeabilize cells,

and stain intracellular cytokines (TNF-α and IFN-γ) in a 4 °C refrigerator for 45 min.

4.2.3. After the final wash in intracellular staining, resuspend cells in 150 µL of flow cytometry buffer (DPBS + 0.5% BSA).

4.2.4. Acquire flow cytometry data on a flow cytometer.

4.3. Analysis of flow cytometry results

4.3.1. Definition of positive well: consider a well as positive if they have a frequency of cytokine secreting T cells at least two times of the background control well (**Figure 1**).

4.3.2. According to the following formula, calculate the response rate for each cytokine analyzed (**Figure 2**):

$$\text{Reponse rate} = \frac{\sum(\text{frequency of cytokine secreting cells in postive well} - \text{background})}{2}$$

NOTE: The row peptide pool and the column peptide pools in the matrix represent 2 separate formations of HBV core antigen, so the final response rate should be divided by 2.

5. Identification of HBV-specific HLA-DR Restricted CD4 T-cell Epitopes

5.1. Thaw and maintain allogenic B lymphoblastoid cell lines (BLCLs) in T-75 flask (5-20 $\times 10^6$ cells, 20 mL of complete culture medium).

NOTE: To guarantee the good state of BLCLs, this step should be initiated 2 weeks before thawing of patients' PBMCs. BLCLs must be homozygous in HLA-DRB1 allele. According to the genotyping result, patients should share the same HLA-DRB1 allele as BLCLs.

5.2. Screening of candidate peptides for identification (**Figure 3**)

5.2.1. According the T-cell response rate results at day 10, screen out 2 peptide pools with the highest response rate (1 row peptide pool and 1 column peptide pool).

5.2.2. Set the peptide in those 2 pools as a candidate peptide if the other peptide pool containing this peptide also shows a positive result in T-cell response analysis. Use the PBMCs expanded with the other peptide pool for epitope identification later.

5.3. Pulsing BLCLs with peptide

5.3.1. At day 12, count the number of viable BLCLs, transfer cells to 15 mL centrifuge tubes, centrifuge (350 × g, 10 min) and remove the supernatant. Resuspend the cell pellet in complete culture medium, and aliquot BLCLs to a 96-well plate (round bottom) at 4×10^4 cells/well in 80 µL complete culture medium.

5.3.2. Add a single peptide (10 µg/mL), incubate at 37 °C and 5% CO_2 for 2 h. Set 2 background control: peptide pulsing with HLA-DR blocking (pretreatment with anti-HLA-DR (10 µg/mL) for 1 h); DMSO (1 µL/mL) pulsing. The final volume of complete culture medium in each well is 100 µL.

5.3.3. Add mitomycin C (100 µg/mL), incubate at 37 °C and 5% CO_2 for 1 h.

5.3.4. Wash 3 times with 200 µL of RPMI 1640 (550 × g, 3 min) in a plate to remove un-pulsed peptide and mitomycin C. For the first wash, supplement the incubation culture with 100 µL of RPMI 1640.

5.3.5. Resuspend cells in 120 µL of complete culture medium.

5.4. Stimulating PBMCs with peptide pulsed BLCLs.

5.4.1. At day 12, transfer PBMCs to a 96-well plate (round bottom).

5.4.2. Remove the supernatant after centrifugation (550 × g, 3 min) in a plate, an wash twice with 200 µL of RPMI 1640 (550 × g, 3 min) in a plate.

NOTE: Removal of residual cytokines and peptides in the culture by repeated washing is the key step to decrease the background in intracellular flow cytometry analysis. Especially for residual peptides, it will bind to BLCLs and significantly increase the background.

5.4.3. Resuspend PBMCs at each well with 210 µL of complete culture medium.

5.4.4. For the well of PBMCs chosen for epitope identification, aliquot th cell suspension (70 µL each) and mix with peptide pulsed BLCLs (3 wells, including 2 background controls).

NOTE: At day 12, the number of peptide pools expanded PBMCs will usually reach to above $5\text{-}7\times10^5$ per well, so the ratio of PBMCs/BLCLs is about 6/1 to 4/1.

5.4.5. Incubate at 37 °C and 5% CO_2 for 6 h.

5.4.6. After 1 h of incubation, add Monensin (1.37 µg/mL) to the culture. The final volume of complete culture medium in each well is 200 µL.

5.5. Flow cytometry

5.5.1. Repeat the same operations as in step 4.2.

5.6. Analysis of flow cytometry results

5.6.1. Verify a peptide as an HLA-DR restricted CD4 T-cells epitope if PBMCs incubated with this peptide pulsed BLCLs show a frequency of cytokine secreting CD4 T cells at least two times of the PBMCs incubated with background controls (peptide pulsing with HLA-DR pre-blocking; DMSO pulsing) (**Figure 4**).

REPRESENTATIVE RESULTS:

The frequency of cytokine secreting CD4 T cells are calculated as the sum of both single producers and double producers. As demonstrated in **Figure 1**, the frequency of TNF-α secreting CD4 T cells and the frequency of IFN-γ secreting CD4 T cells in background control (DMSO) are 0.154% and 0.013% respectively. The frequency of TNF-α secreting CD4 T cells and the frequency of IFN-γ secreting CD4 T cells specific for peptide pool Core11 are 0.206 and 0.017 respectively, so both TNF-α secreting CD4 T-cell response and IFN-γ secreting CD4 T-cell response for this peptide pool are considered as negative. The frequency of TNF-α secreting CD4 T cells and the frequency of IFN-γ secreting CD4 T cells specific for peptide pool Core09 are 2.715% and 0.973% respectively, so both TNF-α secreting CD4 T-cell response and IFN-γ secreting CD4 T-cell response for this peptide pool are considered as positive.

As demonstrated in **Figure 2**, positive wells are indicated with gray background. When calculating HBV core-specific TNF-α secreting CD4 T-cell response rate, data of peptide pools Core01, Core02, Core04, Core05, Core06, Core07, Core08, Core09, and Core10 should be included. When calculating HBV core-specific IFN-γ secreting CD4 T-cell response rate, data of peptide pools Core01, Core02, Core03, Core04, Core05, Core06, Core07, Core08, Core09, and Core10 are included.

As demonstrated in **Figure 3**, candidate peptides for epitope identification are indicated in red. Core01 has the highest response rate for both TNF-α secreting CD4 T cells and IFN-γ secreting CD4 T cells in column peptide pools. Peptides C1-15, C31-45, C61-75, and C91-105 in this peptide pool are set as candidate peptides as the row peptide pools containing those peptides also shows positive results in T-cell response. The PBMCs expanded with the peptide pools Core07, Core08, Core09, and Core10 are used for epitope identification of peptides C1-15, C31-45, C61-75, and

C91-105, respectively. Core09 has the highest response rate for both TNF-α secreting CD4 T cells and IFN-γ secreting CD4 T cells in row peptide pools. Peptides C61-75, C66-80, C71-85, C76-90, C81-95, and C86-100 in this peptide pool are set as candidate peptides as the column peptide pools containing those peptides also shows positive result in T-cell response. The PBMCs expanded with the peptide pools Core01, Core02, Core03, Core04, Core05, and Core06 are used for epitope identification of peptides C61-75, C66-80, C71-85, C76-90, C81-95 and C86-100, respectively.

As demonstrated in **Figure 4**, for peptide pool Core08 expanded PBMCs, after stimulation with peptide C31-45 pulsed BLCLs, the frequency of TNF-α secreting CD4 T cells and the frequency of IFN-γ secreting CD4 T cells are 0.995% and 0.131% respectively, which are more than 2 times higher than background controls (peptide C31-45 pulsed BLCLs with HLA-DR pre-blocking, DMSO pulsed BLCLs). Thus, peptide C31-45 is verified as a HLA-DR restricted CD4 T-cell epitope. For peptide pool Core10 expanded PBMCs, after stimulation with peptide C91-105 pulsed BLCLs, the frequency of TNF-α secreting CD4 T cells and the frequency of IFN-γ secreting CD4 T cells are 0.221% and 0.000% respectively, which do not exceed the 2 times of background controls (peptide C91-45 pulsed BLCLs with HLA-DR pre-blocking, DMSO pulsed BLCLs), so peptide C91-105 is not verified as HLA-DR restricted CD4 T-cell epitope.

FIGURE AND TABLE LEGENDS:

Figure 1: **Flow cytometry demonstration of TNF-α/IFN-γ secreting CD4 T cells in peptide pools expanded PBMCs after stimulated with their respective peptide pools.**

Figure 2: **Demonstration of the analysis HBV core-specific TNF-α/IFN-γ secreting CD4 T cells.** The TNF/DMSO and IFN-Y/DMSO indicate the ratios of the frequencies of TNF-α/IFN-γ secreting CD4 T cells in each well of peptide pool stimulated PBMCs divided by the frequency of TNF-α/IFN-γ secreting CD4 T cells in the well of DMSO control. Gray background indicates wells with positive CD4 T-cell response judged by comparison with background control.

Figure 3: **Demonstration of the screening of candidate peptides for epitope identification.** The TNF/DMSO and IFN-Y/DMSO indicate the ratios of the frequencies of TNF-α/IFN-γ secreting CD4 T cells in each well of peptide pool stimulated PBMCs divided by the frequency of TNF-α/IFN-γ secreting CD4 T cells in the well of DMSO control. Gray background indicates wells with positive CD4 T-cell response judged by comparison with background control. Peptides in red indicate candidate peptides according to the screening criteria.

Figure 4: Flow cytometry demonstration of epitope identification results.

DISCUSSION:

The most critical steps in this protocol are listed as follows: 1) enough PBMCs of high viability to start PBMCs expansion; 2) appropriate environment for PBMCs expansion; and 3) complete removal of residual peptide pools in PBMCs culture before epitope identification.

All the analysis in this protocol depends on the robust proliferation of CD4 T cells. In general, the number of PBMCs after 10-day expansion will be 2-3 times of the initial number. The cell number and the viability of PBMCs are 2 key factors in PBMCs expansion. If the purpose is only to analyze HBV-specific CD4 T cells without epitope identification, it is reasonable to reduce the initial PBMCs number, especially when the volume of blood sample is limited. While, in our experience, successful PBMCs expansion could barely be obtained if the start number of PBMCs is below 1.5×10^5 cells/well. When using fresh PBMCs for expansion, the cell viability will not be a problem. While when using cryopreserved PBMCs for expansion, the cryopreservation and thawing of PBMCs should be conducted very carefully to maintain the viability of PBMCs.

In functional analysis of HBV-specific T cells, IL-12 is usually used in PBMCs expansion to enhance the function of CD8 T cells. As IL-12 could induce differentiation of CD4 T cells towards CD4 T follicular helper cells, this cytokine should be avoided in functional analysis of HBV-specific CD4 T cells. In our protocol, only IL-2 (for T-cell expansion) and IL-7 (for T-cell survival) are supplemented to maintain the functional profile of HBV-specific CD4 T cells during expansion as intact as possible. We have tested 5 cytokines for functional analysis of HBV-specific CD4 T cells: TNF-α, IFN-γ, IL-4, IL-17, and IL-21. In our analyzed samples, TNF-α and IFN-γ are 2 major cytokines secreted by HBV-specific CD4 T cells[16]. In analyzing the functional profile of HBV-specific CD4 T cells, it is recommended to test as many as possible cytokines to obtain the detailed functional profile information. While in epitope identification, it is recommended to analyze only TNF-α and IFN-γ, for economic consideration.

Enough HBV-specific CD4 T cells are vital for successful epitope identification, so epitope identification should be considered in patients with high HBV-specific CD4 T-cell response, such as hepatitis B flare patients (strong HBV-specific TNF-α secreting CD4 T-cell response) and patients with viral clearance (strong HBV-specific IFN-γ CD4 T-cell response)[16]. It is very important to remove residual peptides in the peptide pool expanded PBMCs by repeated washing before incubating these cells with BLCLs for epitope identification. The residual peptides will bind to DMSO pulsed BLCLs, activate peptide-specific CD4 T cells, hereby increase the background to a great extent.

Some HBV antigen has variable sequences in different HBV genotypes (e.g., HBV surface antigen). A solution is to pre-determine the specific HBV genotypes in patients and design HBV genotype-specific peptide pools for patients with different HBV genotypes. While the HBV genotype is un-measurable in patients with low HBV viral loads (e.g., HBeAg negative patients with regular anti-viral treatment), in this scenario, the solution is to mix peptides from different HBV genotypes together into the same peptide pools, as we did in a previous study[16]. A drawback of this mixture strategy is that epitope might be identified as a peptide pair but not a single peptide, as some positions in the peptides matrix contain a peptide pair in the same fragment of the antigen.

A major drawback in this method is the time-consuming 10-day PBMCs expansion. Currently, ex vivo analysis of cytokine secretion could not detect HBV-specific CD4 T cells in a reliable way. Using peptide pulsed allogeneic BLCLs as stimulators usually detected more peptide-specific CD4 T cells in peptide expanded PBMCs, compared to simply stimulating with peptides[16]. It is worth investigating whether using peptide pulsed autologous B cells as antigen presentation cells could help to reliably detect HBV-specific CD4 T cells ex vivo.

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DISCLOSURES:

The authors have nothing to disclose.

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18. Anthony, D. D., Lehmann, P. V. T-cell epitope mapping using the ELISPOT approach. *Methods*. **29**, 260-269 (2003).

Figure 1

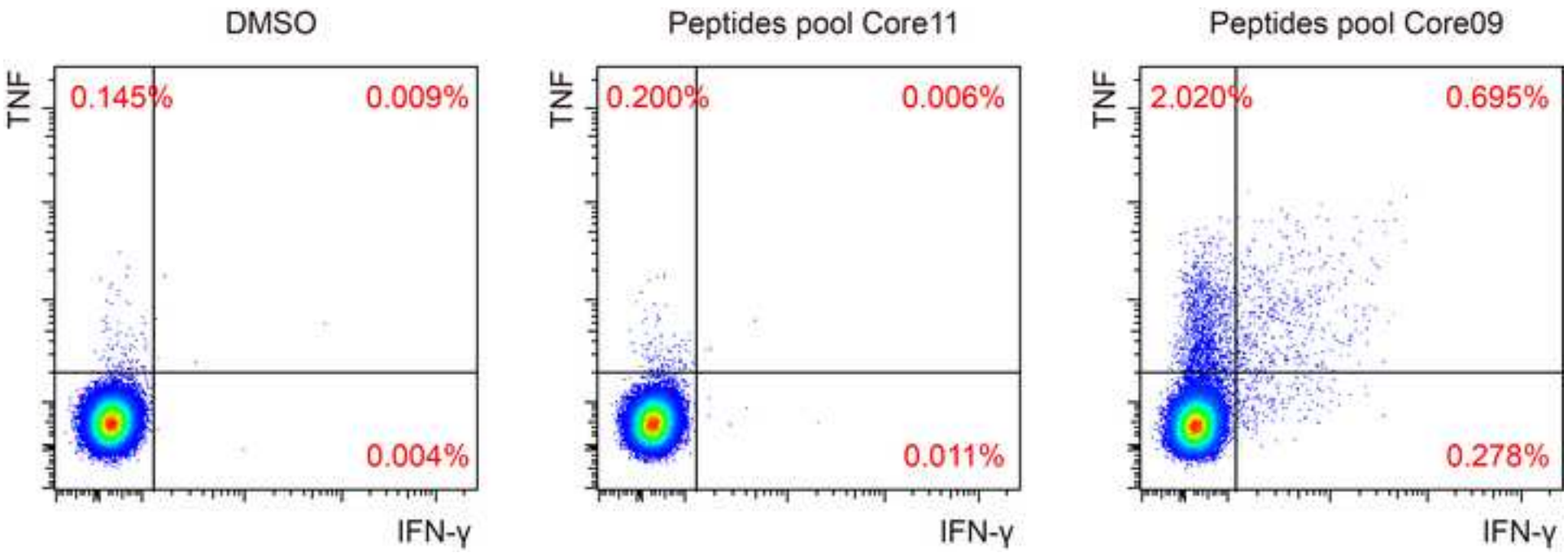

Figure 2

Core peptides pools	Core01	Core02	Core03	Core04	Core05	Core06	% TNF-α secreting cells	TNF/ DMSO	% IFN-γ secreting cells	IFN-γ/ DMSO
Core07	C1-15	C6-20	C11-25	C16-30	C21-35	C26-40	0.977	6.344	0.117	9.000
Core08	C31-45	C36-50	C41-55	C46-60	C51-65	C56-70	0.429	2.786	0.080	6.154
Core09	C61-75	C66-80	C71-85	C76-90	C81-95	C86-100	2.715	17.630	0.973	74.846
Core10	C91-105	C96-110	C101-115	C106-120	C111-125	C116-130	0.552	3.584	0.084	6.462
Core11	C121-135	C126-140	C131-145	C136-150	C141-155	C146-160	0.206	1.338	0.017	1.308
Core12	C151-165	C156-170	C161-175	C166-180	C169-183	DMSO	0.224	1.455	0.021	1.615

% TNF-α secreting cells	0.912	0.540	0.218	0.375	0.811	1.101
TNF/ DMSO	5.922	3.506	1.416	2.435	5.266	7.149
% IFN-γ secreting cells	0.398	0.086	0.032	0.065	0.036	0.174
IFN-γ/ DMSO	30.615	6.615	2.462	5.000	2.769	13.385

Background control (DMSO)	
% TNF-α secreting cells	% IFN-γ secreting cells
0.154	0.013

Figure 3

Core peptides pools	Core01	Core02	Core03	Core04	Core05	Core06	% TNF-α secreting cells	TNF/ DMSO	% IFN-γ secreting cells	IFN-γ/ DMSO
Core07	C1-15	C6-20	C11-25	C16-30	C21-35	C26-40	0.977	6.344	0.117	9.000
Core08	C31-45	C36-50	C41-55	C46-60	C51-65	C56-70	0.429	2.786	0.080	6.154
Core09	C61-75	C66-80	C71-85	C76-90	C81-95	C86-100	2.715	17.630	0.973	74.846
Core10	C91-105	C96-110	C101-115	C106-120	C111-125	C116-130	0.552	3.584	0.084	6.462
Core11	C121-135	C126-140	C131-145	C136-150	C141-155	C146-160	0.206	1.338	0.017	1.308
Core12	C151-165	C156-170	C161-175	C166-180	C169-183	DMSO	0.224	1.455	0.021	1.615

% TNF-α secreting cells	0.912	0.540	0.218	0.375	0.811	1.101
TNF/ DMSO	5.922	3.506	1.416	2.435	5.266	7.149
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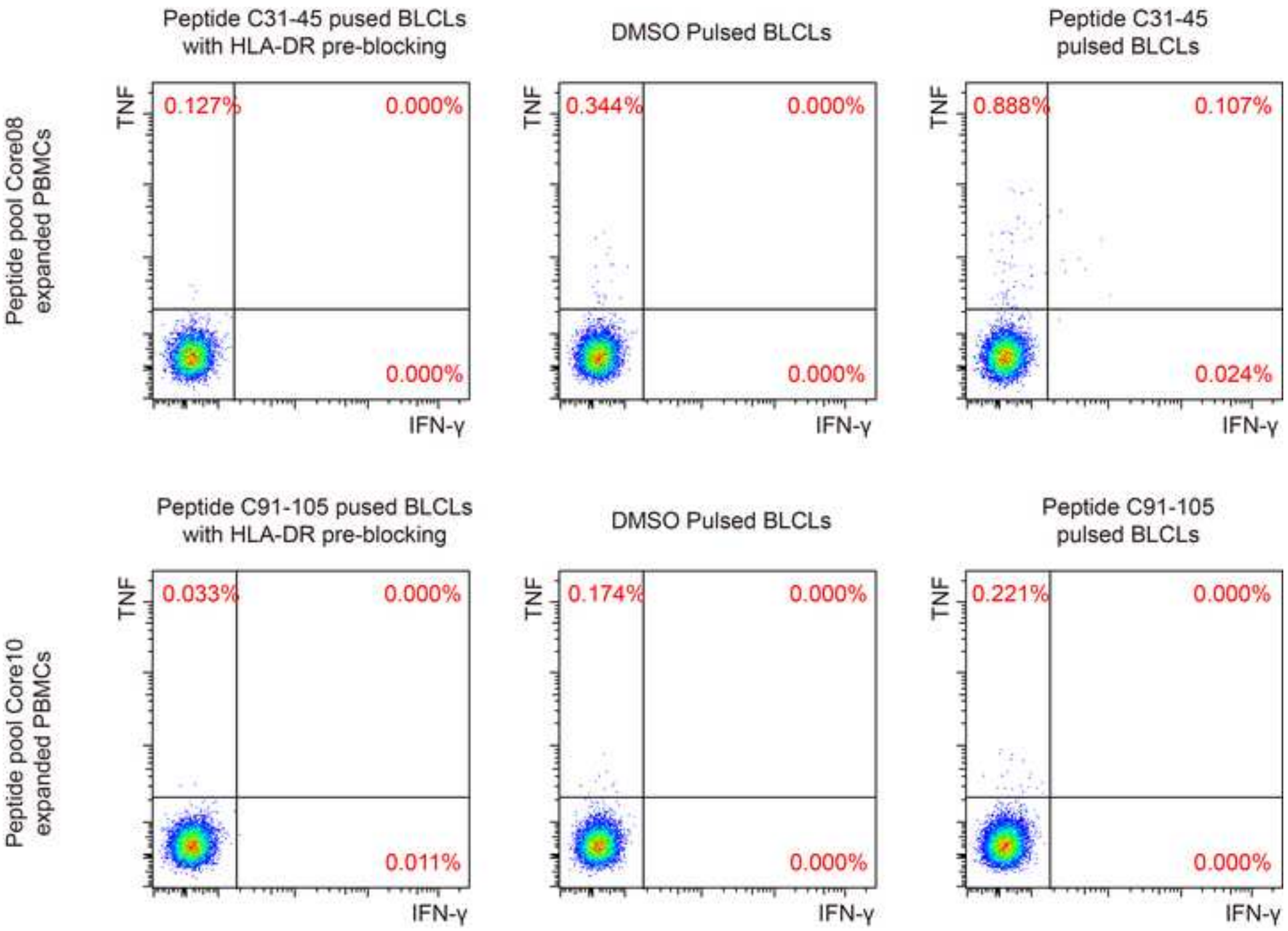
IFN-γ/ DMSO	30.615	6.615	2.462	5.000	2.769	13.385

Background control (DMSO)	
% TNF-α secreting cells	% IFN-γ secreting cells
0.154	0.013

Figure 4



Name of Material/ Equipment	Company	Catalog Number
Albumin Bovine V (BSA)	Beyotime	ST023
APC-conjugated Anti-human TNF-α	eBioscience	17-7349-82
Benzonase Nuclease	Sigma-Aldrich	E1014
B lymphoblastoid cell lines (BLCLs)	FRED HUTCHINSON C	IHW09126
B lymphoblastoid cell lines (BLCLs)	FRED HUTCHINSON C	IHW09121
Cell Culture Flask (T75)	Corning	430641
Cell Culture Plate (96-well, flat bottom)	Corning	3599
Cell Culture Plate (96-well, round bottom)	Corning	3799
Cell Strainer	Corning	CLS431751
Centrifuge Tube (15 mL)	KIRGEN	KG2611
Centrifuge Tube (50 mL)	Corning	430829
Centrifuge, Refrigerated	Eppendorf	5804R
Centrifuge, Refrigerated	Thermo	ST16R
Centrifuge, Refrigerated	Thermo	Legend Micro 21R
Cytofix/Cytoperm Kit (Transcription Factor Buffer Set)	BD Biosciences	562574
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco’s Phosphate Buffered Saline		
Ficoll-Paque Premium	GE Healthcare	17-5442-03
Filter Tips (0.5-10)	Kirgen	KG5131
Filter Tips (100-1000)	Kirgen	KG5333
Filter Tips (1-200)	Kirgen	KG5233
FITC-conjugated Anti-human CD4	BioLegend	300506
Fixable Viability Dye eFluor780	eBioscience	65-0865-14
GolgiStop Protein Transport Inhibitor (Containing Monensin)	BD Biosciences	554724
Haemocytometer	Brand	718620

HBV Core Antigen Derived Peptides	ChinaPeptides	
HEPES	Gibco	15630080
Human Serum AB	Gemini Bio-Products	100-51
Ionomycin	Sigma-Aldrich	I0634
KCl	Sangon Biotech	A100395-0500
KH2PO4	Sangon Biotech	A100781-0500
LSRFortessa Flow Cytometer	BD	
L-glutamine	Gibco	25030081
Microcentrifuge Tube (1.5 mL)	Corning	MCT-150-C
Microplate Shakers	Scientific Industries	MicroPlate Genie
Mitomycin C	Roche	10107409001
Na2HPO4.7H2O	Sangon Biotech	A100348-0500
NaCl	Sangon Biotech	A100241-0500
PCR Tubes (0.2 mL)	Kirgen	KG2331
PE/Cy7-conjugated Anti-human CD8	BioLegend	300914
PE-conjugated Anti-human IFN-γ	eBioscience	12-7319-42
Penicillin Streptomycin	Gibco	15140122
PerCP-Cy5.5-conjugated Anti-human CD3	eBioscience	45-0037-42
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	P1585
Recombinant Human IL-2	PeproTech	200-02
Recombinant Human IL-7	PeproTech	200-07
RPMI Medium 1640	Gibco	C11875500BT
Sodium pyruvate,100mM	Gibco	15360070
Trypan Blue Stain (0.4%)	Gibco	15250-061
Ultra-LEAF Purified Anti-human HLA-DR	BioLegend	307648
Wizard Genomic DNA Purification Kit	Promega	A1125

Comments/Description

Keep protected from light
Limit cell clumping
HLA-DRB1*0803 homozygote
HLA-DRB1*1202 homozygote

Flat bottom
Round bottom
Pore size 70 µm, white, sterile
Sterile
Sterile

Prepare solution before use
Keep at room temperature to prevent crystallization
Prepare ddH_2O (1000 ml) containing NaCl (8000 mg), KCl (200 mg), KH2PO4 (200 mg), and Na2HPO4.7H2O (2160 mg). Adjust

Sterile
Sterile
Sterile
Keep protected from light
Keep protected from light
Protein Transport Inhibitor

100 ml
100 ml

100 ml
Autoclaved sterilization before using

Keep protected from light
Keep protected from light
100 ml
Keep protected from light

500 ml

PH to 7.4. Sterilize through autoclave.

Editorial comments:

Response: Dear Vineeta Bajaj, **t**hanks you for the valuable suggestions to our manuscript. We have made some alterations to our manuscript. All changes in the manuscript have been indicated in red. The point by point responses to your comments are listed below.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have proofread the manuscript and corrected the spelling and grammar errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points. Please ensure that the highlight is no more than 3 pages including headings and spacings.

Response: We have reformatted the manuscript accordingly.

3. Please provide the institutional mailing address of all the authors.

Response: The institutional mailing address is Department of Infectious Diseases, Southwest Hospital, Third Military Medical University (Army Medial University), 29 Gaotanyan Street, Shapingba District , Chongqing 400038, China (the first affiliations in title page)

4. Please define all abbreviations before use (i.e., MHC, DMSO, etc.)

Response: All the abbreviations have been defined before the first use.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Personal pronouns have been removed in the revised manuscript.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: An ethics statement has been added.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent(Examples: Ficol-plaque, QIAamp DNA Mini Kit, GolgiStop). Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Response: All the commercial language has been removed.

8. Line 102: Please provide details of the density gradient centrifugation. How much is the centrifugation speed? How long is the centrifugation performed?

Response: Details of the density gradient centrifugation has been added (Protocol 2.2).

9. Line 104: Please include details on how the granulocyte is collected?

Response: Details of the collection of granulocytes has been added (Protocol 2.3)

10. Line 105: Please provide more details on genomic DNA extraction. How is it performed? What are the parameters involved?

Response: Genomic DNA extraction is performed using a commercial kit (Protocol 2.3). In our opinion, there are no secret tricks in this procedure. Good results could be obtained by simply following providers' guide.

11. Line 112/116/119/124/129: Please define the term warm. What is the temperature?

Response: The temperature has been added to replace the term "warm".

12. Line 123: Please define the centrifugation speed (in x g) and time.

Response: The details of the centrifugation has been added (Protocol 3.1.4).

13. Line 127: Please elaborate on the enumeration step using a hemocytometer.

Response: The procedure of cell counting has been updated (Protocol 3.1.5).

14. Line 146: Please include details on pipetting. How much of what is pipetted?

Response: The details of pipetting has been added (Protocol 3.6).

15. Line 159: Please provide more details on the washing step. How much medium is used to wash?

Response: The details of cell washing has been added (Protocol 4.1.1).

16. Please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Lines: 174,175,177, 211,213, 214, 230,231)

Response: All the abbreviated time units less than one day have been modified accordingly.

17. Line 183: Please describe the steps involved in flow cytometry.

Response: Flow cytometry analysis is a complicated technique and includes many key steps, e.g, panel design, staining, data acquisition, and data analysis. It is impossible to clarify all the key steps in this manuscript and the main purpose of this protocol is not about flow cytometry. In this protocol, we emphasize on the staining procedure of flow cytometry (Protocol 4.2.1-4.2.3) which require the least knowledge of flow cytometry and the other steps could be done with assistance from a specialized expert.

18. Line 190: Please use consistent font style and size when formatting equations.

Response: The equation has been reformatted accordingly (Protocol 4.3.2).

19. Line 195: Please provide more details on the maintenance of allogenic lymphoblastoid cell lines. How much medium is required for how many cells?

Response: The details on the maintenance of BLCLs has been added (Protocol 5.1).

20. Line 209: Please provide details of the harvesting step.

Response: The details on BLCLs harvesting has been added (Protocol 5.3.1).

21. Line 212: Please provide details on peptide pulsing. What is the dilution of anti-HLA-DR used?

Response: The details on peptide pulsing has been added (Protocol 5.3.2).

22. Line 213: Please elaborate on DMSO pulsing. What is the concentration of DMSO used?

Response: The details on DMSO pulsing has been added (Protocol 5.3.2).

23. Line 215: Please specify the volume of RPMI used for washing.

Response: The details on BLCLs washing has been added (Protocol 5.3.4)

24. Figure 2 and Figure 3: Please define the columns and rows labeled "TNF/DMSO" and "IFN-Y/DMSO". Are these expressed as a percentage?

Response: The meanings of "TNF/DMSO" and "IFN-Y/DMSO" have been explained in Figure and Table Legends section of revised manuscript.

25. Figure 4: Please ensure the titles of the figures are correct and consistent. Please revise the misspelled words.

Response: The spelling errors in Figure 4 have been corrected.

26. Please sort the table of materials in alphabetical order.

Response: An updated version of Table of Materials consistent with the requirement has been provided.

27. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: An updated version of Table of Materials consistent with the requirement has been provided.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Understanding the immunopathogenesis of chronic hepatitis B is nowadays believed to be a key to deliver new treatment strategies and the participation of CD4+ T cell subsets has gained more attention. The authors desribe a more high-throughput method to identify HLA-DR-restricted epitopes from a single or more HBV-encoded proteins, combined with studying the T cell responses. It is a valuable method that should be useful to readers. In fact, the protocol has been already applied by the authors in a study published in a high-ranked journal (J Hepatol. Reference 15). As the authors quote, the peptide librares have been used to study T cell responses (like cytokine profiles); however combining them with peptide-pulsed allogenic BLCLs as stimulators seems to be the novel and original approach proposed by the authors; the reviewer has not found a similar method described by other authors. For these reasons the protocol deserves to be shared in JOVE to other scientists. Unfortunately, not it its current form as the text requires a thorough revision, containing numerous errors in English. If JOVE provides the editing sevice, this should solve the problem; however in the current form the text is hard to read and reproduce the results. The low quality of English language is surprising as the J.Hepatol. paper involving the described technique, with a similar vocabulary, is English error-free. The mistakes are both typographical and grammar (for instance, plural vs singular, use of tenses, comas, fullstops inside sentences).

Positive and negative controls are included, there are appropriate.

The Introduction is proper and interesting, describing three types of approach to study T-cell epitopes and responses in a comparative way, justifying the need for a novel method like the one proposed by the authors. Discussion is almost sufficent - I

have a question on the variability of HBV sequence - how to deal with the regions that are more variable and may not be represented by the designed peptide pools; I recommend adding such a comment to the Discussion.

Response: Dear reviewer, **t**hanks you for the valuable suggestions to our manuscript. With consideration of your comments and academic meaning of this study, we have made some alterations in our manuscript. All changes in the manuscript have been indicated in red.

We have proofread the manuscript and corrected the spelling and grammar errors.

Some HBV antigen has variable sequences in different HBV genotypes (e.g., HBV surface antigen). One solution is to pre-determine the specific HBV genotypes in patients and design HBV genotype-specific peptides pools for patients with different HBV genotypes. While HBV genotype is un-measurable in patients with low HBV viral loads (e.g., HBeAg negative patients with regular anti-viral treatment), in this scenario, the solution is to mix peptides from different HBV genotypes together into the same peptides pools, as we did in a previous study (Ref. 16). A drawback of this mixture strategy is that epitope might be identified as a peptides pair but not a single peptide, as some positions in the peptides matrix contain a peptides pair in the same fragment of the antigen. We have added a comment concerning variable HBV sequence to the Discussion section of the manuscript.

The point by point responses to your comments are listed below.

Major Concerns:

1. The protocol was published by the authors in Journal of Hepatology, reference 15; The main body of the paper and this submission to JOVE contain different representative data, so this is not a republished data. The information on the published protocol should be, however, stated more clearly.

Response: Thank you for the advice. We have pointed out the published protocol in a more clear way in the Introduction section of the manuscript.

2. The Title: A Method to Analyze HBV-specific CD4 T-cell Response and Identify HLA-DR Restricted CD4 T-cell Epitopes Simultaneously Based on Peptides Matrix: Apart from the fact it contains mistakes (the title should be, most probably, read as: A Method to Analyze HBV-specific T-cell responses and Identifiy HLA-DR-restricted CD4 T-cell epitopes simutaneuosly based on a peptide matrix) it is unclear why the authors use the term peptide MATRIX. Matrix is usually associated with reagents on a solid support, like conjugated, linked to a support; whereas in the paper resuspended peptides are added to cell cultures. Please explain what is the reason to use the term "matrix", or, alternatively, it would be more proper to use the term "a peptide LIBRARY" and indeed in one of the quoted papers (16) such a term is used.

Response: Thank you for the advice about the title. We have changed the title into "A Method to Analyze HBV-specific CD4 T-cell Responses and Identify HLA-DR-restricted CD4 T-cell Epitopes Based on Peptide Matrix". The word "matrix" has been used to indicate a rectangular array of elements in Mathematics. We used the "matrix" to indicate the rectangular array of peptides like previously described (Ref. 17 and 18). The term "peptide library" could not convey the rectangular arrangement of peptides in this method.

3. Using peptide libraries covering the whole HBV genome to study T cell responses (both CD8+ and CD4+) by intracellular cytokine staining was used in another reference that should be included :Chang et al., Reduced Hepatitis B Virus (HBV)-Specific CD4+ T-Cell Responses in Human Immunodeficiency Virus Type 1-HBV-Coinfected Individuals Receiving HBV-Active Antiretroviral Therapy, J Virol. 2005 Mar; 79(5): 3038-3051.doi: 10.1128/JVI.79.5.3038-3051.20054.

Response: This is a good pioneering study in HBV-specific T-cell responses. We have included it in the Introduction section of the manuscript (Ref. 11).

4. Lines 89-95: the setup of the peptide matrix is unclear, it will be perhaps more clear when visualized as a movie, for instance, line 87: we purchase a panel of 35 15-mer peptides, but there are 12 peptide pools by 5-6 peptides each, and judging

from the peptide numbers in Figure 2, there are 183 peptides? How you can mix peptides before dissolving them in DMSO, line 92-93?

Response: This peptide matrix is a 2-dimension (row/column) peptide library. The row peptide pools and the column peptide pools in the matrix represent 2 separate formations of HBV core antigen. For a 1-dimension peptide library, 6 peptide pools with 5-6 peptides in each pool is enough to cover the whole antigen; while for a 2-dimension peptide library, 6×2 peptide pools with 5-6 peptides in each pool are required to cover the whole antigen twice. We have modified Protocol 1.3. to convey the setup of peptide matrix in a more clear way.

For the Figure 2/3, the number indicates the sequence information of peptides, i.e., C169-183 indicates the peptide (position: amino acid 169 to amino acid 183 of HBV core antigen).

We mix peptides by dissolving them together in DMSO. The original description in Protocol 1.4 is not accurate, and modification has been made accordingly.

5. Cell washing is stated as a crucial step in the protocol -therefore washing should be described in more details, e.g., 4.1.1. line 159-160 - "and Wash 3 times with RPMI 1640 to remove un-pulsed peptide and mitomycin C." - the info is missing how the cells are washed - by plate centrifugation?

Response: Cells are washed by plate centrifugation. Detailed description of cell washing is added to Protocol 4.1.1.

6. 4.3.1. in flow cytometry and in other places in the text and the formula - the term "frequency" is used, whereas "percentage" is commonly used to name the % of positive cells (as shown in Figure 1).

Response: The term "percentage" is ok, but the term "frequency" is also commonly used to indicate the magnitude of HBV-specific T-cell responses (Ref. 12-16).

Minor Concerns:

1. Line 56: The commonly used markers include 56 CD69, CD25, OX40, CD40L, PD-L1, 4-1BB, and etc. would sound better: The commonly used markers include, e.g., CD69, CD25, OX40, CD40L, PD-L1, 4-1BB.

Response: Thank you for the advice and the alteration in the manuscript has been made accordingly.

2. Paragraph 3: Although the use of benzonase in PBMC isolation is explained in standard protocols, the info that it reduces cell clumping may appear earlier as it explains better the benefit of using the nuclease.

Response: Thank you for the advice and the alteration in the manuscript has been made accordingly (Protocol 3.1.1 Note).

3. In paragragh 3.5 and the Note, line 152-153, maybe it would be a good idea to make a note that the cells should be settled at the bottom of the plates when replacing the half of the medium.

Response: Thank you for the advice and the alteration in the manuscript has been made accordingly (Protocol 3.5 Note).

4. Line 175 and throughout the text: GolgiStop instead of GlogiStop, this is an important mistake.

Response: Thank you for the kind reminder. The term “Golgistop” (commercial name) has been replaced with the term “Monensin” (generic name) accordingly (Protocol 4.1.4 and 5.4.6).

5. 4.2.1 "stain surface markers and viability marker": the markers and the dye used for viability analysis should be stated here.

Response: Thank you for the advice and the alteration in the manuscript has been made accordingly (Protocol 4.2.1).

6. The list of Reagents: GemCell™ U.S. Origin Human Serum AB is stated as a reagent and it does not appear in the text. COBAS TaqMan 48 - what is is needed for?

HBV core and S antigen derived peptide - there is no need to state S-derived peptides if only core was used as an example to obtain representative data.

Response: Thank you for the scrutiny. The original Table of Materials is not well-prepared. We have updated a new version of Table of Materials with all the mistakes corrected.

7. The source or examples of allogenic B lymphoblastoid cell lines (BLCLs) would be given.

Response: Thank you for the advice. The information about the source of BLCLs has been added to the Table of Materials.

Why some parts are highlighted in yellow?

Response: The parts highlighted in yellow are procedures which we deem suitable for visual demonstration.

Why there is a remark: "revised November 2017"?

Response: This is a marker in the manuscript template provided by JOVE. We have deleted it in the revised manuscript.

Reviewer #2:

Manuscript Summary:

A Method to Analyze HBV-specific CD4 T-cell Response and Identify HLA-DR Restricted CD4 T-cell Epitopes Simultaneously Based on Peptides Matrix
In this manuscript, the authors Xiao et al used fluorescent staining and flowcytometry to categorize MHC-II associated CD4 T Cell functionality against Hepatitis as FN-γ for CD4 T helper 1 cells, IL-4 and IL-13 for CD4 T helper 2 cells, IL-21 for CD4 T follicular helper cells, and IL-17 for CD4 T helper 17 cells.

The authors address an important methodological approach to inform analyses of simultaneously detecting pathogen-induced MHC-II restricted CD4 T-Cell functional T Cell immune responses. This methodology builds onto existing research in this area.

The authors will need to clarify a few grey areas below
Response: Dear reviewer, **t**hanks you for the valuable suggestions to our manuscript. With consideration of your comments and academic meaning of this study, we have made some alterations in our manuscript. All changes in the manuscript have been indicated in red. The point by point responses to your comments are listed below.

Major Concerns:

1. The methodology is very relevant for distinguishing vaccine-induced response outcomes especially where TH1 responses might be preferred to TH2 responses. The authors could discuss a little more on the relevance of analysing for the categories of functional responses in natural infection and in vaccinees.
Response: Thanks you for the valuable suggestions to the academic meaning of this study. Theoretically this method could be applied to analyze T-cell responses in natural infection of other virus and in vaccination. While for acute virus infection or vaccination in which antigen-specific T-cell responses are readily detectable after

antigen stimulation ex vivo, there is no need for a 10-day expansion of antigen-specific T cells in vitro like we did in chronic HBV infection.

2. It is recommended that cryopreserved cells are allowed to go through an overnight recovery incubation at 37C, 5% CO2 rather than just use them immediately after thawing for assays; this allows the true quantitation of the health of the cells and number of viable cells used at the onset of this assay. In this manuscript, the assay is set up without proper computation of the cell number; starting cells may not be equivalent. This makes the comparability of the cells across different cell recoveries difficult. The authors should show comparative data of the assay set up with and without overnight resting of cells

Response: Thank you for the advice. Resting thawed cells overnight before functional analysis has 2 advantages: firstly, resting overnight allow the quantitation of true viable cells like you stated in the comment; more importantly, this could relieve the functional suppression of T cells by residual DMSO (a component of cryopreservation medium). The suppression of DMSO on T-cell function is not only observed in our lab but also reported by other study (Lucas de Abreu Costa et al., Dimethyl Sulfoxide (DMSO) Decreases Cell Proliferation and TNF-α, IFN-γ, and IL-2 Cytokines Production in Cultures of Peripheral Blood Lymphocytes, Molecules. 2017; 22(11): 1789. doi: 10.3390/molecules22111789.). So, for direct functional analysis of thawed cells, it is necessary to rest cell overnight before analysis. However, in our method, HBV-specific T cells are analyzed after 10-day in vitro expansion. In that scenario, the suppression role of DMSO is no longer a problem, and the flow cytometry analysis has gated out dead cells.

3. Under pulsing with peptides (section 5.3), the peptide concentration of 10 ug/ml used to stimulate the cells is too high, the authors should show optimisation data that led to recommending this concentration. Likewise, optimisation data for use of 100 ug/ml of Mitomycin should be presented, this concentration seems too high

Response: For the peptide dose in epitope identification: It is reasonable to enhance the dose of peptides for optimum pulsing. Actually, the dose (10 μg/mL) of a single peptide for epitope identification is not higher than the dose of a peptide pool (2 μg/mL for each peptide, 5-6 peptides, 10-12 μg/mL in total) for PBMCs stimulation. As we indicate in the manuscript, throughout the protocols, the dose of DMSO (solvent of peptides) is not allowed to exceed 1 μL/mL which exerts no obvious suppression on T-cell function in our preliminary experiments. For the dose of Mitomycin C in BLCLs treatment: the BLCLs in our methods showed high tolerance to Mitomycin C. In our preliminary experiments, BLCLs proliferate robustly with the dose of Mitomycin C at 50 μg/ml; with the dose of Mitomycin C at 100 μg/ml, BLCLs cease robust proliferation while remain viable, which is a good state for peptides pulsing.

Minor Concerns:

1. The title and abstract are appropriate for this manuscript.
Response: Some minor modifications have been made to the title.

2. Analysis templates are clear, but only reflecting the ability to detect IFN-g/TNF-a. It would be more useful for the reader to see data that shows ability of this methodology to distinguish the different CD4 functional profiles.
Response: In the Discussion section, we have addressed the analysis of secretion of other cytokines by HBV-specific CD4 T cells based on our previous study (Ref. 16).

3. It would be helpful if relevant references or links are provided for the mentioned NCBI database (line 86) and the procedure for Ficoll Paque gradient centrifugation
Response: Thank you for the advice. The GenBank reference number of HBV core antigen has been added (Protocol 1.1). Also, the condition of Ficoll density gradient centrifugation has been added (Protocol 2.2).

4. There are a few typos to address

Response: Thank you for the kind reminder. We have proofread the manuscript and corrected the spelling and grammar errors.