

Tetramer-Guided Epitope Mapping: A Rapid Approach to Identify HLA-Restricted T-Cell Epitopes from Composite Allergens

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Abstract

Tetramer-guided epitope mapping (TGEM) is a technique in immunology that permits the rapid identification of allergenic epitopes through peptide screening procedures utilizing human lymphocyte antigen (HLA) class II tetramers as staining reagents for detection. The identification of allergenic epitopes is a prerequisite for the accurate characterization of allergen-specific CD4⁺ T cells without in vitro stimulation. Additionally, these MHC-II/peptide complexes that interact with T-cell receptors (TCR) of pathogenic CD4⁺ T cells are compatible with a different number of assays like Intracellular Cytokine Staining (ICS), and Carboxyfluorescein succinimidyl ester (CFSE) making it a robust technology to study the functionality of allergen-specific CD4⁺ T cells.

Key words Food allergy, Epitopes, MHC class II tetramers, T cells

1 Introduction

An epitope is part of a protein that is recognized by the immune system [1]. In the allergy context, allergens are considered to have antigenic properties if they bind specific IgE antibodies [2] and induce IgE-mediated reactions [3]. On the other hand, it is now firmly established that allergen-specific CD4⁺ T cells play an important role in the cycle that perpetuates allergic inflammation [4, 5]. In this regard, linear epitopes within the allergen can trigger T-cell reactivity and their identification is essential to develop novel immunotherapies that could target these T cells directly. In addition, identification of epitopes will allow the tracking of allergen-specific CD4⁺ T cells in both allergic and non-allergic subjects. Several approaches have been utilized to identify T-cell epitopes, such as: ELISPOT, CD137 activation assay [6] and by intracellular cytokine staining [7]. Each of these approaches has its own advantages and disadvantages. Here, we describe another approach designated

as Tetramer-Guided Epitope Mapping (TGEM). In TGEM, epitope identified will have known MHC restriction, and antigen-specific T-cell clones can be isolated in the process of epitope identification.

For the TGEM approach, soluble class II monomers with leucine zipper regions attached to the carboxyl terminals of the molecules loaded with specific peptides are assembled into “tetramer” complexes through fluorochrome-labeled streptavidin [8]. HLA/peptide complexes can engage $\alpha\beta$ TCR and permit the analysis of allergen-specific CD4⁺ T cells. The concept of using soluble HLA/peptide-labeled ligands to study these rare T-cell populations has been a successful strategy applied in numerous translational and clinical contexts [9]. As mentioned earlier, Class II tetramers can be used to identify novel T-cell epitopes. TGEM was first described to identify Herpes virus simplex virus VP16 [10] and has since been utilized to describe a variety of HLA-DR-restricted T-cell epitopes derived from multiple allergens, including seasonal allergens [11, 12], perennial allergens [13], and food allergens [14–17]. The protocol described below will focus on identifying of T-cell epitopes for allergens. Although tetramer assays are highly specific and sensitive, the HLA type of the subject and a library of overlapping peptides for a known antigen of interest is a prerequisite to apply TGEM.

The basis of this approach (Fig. 1) is to stimulate PBMC (Protocol can be carried out with either PBMC or isolated CD4⁺ T cells with antigen presenting cells. Both protocols will be described below.) from the subject with peptide mixtures. These mixtures are composed of multiple pools of peptides where each pool consists of 5 to 10 different overlapping peptides. Soluble class II molecules that match the HLA of the subject are loaded with each mixture of peptides, and matching tetramers are used to screen individual wells of stimulated PBMC usually at day 14 post-stimulation. The particular pooled peptide tetramer that provides a positive staining is identified. Next, individual peptides from the pool are then utilized to generate single peptide tetramers. Positive staining in a second round of FACS analysis with a particular tetramer will identify the peptide containing the allergenic epitope. Following up the identification of allergenic epitopes additional tetramer complimentary assays can be utilized for further analysis. Besides the fast identification of novel epitopes, tetramer assays offer important advantages over other methods by enabling the recovery of allergen-specific T cells through sorting of tetramer-positive T cells with flow cytometry. Epitope-specific T-cell clones can then be generated and permit further analysis. Additionally, the tetramer technology can be compatible with a number of assays such as intracellular cytokine assay that permit the accurate characterization of phenotype and functionality of CD4⁺ T cells [11]. Here, we will demonstrate how to efficiently use the TGEM approach to identify novel epitopes from food

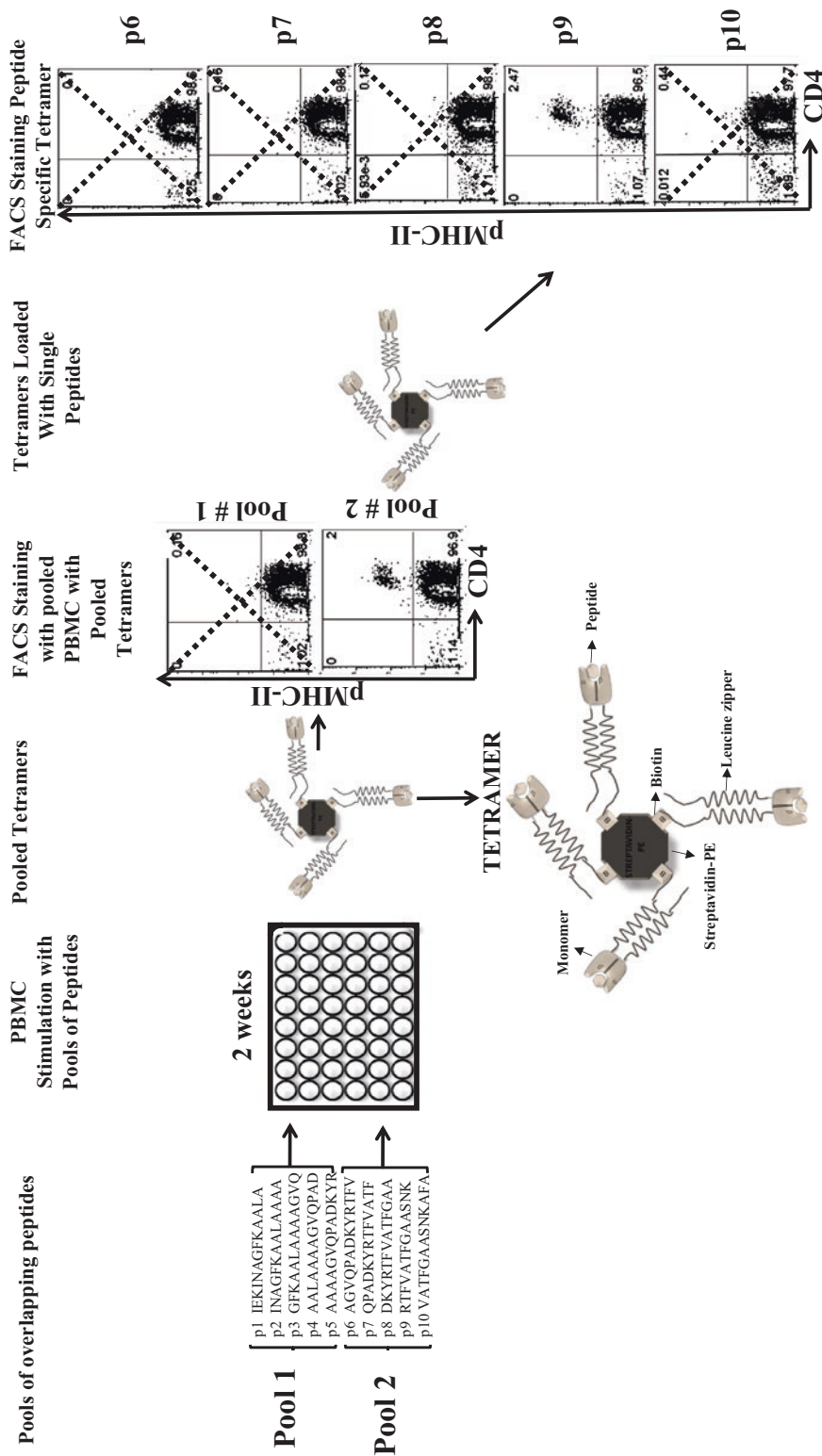


Fig. 1 Tetramer-guided epitope mapping. A panel of overlapping peptides that span the allergen of interest are divided into pools that consist of five peptides per pool. Each peptide pool is then utilized to stimulate individual wells plated with PBMC and cells will be cultured for 14 days. Peptide pools are also loaded onto soluble HLA class II molecules to generate pool peptide tetramers of the appropriate allergen. The pooled peptide tetramer that gives a positive staining is identified and peptides from that particular pool are utilized to generate tetramers loaded with individual peptides. Protocols for monomer production, assembly of pooled peptide tetramers, and single peptide loaded tetramers from monomers have been described elsewhere, and will not be described below. The particular tetramer that gives a positive staining is the allergenic HLA-restricted epitope

allergens in previously studied food allergens and will provide a step-by-step description of the methodological approach of using HLA class II tetramers in mapping T-cell epitopes.

2 Materials

2.1 Reagents

1. Class II tetramer at 0.5 mg/mL, BRI-A009.
2. RPMI 1640.
3. Penicillin-Streptomycin (P/S).
4. Pooled human serum.
5. IL-2 Stock (10,000 U), Roche.
6. Phytohaemagglutinin, Remel.
7. Running buffer: 2 mM EDTA, 0.5% BSA in 1× PBS.
8. Hemolytic buffer: 8.3 g NH₄Cl, 1.0 g NaHCO₃, 0.04 g disodium EDTA in 1 L of H₂O.
9. Ficoll-Paque, GE Healthcare.
10. Dulbecco's Phosphate Buffered Saline (1× PBS).
11. CD4⁺ T-cell isolation kit human, Miltenyi Biotect.
12. *n*-Dodecyl-β-maltoside.
13. 1 mM Pefabloc SC.
14. R-PE streptavidin.

2.2 Equipment and Consumables

1. 50 mL polypropylene conical tubes.
2. 48 and 96-well culture cluster.
3. 37 °C, 5% CO₂, Incubator.
4. Centrifuge.
5. FACS tubes, Falcon.
6. FACS Calibur, BD Biosciences.
7. FACS ARIA II, BD Biosciences.
8. Cell irradiator.
9. Easysep™ Magnet, Stem cell technologies.

3 Methods

Carry out all procedures at room temperature and in a sterile Class II Biological Safety Cabinet unless otherwise specified.

3.1 PBMC Isolation

1. Cover work area in Class II Biological Safety Cabinet with an absorbing pad. Gather enough 50 mL conical tubes in a rack for each to contain 20 mL of whole blood per tube and dilute the blood to 40 mL with 1× PBS.

2. Underlay Ficoll utilizing a 10 mL pipette per conical tube. Draw up to 10 mL of Ficoll, insert into conical tube with tip placed on the bottom of tube and remove from pipet aid. Let the Ficoll gradually drain into the bottom.
3. Spin tubes at 448 rcf for 20 min with no brake at RT (above 26 °C). After spinning, the tubes should be layered top to bottom as follows: serum (yellow), PBMC (white), Ficoll (clear), and buffy coat/blood (red). Utilizing a transfer pipette carefully draw up the entire layer of PBMC. Get as many cells as possible and transfer equally to half as many conical tubes (e.g., if you start with eight conical tubes transfer cell suspension into four new conical tubes).
4. Dilute the cells to 50 mL with 1× PBS. Spin at 252 rcf for 15 min with low brake. Dispose of blood waste in a double bag filled with absorbent pellets during this spin.
5. Aspirate supernatant down to ~5 mL. Add 5 mL of pre-warmed hemolytic buffer to each tube. Resuspend by pipetting up and down with transfer pipette. Incubate at room temperature in the dark for 5 min. Dilute up to 50 mL with 1× PBS and spin at 112 rcf for 10 min.
6. Aspirate supernatant and resuspend pellet. Fill up to 50 mL with 1× PBS. Do cell counts. Spin at 112 rcf for 10 min.

3.2 In Vitro Stimulation of PBMC with Peptide

1. Aspirate supernatant and resuspend pellet with fresh medium up to desired cell concentration (2 or 2.5 million cells/mL) (*see* **Notes 1** and **2**). Add 1 mL of fresh medium containing PBMC to each individual well as necessary (*see* **Note 3**).
2. Add 1 μ L (4 μ g per mL final concentration for each individual peptide pool) of appropriate peptide pools to each well. Leave some wells without peptide as controls and incubate at 37 °C for 7 days. Next, carefully draw $\frac{1}{2}$ of the media from each well and gently resuspend cells in remaining medium and transfer to a new well. Add 500 μ L of culture medium and 50 μ L/mL of IL-2 (10 U/mL). Every 2 days cells are fed with 500 μ L of culture medium and 50 μ L/mL of IL-2 and replate into additional wells when necessary.

3.3 Isolation of CD4⁺ T Cells and In Vitro Stimulation of Cells with Peptide

1. Aspirate supernatant and resuspend pellet with running buffer up to desired cell concentration according to CD4⁺ T-cell isolation kit human manufacturer's instructions (40 μ L per every 10 million cells, e.g., if 100 million cells total: 40 μ L $\times$ 10) (*see* **Note 1**).
2. Add CD4⁺ T-cell Biotin-Antibody cocktail (10 μ L per every 10 million cells). Resuspend cells by pipetting up and down with micropipette equivalent to total volume. Incubate at 4 °C for 10 min.

3. Add running buffer (30 μ L per 10 million cells). Add CD4⁺ T-cell microbead cocktail human (20 μ L per 10 million cells) and gently mix with micropipette equivalent to total volume avoiding bubbles. Incubate at 4 °C for 10 min.
4. Get two sterile, capped polypropylene vials in EasySep™ magnet. Transfer about 50 million cells to each vial (100 million cells per vial maximum). Bring volume up to ~4 mL with running buffer. Incubate for 10 min at room temperature in Class II Biological Safety Cabinet in the dark.
5. Keeping polypropylene vials in magnets, quickly dump out unbound CD4⁺ T cells into new 50 mL conical tube (This is a negative selection kit, non-CD4⁺ T cells bind magnetic microbeads, CD4⁺ T cells do not bind microbeads).
6. Add 1 mL of running buffer to polypropylene vial and resuspend non-CD4⁺ T-cell fraction. Transfer to another 50 mL conical tube. Rinse out tube with 1 mL of running buffer and transfer to the 50 mL conical tube to recover cell fraction. Add 1 $\times$ PBS to 50 mL in both CD4⁺ T-cell fraction and non-CD4⁺ T-cell fraction [Antigen presenting cells (APC)] conical tubes to do accurate cell counts. Next, spin both conical tubes at 112 rcf for 7 min. Resuspend non-CD4⁺ T-cell fraction to 10 million cells/mL in T-cell media. Add 200 μ L of non-CD4⁺ T-cell fraction to each well of a 48-well TC treated flat-bottom dish to be used in culture.
7. Incubate for 1 h at 37 °C. During this incubation resuspend CD4⁺ T-cell fraction in culture medium at the following concentration: 1.5 million cells/mL. Store this fraction at 4 °C.
8. After incubation thoroughly wash the plates with culture medium, especially around the edges of each well utilizing a transfer pipette. Remove all culture medium. Quickly check under the microscope to see that you have removed all of the floating cells from each well of the plate.
9. Add 1 mL of CD4⁺ T-cell fraction to each well containing APC. Add 1 μ L (4 μ g per mL final concentration for each individual peptide) of appropriate peptide pools to each well. Leave some wells without peptide as controls and incubate at 37 °C for 7 days. Next, carefully draw ½ of the media from each well and gently resuspend cells in remaining medium and transfer to a new well. Add 500 μ L of culture medium and 50 μ L/mL of IL-2 (10 U/mL). Every 2 days cells are fed with 500 μ L of culture medium and 50 μ L/mL of IL-2 and replated into additional wells when necessary (*see* **Notes 4** and **5**).

3.4 Tetramer-Guided Epitope Mapping

1. For peptide loading into HLA-DR monomer, take the biotinylated monomer into a 1.5 mL Eppendorf tube and load with 0.2 mg/mL of peptide pools and incubate for 72 h at 37 °C in

the presence of 0.2 mg/mL *n*-Dodecyl- β -maltoside and 1 mM Pefabloc SC. Subsequently, add R-PE streptavidin at a molar ratio of 8:1 into the peptide-loaded monomers and gently resuspend.

2. After 14 days of in vitro expansion (*see* **Note 6**), aliquot at least 200 μ L of cell suspension into 5 mL Falcon polystyrene tubes from each well. Add into each of the tubes 1 μ L PE-labeled HLA-DR tetramer loaded with the same peptide pool as used in the specific stimulation (final concentration of 10 μ g/mL). PE-labeled HLA-DR tetramer loaded with a control peptide can be used to monitor unspecific staining.
3. Stain T cells in the dark with HLA-DR tetramers for 2 h at 37 °C in a CO₂ incubator (*see* **Note 7**). Add 2 mL of running buffer and spin at 112 rcf for 5 min. Decant excess of running buffer and add 2 μ L of antibodies specific for human CD4, CD3, and CD25 with fluorochromes other than PE (*see* **Note 8**). Stain cells for 10 min in the dark at room temperature. Wash each tube with 2 mL of running buffer.
4. Decant and add 200 μ L running buffer, mix by tapping, and analyze in a flow cytometer. Perform tetramer analysis utilizing Flow Jo software to assess the tetramer positivity of each stimulated well by comparing positive versus negative tetramer staining. Tetramer staining should be at least one log above background.
5. After identifying the wells that are tetramer positive, generate PE-labeled HLA-DR tetramers loaded with same single peptides that belong to that particular pool and repeat **steps 2** and **3** (Fig. 2).

3.5 Isolation and Culture of Tetramer-Positive T-Cell Clones

Although it is not required, an additional procedure can also be utilized to confirm Tetramer positivity.

1. Repeat **steps 1** and **2** from Subheading 3.4.
2. Irradiate unmatched PBMC in 5 mL of culture medium in a 50 mL conical tube. Wash irradiated PBMC with 30 mL culture medium and spin at 112 rcf for 5 min. Aspirate supernatant and adjust cells at a concentration of 1×10^6 cells per 1 mL. Plate 100 μ L of irradiated PBMC in culture medium per well.
3. Set up FACS Aria II for single cell sorting and sort tetramer-positive T cells into round-bottom 96-well containing 100 μ L of culture medium per well.
4. Add an extra 100 μ L of culture medium and expand sorted T cells by stimulation with 5 μ g per 1 mL of phytohemagglutinin (PHA) and incubation in a CO₂ incubator at 37 °C. On the next day stimulate T cells by adding 10 U/mL of IL-2 and expand for 10 to 14 days.

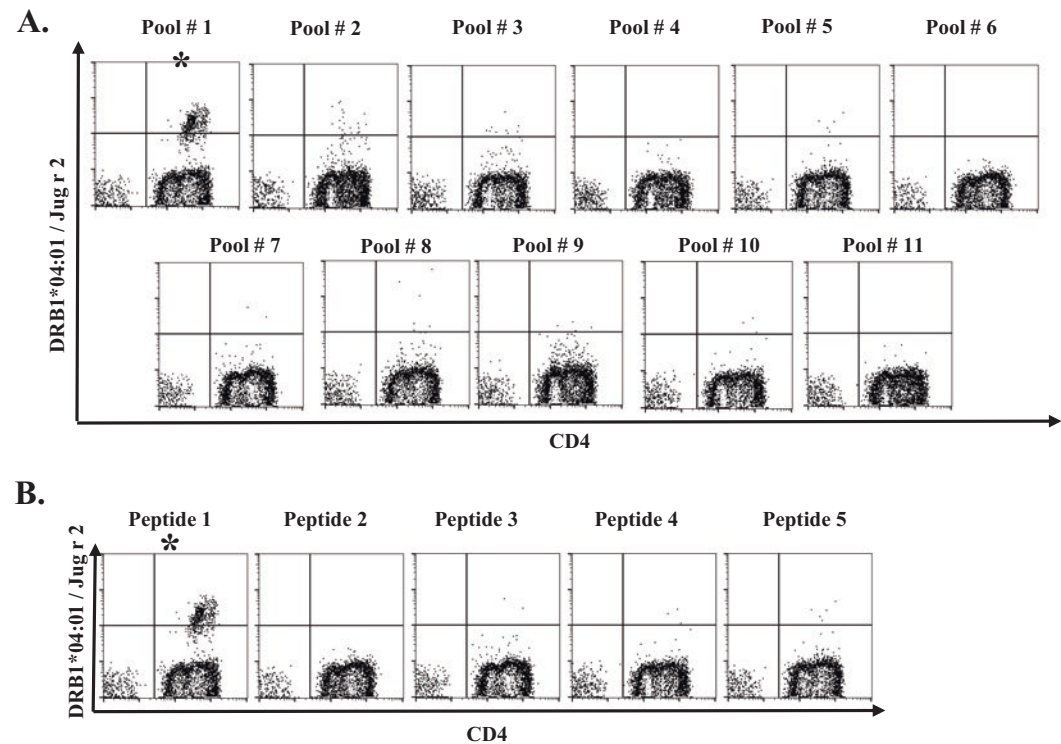


Fig. 2 TGEM studies of DRB1*04:01-restricted Jug r 2-reactive CD4⁺ T-cells. **(a)** PBMC from a DRB1*04:01 subject with walnut allergy were stimulated with 11 pools of Jug r 2 peptides for 2 weeks and subsequently stained with corresponding DRB1*04:01/Jug r 2 pooled peptide tetramers. **(b)** Cells that were stimulated with pool #1 were restained with individual peptides from the corresponding pool. The staining identified p1 (Jug r 2₁₅₂₋₁₇₁) as DRB1*04:01 restricted Jug r 2 T-cell epitopes

5. On day 14 screen cells for growth and split confluent cells as needed by transferring half of the cells from each well into a new well and adding fresh culture medium and IL-2 as needed.
6. On day 20, screen cells by tetramer staining and repeat **steps 1** and **2** from Subheading 3.4. Tetramer-positive T-cell clone staining should be at least one log above negative population (Fig. 3).

4 Notes

1. To map a protein that is 300 amino acids long you will require approximately 28 wells for peptide pool stimulation, which is roughly equivalent to 70 million PBMC.
2. It is very critical to plate 2–2.5 million cells per well in a 48-well culture plate as antigen-specific T cells that are cultured in either too low or high cell confluency will not expand adequately.

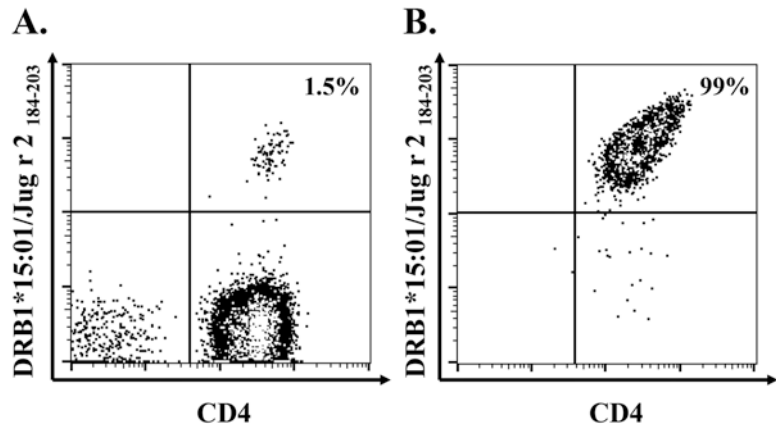


Fig. 3 Comparison of Tetramer staining in Tetramer-guided epitope mapping for walnut allergenic epitope Jug r 2₁₈₄₋₂₀₃ and single cell sorted T-cell clones for the same allergenic epitope from a single walnut allergic subject. (a) Staining of cells with the DRB1*15:01/Jug r 2₁₈₄₋₂₀₃ tetramer. (b) The tetramer-positive T cells were single cell sorted by flow cytometry, expanded with PHA and reexamined for positive tetramer staining

3. PBMC stimulation requires a more stringent every-day observation of your culture, as APC can also be expanded when adding fresh medium with IL-2. Thus, we find that transferring cells to a new well is advantageous the first 2 days after adding fresh medium with IL-2. Do not add new medium when transferring.
4. It is critical to split confluent cells as needed if there is high confluency. Do not split cells into a new well if confluency is too low. Adding fresh culture medium with IL-2 every 2 days is crucial for antigen-specific T-cell growth.
5. Avoid carrying APC plated from the original well of in vitro stimulation as these cells can outcompete antigen-specific T cells. If you encounter over carrying APC, gently pipette up and down and transfer into a new well.
6. TGEM should be performed within 10–16 days after in vitro stimulation, as apoptosis may prevent accurate detection of antigen-specific T cells.
7. Optimal time and temperature of different tetramer specificity can vary.
8. Tetramer-positive T cells express activation marker CD25, which is the α -chain of the IL-2 receptor (Fig. 4).

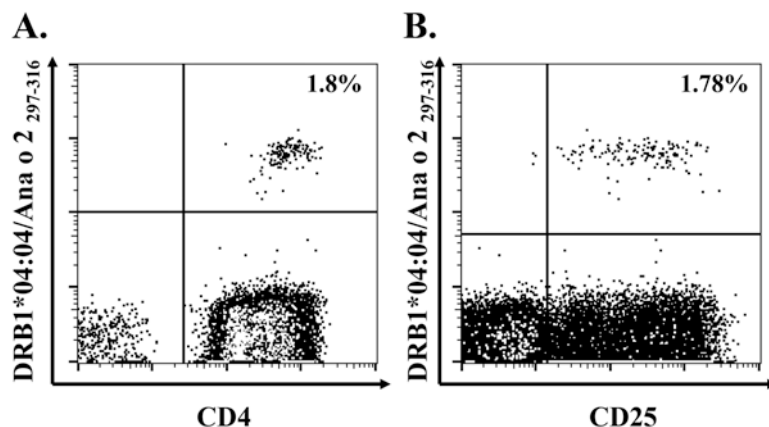


Fig. 4 Surface expression in tetramer-specific T cells. (a) In vitro expression of CD4 of DRB1*04:04/Ana o 2₂₉₇₋₃₁₆ tetramer. (b) In vitro expression of CD25 of DRB1*04:04/Ana o 2₂₉₇₋₃₁₆ tetramer

Acknowledgements

This work was supported by National Institutes of Health (NIH) contract HHSN272200700046C.

Disclosure of potential conflict of interest: L.D. Archila, W.W. Kwok has received research support from the US National Institutes of Health. The rest of the authors declare that they have no relevant conflict of interest.

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