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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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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.
   NOTE: As all the interviewees have finished COVID-19 vaccination recently, they did not wear a facial mask during the interview for the sake of convenience.

4. **Filming location:** Will the filming need to take place in multiple locations? ***Yes, same floor of the building***

Current Protocol Length

Number of Steps: 29
Number of Shots: 54

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Guohong Deng:** In a chronic HBV infection, CD4 T cells play important roles in both viral clearance and liver damage.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Guohong Deng:** This method enables us to evaluate HBV-specific CD4 T-cell responses and identify HBV-specific CD4 T-cell epitopes simultaneously.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Video Editor: Suggested b-roll: 3.2.1, 4.8.2*

Introduction of Demonstrator on Camera

1.3. **Guohong Deng:** Demonstrating the procedure will be Jianmei Xiao, a research assistant, and Xing Wan, a technician from my laboratory.

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrators look up from workbench or desk or microscope and acknowledge the camera.

1.3.2.1 Jianmei Xiao looks up from workbench or desk or microscope and acknowledge the camera.

1.3.2.2 Xing Wan looks up from workbench or desk or microscope and acknowledge the camera.

Ethics Title Card

1.4. Procedures involving human subjects have been approved by the Southwest Hospital's human research committee. Written informed consent was obtained from each patient included in the study.

Protocol

2. Isolation of PBMCs and In Vitro Expansion Using an HBV Peptide Matrix

2.1. To begin, isolate peripheral blood mononuclear cells, or PBMCs *(P-B-M-sees),* from blood using Ficoll density gradient centrifugation at 800 × *g* for 20 minutes **[1]**. Then, collect the granulocytes **[2]** between the Ficoll and the red blood cell layer **[2.1]** using a Pasteur pipette **[2.2]**.

2.1.1. WIDE: Establishing shot of talent using the centrifuge.

2.1.2. ECU: Talent collecting granulocytes.

2.1.2.1 Added shot: Talent demonstrating layers after Ficoll spin.

2.1.2.2 Added shot: benzonase RPMI 1640 in a water bath.

~~2.1.3. Talent thawing the cells in a water bath.~~

2.2. Transfer the thawed cell suspension to a 15-milliliter centrifuge tube **[1]**. Add 1 milliliter of the pre-warmed Benzonase RPMI 1640 dropwise, then slowly add another 6 milliliters **[2].** *Videographer: This step is important!*

2.2.1. Talent transferring the cell suspension to a centrifuge tube.

2.2.2. Talent adding 1 mL of RPMI 1640 dropwise, then another 6 mL.

2.3. Rinse the cryovial with 2 milliliters of Benzonase RPMI 1640 to retrieve the remaining cells **[1].**

2.3.1. Talent rinsing the cryovial.

2.4. Then, centrifuge the tube at 400 × *g* for 10 minutes **[1]**, remove the supernatant **[2]**, and loosen the pellet by tapping the tube **[3]**.

2.4.1. Talent placing the tube in the centrifuge.

2.4.2. Talent removing the supernatant.

2.4.3. Talent tapping the tube.

2.5. Gently resuspend the pellet in 1 milliliter of warm Benzonase RPMI 1640. Mix the cells gently and filter them through a 70-micrometer cell strainer if any cell clumps are visible **[1]**. Count viable cells using Trypan blue and a hemocytometer **[2.1][2.2-TXT]**.

2.5.1. Talent resuspending and mixing the pellet.

2.5.2. ~~Talent counting the cells.~~

2.6.2.1 Added shot: Talent loading cell suspension to a hemocytometer.

2.6.2.2: Added shot: Talent counting the cells. **TEXT: Clear cells = Viable cells.**

2.6. Resuspend the PBMCs in complete culture medium containing 10 units per milliliter IL-2 and 10 nanograms per milliliter IL-7 **[1]**. Adjust the cell density to 1.5 x 10^6 cells per milliliter, then plate the cells in 96-well flat bottom plates at a density of 3 x 10^5 cells per well **[2]**.

2.6.1. Talent resuspending the cells.

2.6.2. Talent plating the cells.

2.7. Add hepatitis B virus, or HBV, derived peptide pools to each well. For the background and positive control wells, add the same amount of solvent **[1-TXT]**. Incubate the plates at 37 degrees Celsius and 5% carbon dioxide **[2]**.

2.7.1. Talent adding peptide pools to the wells. **TEXT: Peptide pools: 2 µg/mL of each peptide**

2.7.2. Talent putting the plates in the incubator.

2.8. On day 3, supplement the culture medium with 50 units per milliliter of IL-2 and 10 nanograms per milliliter of IL-7 **[1]**. On day 7, replace half of the culture medium **[2.1]** with fresh medium containing 4 micrograms per milliliter peptides, 100 units per milliliter IL-2, and 20 nanograms per milliliter IL-7 **[2.2]**. *Videographer: This step is important!*

2.8.1. Talent adding IL-2 and IL-7.

~~2.8.2. Talent replacing the culture medium.~~

2.9.2.1 Added shot: ECU: Talent removing half of the culture medium.

2.9.2.2 Added shot: Talent adding fresh culture medium.

2.9. On day 10, gently pipette the cells in each well 7 to 9 times to disaggregate cell clusters **[1]**, count the number of viable cells and transfer 2×10^5 cells into each well of a 96-well round bottom plate for HBV-specific CD4 T-cell response analysis **[2]**.

2.9.1. Talent pipetting the cells.

2.9.2. Talent transferring cells to a 96-well round bottom plate.

2.10. For the residual cells in the 96-well flat bottom plate, adjust the volume of the culture medium to 100 microliters by discarding excessive medium **[1]**.

2.10.1. Talent discarding excessive medium.

2.11. Then, supplement the culture with 100 microliters of fresh complete culture medium containing 4 micrograms per milliliter peptides, 100 Units per milliliter IL-2 and 20 nanograms per milliliter IL-7 **[1].** Continue culturing the cells at 37 degrees Celsius and 5% carbon dioxide for epitope identification on day 12 **[2]**.

2.11.1. Talent adding fresh media.

2.11.2. Talent placing the plate in the incubator.

3. Analysis of HBV-specific CD4 T-Cell Responses by Intracellular Flow Cytometry

3.1. Wash the cells in the 96-well round bottom plate by adding 200 microliters of RPMI 1640 **[1]**, centrifuging **[2]** the plate at 550 × *g* for 3 minutes **[3.1]**, and discarding the supernatant. Repeat the wash twice, using complete culture medium for the last wash **[3.2]**. *Videographer: This step is important!*

3.1.1. Talent adding RPMI 1640 to the wells.

3.1.2. Talent centrifuging the plate.

~~3.1.3. Talent discarding the supernatant.~~

3.1.3.1 Added shot ECU: Talent demonstrating cell pellet well after centrifugation.

3.1.3.2 Added shot: Talent discarding the supernatant.

3.2. For each well of cells stimulated with a specific peptide pool, add 200 microliters of complete culture medium supplemented with the same peptide pool **[1-TXT]**. *Videographer: This step is important!*

3.2.1. Talent adding culture medium containing peptides in the wells. TEXT: **Peptide pools: 2 µg/mL of each peptide**

3.3. For the background control well, add complete culture medium supplemented with1 microliter per milliliter of DMSO **[1]**. For the positive control well, add complete culture medium supplemented with 1 microliter per milliliter DMSO, 150 nanograms per milliliter PMA, and 1 micromole per liter of ionomycin **[2-TXT]**.

3.3.1. Talent adding medium to background control well.

3.3.2. Talent adding medium to positive control well. TEXT: **PMA = phorbol 12-myristate 13-acetate**

3.4. Incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 6 hours **[1]**. After 1 hour of incubation, add 1.37 micrograms per milliliter Monensin to the culture **[2]**.

3.4.1. Talent placing the plate in the incubator.

3.4.2. Talent adding Monensin.

3.5. After the 6-hour incubation is complete, centrifuge the plate at 550 × *g* for 3 minutes **[1]**, remove the supernatant, and wash the cells once with 200 microliters of DPBS as previously demonstrated **[2]**.

3.5.1. Talent placing the plate in the centrifuge.

3.5.2. Talent removing supernatant.

3.6. Stain for surface markers CD3, CD4, and CD8 and a viability marker **[1.1]**, after suspending the cells with vibrator **[1.2]**, then refrigerate the plate at 4 degrees Celsius for 30 minutes **[2]**.

~~3.6.1. Talent adding surface marker antibodies.~~

3.6.1.1 Added shot: Talent suspending cells by a vibrator.

3.6.1.2 Talent adding surface marker antibodies.

3.6.2. Talent placing the plate in the refrigerator.

3.7. After washing the plate once with 200 microliters of DPBS, fix and permeabilize the cells **[1]**, stain for intracellular cytokines, TNF-alpha and interferon-gamma, and refrigerate the plate at 4 degrees Celsius for 45 minutes **[2].**

3.7.1. Talent permeabilizing the cells.

3.7.2. Talent adding antibodies for TNF-α and IFN-γ.

3.8. Wash the cells once again and resuspend them in 150 microliters of flow cytometry buffer. Then, acquire the flow cytometry data using a flow cytometer **[1-TXT]**.

3.8.1. Talent resuspending cells in flow cytometry buffer. **TEXT: Flow cytometry buffer: DPBS + 0.5% BSA**

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

4.1. Maintain allogenic B lymphoblastoid cell lines, or BLCLs, in a T-75 flask **[1]**.

4.1.1. Talent adding BLCLs to a T-75 flask.

4.2. On day 12 of the PBMC expansion, count the number of viable BLCLs and transfer them to 15-milliliter centrifuge tubes **[1]**. Centrifuge the cells at 350 × *g* for 10 minutes and remove the supernatant **[2]**.

4.2.1. Talent transferring the cells to centrifuge tubes.

4.2.2. Talent placing the tube in the centrifuge.

4.3. Resuspend the cell pellet in complete culture medium **[1]**, then aliquot the BLCLs to a 96-well round bottom plate at 4×10^4 cells per well in 80 microliters of complete culture medium **[2]**.

4.3.1. Talent resuspending the cell pellet.

4.3.2. Talent adding cells to the 96-well round bottom plate.

4.4. Add 10 micrograms per milliliter of a single peptide and set 2 background controls: peptide pulsing with HLA-DR blocking and DMSO pulsing **[1]**. Incubate the plates at 37 degrees Celsius and 5% carbon dioxide for 2 hours **[2]**.

4.4.1. Talent adding peptide to the wells.

4.4.2. Talent placing the plate in an incubator.

4.5. Add 100 micrograms per milliliter mitomycin C **[1]** and incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 1 hour **[2]**.

4.5.1. Talent adding mitomycin C.

4.5.2. Talent placing the plate in an incubator.

4.6. Wash the plate 3 times with 200 microliters of RPMI 1640 to remove un-pulsed peptide and mitomycin C, then resuspend the cells in 120 microliters of complete culture medium **[1.1]** using vibrator **[1.2]**.

~~4.6.1. Talent resuspending the cells.~~

4.6.1.1 Added shot: Talent adding 120 microliters of complete culture medium.

4.6.1.2 Added shot: Talent resuspending the cells by a vibrator.

4.7. On day 12 of the PBMC expansion, transfer the PBMCs to a 96-well round-bottom plate **[1]**. Centrifuge the cells in the plate, remove the supernatant, and wash them twice with 200 microliters of RPMI 1640 as previously demonstrated **[2].**
Videographer: This step is difficult and important!

4.7.1. Talent transferring PBMCs to a 96-well round-bottom plate.

4.7.2. Talent placing the plate in the centrifuge.

4.8. Resuspend PBMCs in each well with 210 microliters of complete culture medium **[1]**. For the PBMC wells chosen for epitope identification, aliquot 70 microliters of the cell suspension and mix with peptide pulsed BLCLs in 3 wells, including 2 background controls **[2]**.

4.8.1. Talent resuspending the PBMCs.

4.8.2. Talent mixing PBMCs with peptide pulsed BLCLs.

4.9. Incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 6 hours **[1]**. After 1 hour of incubation, add 1.37 micrograms per milliliter Monensin to the culture and bring the final volume in each well to 200 microliters with complete culture medium **[2].**

4.9.1. Talent placing the plate in the incubator.

4.9.2. Talent adding Monensin to the plates.

Results

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

5.1. In this representative example, the TNF-alpha and interferon-gamma secreting CD4 T-cell responses for peptide pool Core11 are lower than two times the background values, hence considered negative **[1]**. Meanwhile, the responses for peptide pool Core09 are higher than two times the background and considered positive **[2].**

5.1.1. LAB MEDIA: Figure 1. *Video Editor: Emphasize middle panel*

5.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize right-most panel*

5.2. The gray background indicates wells with a positive CD4 T-cell response **[1]** and candidate peptides for epitope identification are indicated in red **[2]**.

5.2.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the gray background cells*

5.2.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the peptides in red*

5.3. Core01 has the highest response rate judging from both TNF-alpha and interferon-gamma secreting CD4 T cells in column peptide pools **[1]**.

5.3.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the Core01 column*

5.4. Peptides C1-15 *(pronunciation example: C-one-to-fifteen)*, 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 show positive results **[1]**.

5.4.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize peptides C1-15, C31-45, C61-75, and C91-105*

5.5. PBMCs expanded with peptide pools Core07, 08, 09, and 10 are used for epitope identification of these peptides **[1]**.

5.5.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize Core07, Core08, Core09, and Core10*

5.6. Similarly, Core09 has the highest response rate in row peptide pools **[1].** All the peptides in this pool are set as candidate peptides **[2]** and PBMCs expanded with peptide pools Core01, 02, 03, 04, 05, and 06 are used for epitope identification of these peptides **[3]**.

5.6.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the Core09 row*

5.6.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize peptides C61-75, C66-80, C71-85, C76-90, C81-95, and C86-100.*

5.6.3. LAB MEDIA: Figure 3. *Video Editor: Emphasize Core01, 02, 03, 04, 05, and 06*

5.7. For peptide pool Core08 expanded PBMCs, after stimulation with peptide C31-45 pulsed BLCLs, the frequencies of TNF-alpha and interferon-gamma secreting CD4 T cells are 2 times higher than background controls. Thus, peptide C31-45 is a verified HLA-DR restricted CD4 T-cell epitope **[1].**

5.7.1. LAB MEDIA: Figure 4. *Video Editor: Only the top 3 panels (Core08 expanded PBMCs) need to be shown. Emphasize 0.888%, 0.107%, and 0.024% in the right-most panel.*

Conclusion

6. Conclusion Interview Statements

6.1. **Haoliang Wang:** If there are additional expanded PBMCs remaining after epitope identification, fine mapping of the identified epitopes can be performed using a panel of shortened peptides.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Video Editor: Suggested b-roll: 4.7.1*