An Optimized Ex Vivo ELISpot assay to identify IFN gamma positive, HBV-specific T cells in Chronic Hepatitis B patients

Cell Cultures, Immunology Assays

Authors Information

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Introduction

During a traditional ELISpot assay, low HBV Specific T cell frequencies have hindered effective ex vivo analysis. We overcame this obstacle to measure ex vivo T cell responses in CHB patients, by modifying the key variables of cell number and the peptide pulsing method to improve ex vivo detection of HBV-specific T cells.

Materials and Reagents

- 10^7 frozen PBMCs (per donor/ per time point)
- 30ml polypropylene tube (one per donor/ per timepoint sample)
- 7ml Eppendorf tubes
- Human IFN-γ single Color ELISPOT(Capture antibody Catalog: hT420; detection antibody: Catalog: hT428)(ImmunoSpot)
- Streptavidin-ALP: MABTEC AB; Code 3310-10
- Developing Buffer: KPL BCIP/NBT Phosphate substrate (KPL Substrate) (Sera Care: Cat No: 5420-0038)
- HBSS medium (Gibco, Ref# 24020-117)
- AIM V medium (Gibco, Ref# 12055-091)
- Knockout serum Replacement (KSR) (Lifetech: 10828028)
- Primocin (1ml of 50mg/ml: InvivoGen: Cat: ANT-PM-05)
- Human Blood type-AB serum (VWR, CA45001-062)
- DMSO solution (Sigma, Ref# D8418)
- HBV overlapping peptides (OLP), genotype C (GenScript) (Make Superstock:50mg/ml in 100% DMSO)
- 250ug/mL (50X) OLP pools: (made from superstock)
- 50X DMSO control media: (38% DMSO in AIM V)
- CEF purified peptide library (GenScript):
- Dynabeads™ Human T-activator CD3/28 (Gibco, Ref# 11131D)
- ImmunoSpot S6 Universal Analyzer
- 50ml multi-pipette troughs *(Optional)*
- Multi-channel pipette *(Optional)*
- Repeater pipette *(Optional)*
- Plate washer
- 70% Ethanol (in PBS)
- Blocking solution (AIM V media with 10% KSR)
- AIM V with Primocin (add 1ml of Primocin to 500ml bottle of AIM V)

Experimental Procedures

Preparation of OLP peptide pools:

313 peptides in total

Spans 15 amino acids (ie. 15-mers)
Peptide offset: 5 amino acids apart

- Peptide pools:
  - PreCore/Core = 41 peptides
  - X = 29 peptides
  - Env - 1 = peptides 1 – 181 (37 peptides)
  - Env – 2 = peptides 186 - 376 (36 peptides - 3 peptides not synthesized: 241, 246, 251)
  - Pol-1 = peptides 1 – 206 (42 peptides)
  - Pol-2 = peptides 211 – 416 (42 peptides)
  - Pol-3 = peptides 421 – 626 (42 peptides)
  - Pol-4 = peptides 631 – 831 (41 peptides)

Preparation of 50X OLP peptide pool

- PreCore/Core 50x
  - 41 peptides x 10 µl /peptide = 410 µl of peptides + 590 ml Aim-V + Prim
- X 50x stimulation pool
  - 29 peptides x 10 µl /peptide = 290 µl of peptides + 710 ml Aim-V + Prim
- Envelope 50x stimulation pool
  - Env-1 37 peptides x 10 µl /peptide = 370 µl of peptides + 630 ml Aim-V + Prim
  - Env-2 36 peptides x 10 µl /peptide = 360 µl of peptides + 640 ml Aim-V + Prim
- Polymerase 50x stimulation pool
  - Pol-1 42 peptides x 10 µl /peptide = 420 µl of peptides + 580 ml Aim-V + Prim
  - Pol-2 42 peptides x 10 µl /peptide = 420 µl of peptides + 580 ml Aim-V + Prim
  - Pol-3 42 peptides x 10 µl /peptide = 420 µl of peptides + 580 ml Aim-V + Prim
  - Pol-4 41 peptides x 10 µl /peptide = 410 µl of peptides + 590 ml Aim-V + Prim

Ex Vivo IFNγ ELISpot – Cell prepping

(General Protocol)

Materials:

- 10^7 frozen PBMCs (per donor; maximum 6 donors per plate)
- 30ml polypropylene tube (one per donor sample)
- Eppendorf tubes
- HBSS medium (Gibco, Ref# 24020-117)
- AIM V medium (Gibco, Ref# 12055-091)
- Knockout serum Replacement (KSR) (Lifetech: 10828028)
- Primocin (1ml of 50mg/ml: InvivoGen)
- Human Blood type-AB serum (VWR, CA45001-062)

- 50X HBV overlapping peptides, genotype C (GenScript)
- CEF purified peptide library (GenScript): (**USED to monitor treatment effect on unrelated virus-specific T cells**)
- DMSO solution (Sigma, Ref# D8418)
- Dynabeads^{TM} Human T-activator CD3/28 (Gibco, Ref# 11131D) (**USED as positive/Assay Control**)

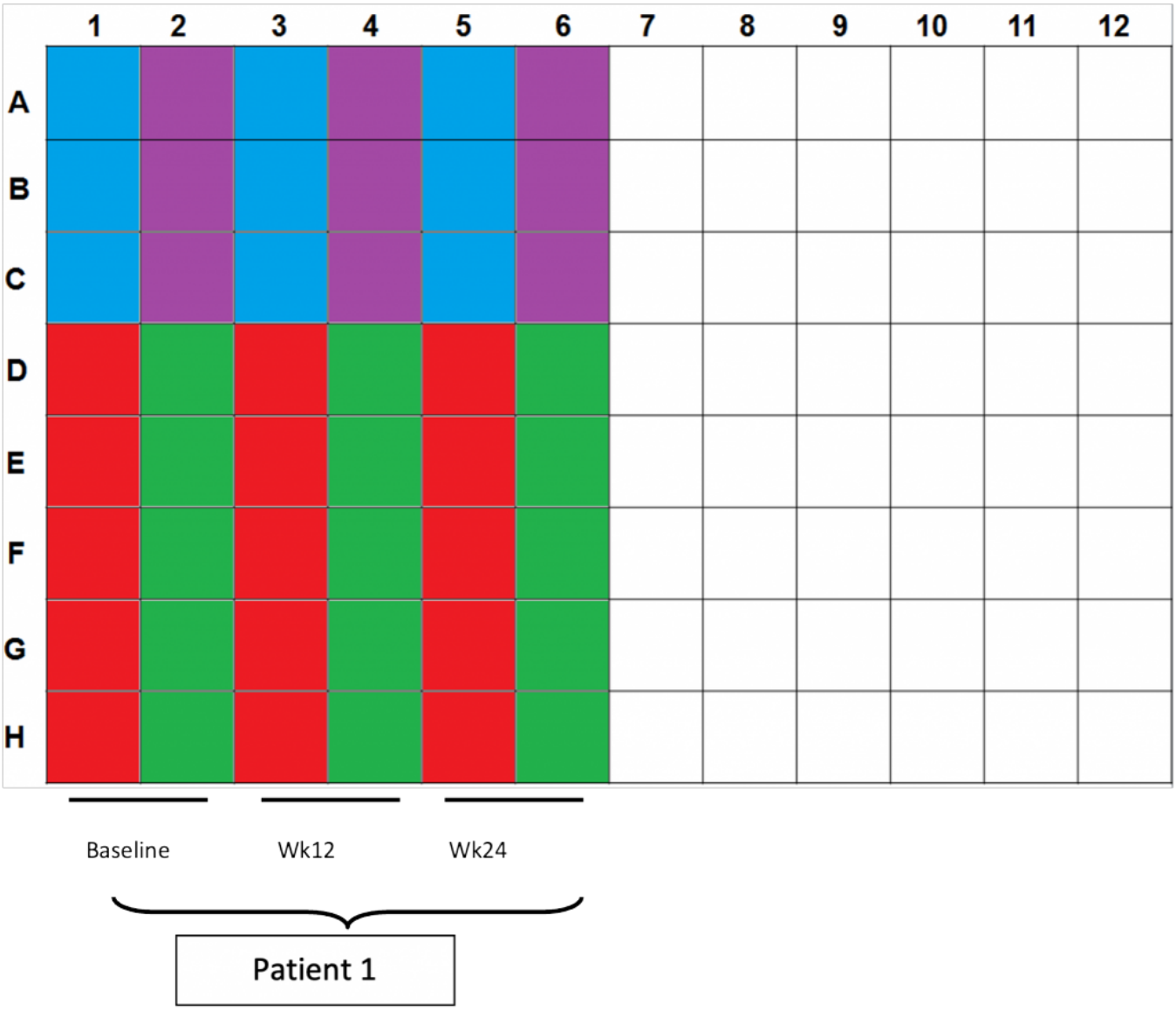
Day 1: Resting cells (pre-warm media)

1. Thaw 10^7c with HBSS medium (as per protocol) in a 30ml polypropylene tube a. Minimum count required for experiment: 6 x 10^6cells/donor
2. Centrifuge at 300xg for 5mins., aspirate
3. Resuspend at approximately 4x10^6 cells/ml with AIM V (+2% human serum)
4. Take counting aliquot for pre-rest counts *a. fix cell concentration if necessary (3.5-4.5x10^6 cells/ml are suitable as well)*
5. Incubate O/N in 37^{o}C incubator @ 5% CO_2
6. Coat IPFL plates, fridge O/N in 4^{o}C (as per manufacturer's, see next pages)

Day 2a: Pulsing cells (pre-warm media)

1. **16-18h** later, resuspend samples and take counting aliquot for post-rest counts
2. Aliquot two Eppendorf tubes with 4.5 x 10^5cells each (per donor) *a. 1 tube for HBV OLP stimulation, another for DMSO vehicle control*
3. Centrifuge at 300xg for 5mins., aspirate
4. Resuspend both tubes with 84µl **AIM V (+2% human serum)** each
5. **Pulsing scheme:** *final volume of 100µl* **a. HBV OLP sample:** add 2µl of 250µg/ml/peptide per OLP pool (8 pools: 16µl) i. HBV OLP final concentration: 5µg/ml/peptide **b. DMSO control sample:** add 16µl of 19.38% DMSO (in AIM V) i. DMSO final concentration: 3.1% DMSO (equivalent to OLP sample)
6. Incubate for 1h in 37^{o}C incubator @ 5% CO_2
7. Block IPFL plate simultaneously (as per protocol, see next page)
8. Centrifuge **both tubes of pulsed cells** at 300xg for 5mins., aspirate a. Resuspend cells with 225µl **AIM V (no serum)** *(ie. 2 x 10^6cells/ml)*
9. Aliquot two Eppendorf tubes with **8 x 10^6cells** each (per donor); centrifuge, aspirate a. Resuspend cells with 450µl **AIM V (no serum)** *(ie. 4 x 10^6cells/ml)* b. Pool respectively with **pulsed cells** c. Wash tube with another 450µl **AIM V (no serum)** and pool respectively d. **Final volume:**25x10^6 PBMCs in 1.125ml AIM V *(ie. 2 x 10^6cells/ml)*
10. Aliquot 1.2 x 10^6cells for **CEF controls**; centrifuge, aspirate a. Resuspend cells with 588µl **AIM V** and 12µl 50x CEF *(ie. 2 x 10^6 cells/ml)*
11. Aliquot 1 x 10^5cells for **CD3/28 controls**; centrifuge, aspirate a. Resuspend cells with 400µl **AIM V** and 0.4µl CD3/28 *(ie. 2.5 x 10^5 cells/ml)*

Day 2b: Plating cells



1. **Color scheme for plating:** a. Blue wells: 100µl **CD3/28 controls** ($2.5x10^4$cells per well) b. Purple wells: 200/200/100µl **CEF controls** ($4x10^5$cells/$2x10^5$cells per well) c. Red wells: 200µl **DMSO controls** ($4x10^5$cells per well) d. Green wells: 200µl **OLP pulsed cells** ($4x10^5$cells per well)
2. **Plating totals:** a. CD3/28: $5x10^4$cells in 3 wells b. CEF: $1x10^6$cells in 3 wells c. DMSO:$2x10^6$cells in 5 wells d. OLP: $2x10^6$cells in 5 wells
3. Incubate for **20h** in 37°C incubator @ 5% CO_2

Day 3: Plate development *(20 hours after plating cells)*

1. Develop plate the next day (as per plate prep protocol)

Ex Vivo IFNγ ELISpot –Plate prepping

Materials:

- 96-well multiscreen PVDF filtration plate: Millipore; Cat. Num. MSIPS4W10
- α-Human IFN-γ capture ab: ImmunoSpot
- α-IFN-γ Biotin ab: ImmunoSpot

- Streptavidin-ALP: MABTEC AB; Code 3310-10
- KPL BCIP/NBT Phosphate substrate (KPL Substrate) (Sera Care: Cat No: 5420-0038)

Day 1: Plate coating

1. Prepare coating Ab: **Stock 1mg/ml; Final 5µg/ml**
    - Add **25µl of IFNγ capture Ab in 5ml of sterile PBS** (for 45 wells)
2. Activate wells by adding **15µl of 35% ethanol** *NOTE: While activating the well with ethanol, don't let it sit. No well, should be exposed to ethanol for more than 60 sec.*
3. Wash the plate 6 times with sterile water
4. Place **100µl of coating Ab solution** into each well
5. Parafilm and incubate plate at 4^{o}C O/N

Day 2: Setting up plate

1. Wash the plate 6 times with sterile water
2. Make blocking solution, AIM V – 10% KSR
    - Add **45ml of AIM V to 5ml of KSR**
3. Add **100µl blocking solution** into each well
4. Incubate the plate at room temperature for at least 30 minutes
5. Remove via flicking
6. Add PBMCs to the plate after pulsing (as per Cell prep protocol, Page 1)
7. Incubate the plate @ 37^{o}C O/N

Day 3: Plate development *(24 hours after plating cells)*

1. Prepare α-IFN-γ Biotin: **Stock: 0.5mg/ml; Final: 0.5µg/ml**
    - Add **5µl anti-human IFNγ mAb Biotin in 5ml sterile PBS** (for 45 wells)
2. Wash the plate 6 times with PBS
3. Place **100µl** of 0.5ug/ml α-IFN-γ solution into each well
4. Incubate plate at room temperature for 2 hours
5. Prepare Streptavidin-ALP: **Stock: 0.5µg/ml; Final: 0.25ng/ml**
    - Add **5µl Streptavidin-enzyme in 5ml sterile PBS** (for 45 wells)
6. Wash the plate 6 times with non-sterile PBS
7. Add Streptavidin. Place **100µl** solution into each well
8. Incubate the plate at room temperature for 30 minutes
9. Wash the plate 6 times with non-sterile PBS
10. Add **50µl of KPL Substrate** solution into each well.
11. Incubate in the dark at room temperature for 20 minutes.
12. Remove plate underdrain and wash underside with running water, flick, and repeat ~10 time; flick dry as much as possible ***NOTE:*** *Avoid flicking on paper towels to minimize dust/particulates in wells*
13. Dry ~20-30mins in the BSC (with underside upwards) in the dark
14. Scan and count plates

References

Chua, C. G. *et al.* Optimized ex vivo stimulation identifies multi-functional HBV-specific T cells in a majority of chronic hepatitis B patients. *Sci. Rep.* **10**, (2020).