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Production of Primary Human CD4⁺ T Cell Lines and Clones

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Summary

Tetramer staining of CD4⁺ T cells is a valuable technique in immunology for detecting rare auto-reactive T cells. Generating clones or cell lines from auto-antigen tetramer positive CD4⁺ T cells allows further characterization and phenotyping of auto-reactive cells.

Keywords

CD4⁺; tetramer; clones; cell lines; antigen specific; cell culture; auto-reactive T cells

1. Introduction

MHC class II tetramers provide a powerful tool for cloning of antigen specific CD4⁺ T cells (1–15). The use of tetramers in the search of disease specific CD4⁺ T cells is based on the presumption that the peptide on the tetramer corresponds to an immunogenic epitope of interest that is prevalent in the disease process. Therefore the prerequisite to use class II tetramers in the detection and cloning of autoimmune T cells is knowledge of the autoantigen, its immunodominant antigen-derived peptides, and the binding of these peptides to the class II molecule playing a role in the disease process. Bearing on mind that there are differences in the processing of epitopes between individuals and by different antigen presenting cells, these variables could influence the selection of disease relevant T cells and potentially limit the utility of the approach described here. Nevertheless, utilization of tetramer techniques in the detection of CD4⁺ T cells allows isolation of live cells with a single, defined, antigen specificity. T cell clones used in the functional assays provide an excellent tool to gain insight into mechanisms and molecular basis of the disease which otherwise would only be possible in the experimental animal models. Furthermore, this approach has many clinical applications ranging from the vaccine design to antigen-specific therapies for prevention of and intervention into autoimmunity.

¹⁴Tetramer staining of T cell clone is dependent on the avidity of the T cell and can vary from 0–100%. Some low avidity T cell clones may not stain with a specific tetramer or stain very weakly but display proliferation and cytokine production in a dose-dependent manner when stimulated with a specific peptide. Avidity of tetramer positive T cell clones can be assessed further by staining at decreasing concentrations of tetramer

2. Materials

2.1. Reagents

2.1.1. Cell Culture Reagents

1. 1X PBS (Ca/Mg free)
2. Human PBMCs
3. FICOLL
4. Peptides
5. Tritiated thymidine
6. CD4 Isolation Kit II, Miltenyi Biotec
7. PHA-P
8. IL-2 (Aldesleukin Proleukin) for injection 22 million IU, 1.3 mg from local pharmacy (or other commercial preparation)
9. 15% Pooled Human Serum (PHS) T cell media: RPMI 1640 (with 25mM HEPES), 15% b.v. Human AB Serum, Omega Scientific, 1% b.v. Penicillin-Streptomycin, 1% b.v. Sodium Pyruvate, and 1% b.v. L-Glutamine (see Note 1)

2.1.2. Flow Cytometry Reagents

1. Appropriate PE-labeled class II tetramer (See Note 2)
2. Anti-human CD3 antibodies in all fluors to be used
3. Anti-human CD4-APC
4. Mouse IgG₁ (Isotype match) in all fluors of to be used
5. Running Buffer: 1X PBS, 2mM EDTA, and 0.5% b.v. Neonatal Calf Serum (NCS) (see Note 1)
6. MACS Buffer: 1X PBS, 2mM EDTA, and 0.5% Bovine Serum Albumin (BSA) (see Note 1)
7. FACS Buffer: 1X PBS, 1% b.v. NCS, and 0.1% b.v. sodium azide (see Note 1)

2.2. Equipment and Supplies

1. 48 well plates, 96 well plates
2. Transfer pipette
3. AutoMACS, Miltenyi Biotec
4. Irradiator

¹All buffers and media should be sterile filtered using a 0.2µm bottle top filter and stored in the accompanying 500 mL bottle

²Information for class II tetramers can be obtained from Benaroya Research Institute's Tetramer Core Laboratory website <http://www.benaroyaresearch.org/our-research/core-resources/tetramer-core-laboratory>

5. FACS tubes and sterile capped FACS tubes
6. Flow cytometer with cell sorting facility (FACS Vantage or Aria)
7. TomTec Harvester
8. FACS Buffer: 1X PBS, 1% b.v. FBS, and 0.1% b.v. sodium azide (see Note 1)

1. Methods

1.1. Tetramer Assay *in Vitro*

3.1.1 Primary stimulation of the PBMC (see Note 3)

1. Warm MACS buffer in 37°C water bath for 2–3 hours
2. Isolate PBMC from each blood sample via Ficoll separation
3. If the total cell count for a sample is less than 10×10^6 cells manually separate CD4⁺ T cells from PBMC using magnetic beads
4. Labeling PBMC for AutoMACS purification
 - a. Count cells
 - b. If $< 10 \times 10^6$ cells are present, proceed to **step 6.e** and aliquot PBMC rather than CD4⁺ cells or perform manual CD4⁺ separation
 - c. If $\geq 10 \times 10^6$ Isolate CD4⁺ cells using CD4 Isolation Kit II according to manufacturer's instructions (or perform manual CD4⁺ separation if AutoMACS not available)
5. AutoMACS purification of cells from PBMC
 - a. Label two 15 mL conical tubes for the collection of CD4⁺ and CD4⁻ cell fractions
 - b. Initialize AutoMACS by attaching buffers and running the **Clean Cycle** program. Place the CD4⁺ tube under the negative port and the CD4⁻ under the positive port
 - c. Run the **Deplete** program to separate the cells into CD4⁺ and CD4⁻ fractions
 - d. Just before the AutoMACS takes up the last of the cell sample, rinse sample tube with 1 mL of running buffer
 - e. Bring volume of each fraction up to 10 mL with T cell media and collect a 40 μ L aliquot of each fraction for counting and count cells
 - f. The total number of CD4⁺ cells will determine the number of wells to plate. Each well of a 48 well plate will contain $1-2 \times 10^6$ cells

³*Option 1:* Whole PBMC can be used instead of stimulation of the purified CD4⁺ T cells. If whole PBMC is used plate 3×10^6 PBMC on 48-well and proceed to **section 3.1.1 step 6.f**. *Option 2:* In order to distinguish memory and naïve T cell populations the cells can be further separated by CD45RO⁺/–

6. Plate the cells and stimulate with the peptide(s) of interest (see Note 4)
 - a. Centrifuge at 1000 rpm ($250 \times g$) for 5 min (use low brake setting) at room temperature
 - b. Resuspend $CD4^-$ cells at a concentration of 10×10^6 cells per mL and $CD4^+$ cells at a concentration of 5×10^6 cells per mL
 - c. For each well add the appropriate amount of $CD4^-$ cells ($3-4 \times 10^6$ cells per well for 48 well plates) and incubate for 1 hour in a $37^\circ C$, 5% CO_2 incubator to allow cells to adhere
 - d. Following the 1 hour incubation, fill each well with T cell media and use a transfer pipette to gently wash away all non-adherent cells. The remaining adherent cells are antigen presenting cell population (APC). Optional: the APC can be irradiated (5000 rad)
 - e. For each well add the appropriate amount of $CD4^+$ cells ($1-2 \times 10^6$ cells for each well of a 48 well plate) and bring volume of each well up to 1mL with T cell media
 - f. Add 10 $\mu g/mL$ of the appropriate peptide to each well. If there are only enough cells for a single well, pool the peptides together. Each peptide is used at final concentration of 10 $\mu g/mL$. Do not pool more than maximum of five peptides
 - g. Incubate cells in a $37^\circ C$, 5% CO_2 incubator for 5–7 days
 - h. Between days 5 and 7 of the culture, remove 400 –500 μL of supernatant from each well. Add a corresponding amount of fresh T cell media and 10 IU of IL-2 to every well
 - i. Monitor the expansion of the stimulated cells. If the cells in any well surpass 100% confluence, resuspend the cells and split into two wells. If the media yellows on a well that does not require splitting, remove 400 – 500 μL of supernatant from that well and add a corresponding amount of fresh T cell media with IL-2
 - j. Incubate cells for a total of 13 to 15 days

3.1.2 Tetramer and antibody staining of stimulated cells and controls

1. Harvest cells for tetramer staining
 - a. Remove supernatant from each well until the remaining volume is approximately 50 μL for each staining tube (tetramer) (plus some extra for single color staining controls)

⁴The optimal number of PBMC for T cell cloning by MHC class II tetramers is variable. If the expected tetramer staining is strong (>2%) 2×10^6 $CD4^+$ T cells should be sufficient. If the staining is expected to be weak (<2%), it is advisable to set up multiple culture wells especially if cloning of $CD4^+$ T cells specific for several different peptides is attempted. T cell responses to self-antigens are generally weaker than responses to foreign antigens. Strong tetramer staining can be expected with flu or other vaccine induced T cell responses and with responses to some immunodominant self-epitopes. The peptides of interest used to stimulate the cells should be selected by the HLA class II (DR, DQ, DP) genotype of the donor of the PBMC

- b. Resuspend the cells in the well using a transfer pipette
- c. If wells have been split, combine corresponding samples into 1 well and mix
- d. Transfer 50 μ L aliquots of cell suspension from each well into labeled FACS tubes and designate for staining with the appropriate tetramers. This should include one or more relevant tetramers and an irrelevant tetramer for each sample ID. The relevant tetramers contain the peptides that were used to stimulate the cells in **section 3.1.1 step 6.f**. If more than one class II allele is tested for a given sample ID, an irrelevant tetramer is needed for each allele (see Note 5)
- e. Pool together all remaining cells and aliquot into labeled FACS tubes for the single color staining controls

2. Tetramer Staining

- a. Add 1 μ L of the appropriate PE-labeled Class II tetramer (10 μ g/mL final concentration) into each labeled FACS tube.
- b. Incubate the cells with tetramer for 2 hours in the dark at 37°C

3. Antibody Staining of Single Color Controls

- a. Take the staining control tubes and incubate on ice for 3 min (see Note 6)
- b. Add 5 μ L α -CD3 antibody for each fluor to be used to the appropriate single control tubes. Include 1 tube unstained with antibody
- c. Add 5 μ L isotype control antibodies in each fluor to the isotype control tube
- d. Incubate in the dark at 4°C for 30 min

4. Antibody Staining of PBMC Samples

- a. Place all sample tubes on ice for 3 min
- b. Add 5 μ L of CD4 APC and 5 μ L of CD3 FITC to each tube
- c. Incubate in the dark at 4°C for 30 min
- d. Washing Samples and Controls
- e. Add 2 mL of cold FACS buffer to each tube
- f. Spin down cells at 1000 rpm (250 x g) for 5 min at 4°C, low brake
- g. Remove most of the supernatant, leaving approximately 200 μ L FACS buffer to resuspend the cells by gently tapping the tube

⁵The irrelevant tetramers are used as negative controls in the staining of cells stimulated with the specific peptides. Empty (unloaded) tetramers or tetramers loaded with peptides that bind to the given MHC class II and were not used in the culture can be used as the negative staining controls. The cut-off value (% of CD4+/tetramer+ cells) for staining with a negative control tetramer is usually set at 0.1–0.5%

⁶In cell sorting protocol all cell and reagent handling steps should be performed in a laminar flow biosafety cabinet

- h. Store all tubes in a covered ice container for subsequent FACS acquisition

3.2 Generating CD4⁺ T Cell Clones

3.2.1 Single Cell Sorting of Tetramer Positive Cells

1. Perform tetramer and antibody staining of stimulated cells and controls according to **section 3.1.2 steps 1–4**
2. Single cell sort CD4⁺ tetramer positive cells into a round bottom 96 well plate containing 100 µl media per well using a FACS Vantage or Aria (see Note 7 and Figure 1)
3. After sorting you should have 1 tetramer positive cell/well
4. Add 100,000 irradiated (5000 rad) HLA-mismatched fresh feeder cells/well in a volume of 100 µL 30% PHS T cell media (see Note 8 and Note 9)
5. Incubate cells in a 37°C, 5% CO₂ incubator for 1 day
6. Add IL-2 10 IU/mL and PHA 5 µg/mL the day after sorting in a volume of 25 µL T cell media
7. Incubate cells for 9 more days
8. Check plates every other day for possible contamination during the sorting process

3.2.2 Expansion of Single Cell Sorted Cells

1. After 10 days of incubation stimulate the cells with HLA-matched fresh feeder cells and peptide (see Note 8)
2. Pulse the feeder PBMC with 10 µg/mL of the peptide of interest. Use a small volume when pulsing, example 10×10^6 /mL
3. Pulse for 2–3 hours at 37°C, 5% CO₂
4. Wash the peptide out with 1X PBS and resuspend in T cell media. Calculate the volume, so that you put 100,000 cells/100 µL/well
5. Irradiate the feeder cells at 5000 rad
6. Take out about 120 µL supernatant from the wells and add 100 µL of the peptide pulsed 100,000 feeder cells/well
7. Add 10 IU/mL of IL-2 to the wells next day in a volume of 25 µL of T cell media
8. Incubate cells for 9 more days. Visually inspect: The cell pellets should grow larger.

⁷For flu stimulated CD4⁺ T cells, usually there are 4–15% tetramer positive cells after 14 days of primary culture. For autoreactive CD4⁺ cells the frequency of tetramer binding cells is much lower, 0.5–4%

⁸Feeder cells are PBMC from HLA-matched (=antigen presenting cells =APC, used in specific stimulation with a peptide) or HLA-mismatched (used in unspecific stimulations with PHA/IL-2) blood donors. PBMC isolated from fresh blood is preferred in T cell clone expansion but frozen PBMC can be used in T cell proliferation assays. In unspecific stimulation of the clones the MHC class II of the feeder PBMC should be mismatched for both HLA alleles. Feeder PBMC can be combined from different donors

⁹Use T cell media with a higher concentration of PHS when performing single-cell sorting. Thirty percent PHS is the standard. When expanding cells on subsequent days the cells can be weaned back to 10% PHS T cell media

9. After 10 days test the specificity of the growing clones in a proliferation assay and with tetramer staining FACS analysis if there are enough cells (you can take a small aliquot from some wells for a quick count). If there are not enough cells for a functional assay expand them further
10. Once the cells are dense and look confluent under a microscope transfer them to flat bottom 96-well plate. Remove 100 μ L supernatant and transfer 100 μ L of cells. Add fresh T cell media and IL-2 (see Note 10)
11. Once the cells fill the well and are in contact with each other transfer them to a 48-well plate for a second unspecific stimulation
12. Plate 250,000 clones/well with $0.75\text{--}1.0 \times 10^6$ irradiated HLA-mismatched feeder cells
13. Incubate cells for 1 day
14. Add IL-2 10 IU/mL and PHA 5 μ g/mL to each well
15. Check cells every 2–3 days. Once cells are confluent you may transfer them to a 24-well plate or split them 1:1 and add fresh T cell media and IL-2
16. After 10–14 days in culture the cells should be tested again for specificity, restimulated with peptide, and/or frozen (see Note 11)

3.3 Generating CD4⁺ T Cell Lines

1. Perform Tetramer and antibody staining of stimulated cells and controls according to **section 3.1.2 steps 1–4**
2. Sorting can be done using a FACS Vantage or Aria
3. Sort the entire CD4⁺ tetramer positive into a 5mL sterile capped FACS tube containing 500 μ L of T cell media (see Figure 1)
4. Take a count of the cells, or use the flow sort counts to plate $1\text{--}2 \times 10^6$ cells/well in a 48-well plate
5. Add $3\text{--}4 \times 10^6$ irradiated PBMC/well and bring up volume of each well to 1mL with T cell media
6. Incubate cells for 1 day
7. Add IL-2 10 IU/mL and PHA 5 μ g/mL to each well
8. Check cells every 2–3 days. Once cells are confluent you may transfer them to a 24-well plate or split them 1:1 and add fresh T cell media and IL-2
9. After 10–14 days in culture the cells may be tested for specificity and restimulated according to the methods in **section 3.2.2**

¹⁰After single-cell sorting it is sometimes necessary to incubate the cells for longer than 10 days to generate enough clones to perform specificity testing. Look at the cells under the microscope: Activated and dividing T cells look elongated and form clusters

¹¹It is important that the cells have time to rest (i.e. no IL-2 added) for a minimum 72 hours before the next stimulation or any functional assay. The cells should be also washed well to remove any residual IL-2 from the culture media. Resting T cells look roundish rather than elongated. It is always important to freeze aliquots of the expanded clones early

3.4 Testing CD4+ T Cell Lines and Clones Specificity in a Proliferation Assay

1. Count the clones (see **Section 3.2**) or lines (see **Section 3.3**) to be tested
2. Calculate the total volume of cells needed to get 25–50,000 cells/well x 7 wells (3 with peptide, 3 without peptide, and 1 with purified α -CD3 antibody) (see Note 12)
3. Plate cells (25–50 000/well) in a 96-well round bottom plate. Bring up the volume to 100 μ L with T cell media in each well
4. Pulse antigen presenting cells (APC = HLA-matched PBMC) cells with 10 μ g/mL peptide for 2–3 hours at 37°C, 5% CO₂. As with specific stimulation, use a small volume when pulsing, such as 10×10^6 /mL (see Note 13)
5. Incubate APC to be added to the no antigen wells, but do not add peptide
6. Irradiate the APC at 5000 rad
7. Wash the peptide out of the APC with 1X PBS and resuspend in T cell media at a concentration of 150,000 cells/100 μ L
8. Plate 150,000 APC/well. Use un-pulsed feeders in the α -CD3 well and add 10 μ g/mL purified α -CD3 antibody as a positive control
9. Incubate for 48 hours at 37°C, 5%CO₂
10. Remove 50 μ L of supernatant from the wells and store in clean 96-well plate at –80°C for cytokine assessment
11. Add 25 μ L/well (1 μ Ci/25 μ L/well) of tritiated thymidine (H³)
12. Incubate further 8–18 hours and harvest on TomTec Harvester according to manufacturer instructions

3.5 Testing the specificity of the clones by tetramer staining

1. Count the clones and lines to be tested
2. Take an aliquot of 50–100,000 cells and adjust the volume to 100 μ L with T cell media
3. Transfer 50 μ L aliquots into two vials which will be stained with specific and irrelevant tetramers as in **section 3.1.2 step 2** at 37°C for 45min–1hour (note: a shorter staining time) (see Note 13 and Figure 1)

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¹²Primary specificity testing can be performed in single or duplicate wells if the cell number is limiting. Negative no antigen control should always be included in the assay. When sufficient number of cells is available proliferation assay should be performed in decreasing concentrations of peptide

¹³HLA-matched B-LCL can be used as APC if HLA-matched PBMC from suitable blood donors are not available. Lower number of B-LCL (50–100,000 cells/well) is sufficient. Optimal irradiation time should be tested for each B-LCL since usually higher irradiation dose (>5000 rad) is needed for a complete blocking of proliferation

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Fig. 1.

Summary of methods 3.2 & 3.3, generating CD4⁺ T cell clones and cell lines. A: After primary stimulation of PBMC stain cultures with tetramer and antibody. B: Single cell sort CD4⁺ tetramer positive cells into a 96-well plate to generate a clone or bulk the cells into a sterile capped FACS tube and plate to generate a cell line. C: After stimulation and expansion test antigen specificity of cells with tetramer staining, as well as proliferation assays and cytokine staining (not shown).