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Peptide:MHCII tetramer-based cell enrichment for the study of epitope-specific CD4⁺ T cells.

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Abstract

Epitope-specific CD4⁺ T cells can be labeled in complex cell mixtures from secondary lymphoid organs with fluorophore-labeled p:MHCII tetramers and then detected by flow cytometry. Magnetic enrichment of tetramer-bound cells before flow cytometry increases the sensitivity of detection to the point where epitope-specific cells can be studied even when very rare at early and late times after the host has been exposed to the epitope. This method is very useful for studying polyclonal epitope-specific CD4⁺ T cells under physiological conditions.

Keywords

Magnetic-bead enrichment; epitope-specific; CD4⁺ T cells; naïve CD4⁺ T cells

Introduction

Vertebrates contain millions of unique CD4⁺ T cells, each with a different TCR and MHCII-bound peptide epitope specificity. This tremendous diversity allows these animals to match the universe of pathogens that cause infection and disease (Davis, 1990). But this great TCR diversity comes with the cost of low abundance for individual epitope-specific populations, which exist at frequencies of only 0.1–1 in 10⁶ total CD4⁺ T cells (Jenkins, Chu, McLachlan, & Moon, 2010) before the host encounters the relevant peptide. This low abundance creates a challenge to the experimentalist who wishes to use flow cytometry to detect epitope-specific T cells before and after antigen-driven clonal expansion and contraction. The core problem is that only about a million cells from a sample can be analyzed by flow cytometers at one time. This means that standard flow cytometry experiments on cells from the secondary lymphoid organs of a wild-type mouse involve analysis of only about one percent of the sample. Although this small slice of the total sample normally contains several hundred thousand CD4⁺ T cells, it will likely contain less than ten cells specific for any given p:MHCII epitope in a host that has never been exposed to the epitope. Methods were therefore needed to capture more of the epitope-specific T

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Conflict of Interest

The authors declare no conflicts of interest.

cells in samples from wild-type T cell repertoires or elevate the frequency of epitope-specific T cells by some other means.

One solution involves the use of fluorophore-labeled p:MHCII tetramers and magnetic bead-based cell enrichment. p:MHCII tetramers are produced by mixing biotin-labeled genetically engineered transmembrane domain-less p:MHCII monomers with fluorophore-labeled streptavidin in a 4:1 molar ratio (Altman et al., 1996). The tetrameric structure is important because it enhances the avidity of relatively weak TCR-p:MHCII interactions, allowing stable TCR binding. Rare p:MHCII tetramer-bound T cells can be enriched from all the secondary lymphoid organ cells from a mouse to a detectable level with magnetic particles conjugated with antibodies specific for the fluorophore in the tetramer (Moon et al., 2007).

The Basic Protocol describes the use of magnetic-bead enrichment to detect p:MHCII tetramer-labeled naïve and activated epitope-specific CD4⁺ T cells in the secondary lymphoid organs of mice. Staining panels describing markers for characterizing these cells are also included, as are optional steps for examining intracellular markers of differentiation.

Basic Protocol

Enrichment of murine CD4⁺ T cells with p:MHCII tetramers

The phenomenon of MHCII restriction (Zinkernagel & Doherty, 1974) dictates that tetramers must be matched with the MHCII molecules of the mouse strain under study. For example, because C57BL/6 mice express I-A^b MHCII molecules, peptide:I-A^b tetramers must be used in experiments on this strain. These reagents can be obtained from the NIH Tetramer Core Facility, which also provides a protocol for flow cytometric detection of CD8⁺ T cells labeled with peptide:MHCI tetramers (Altman & Davis, 2016).

Materials

Six well plate (Corning, cat# 3506)

Sorter buffer (see recipe)

Nylon mesh – 100 µm (Small Parts, cat# CMN-0105–10YD purchased through Amazon.com)

Plunger from a three mL syringe (Covidien, cat# 8881513934)

Five mL Polystyrene round-bottom tube 12 X 75 mm (Falcon, cat# 352008)

15 ml polypropylene conical tube (Sarstedt, cat# 62.554.205)

Round bottom 13 mL 100 X 16 mm polypropylene tube (Sarstedt, cat# 62.515.006)

Fc block (anti-mouse CD16/CD32 clone 2.4G2; Bio X Cell cat# BE0307)

p:MHCII Tetramer in APC and PE at 1 µM concentration (Available from the NIH Tetramer Core Facility, <http://tetramer.yerkes.emory.edu/>)

EasySep Mouse PE Positive Selection Kit (Stemcell Technologies, cat# 18554) or EasySep Mouse PE Positive Selection Kit II (Stemcell Technologies, cat# 17666)

EasySep Mouse APC Positive Selection Kit (Stemcell Technologies, cat# 18453) or EasySep Mouse APC Positive Selection Kit II (Stemcell Technologies, cat# 17681)

AccuCheck counting beads (Life technologies, cat# PCB100)

Ultracomp eBeads (ThermoFisher Scientific, cat# 01-2222-42)

Fixation/Permeabilization Solution Kit (BD Biosciences, cat# 554714)

eBioscience Foxp3 Transcription Factor Fixation/Permeabilization Kit (ThermoFisher Scientific, cat# 00-5523-00)

Fluorescently labeled antibodies for flow cytometry (Listed in Tables 1 and 2)

Mice: C57BL/6 strain, 6 to 10 weeks old (The Jackson Laboratory, cat# 000664)

2W peptide (peptide sequence: EAWGALANWAVDSA; purchased from GenScript)

Poly I:C (InvivoGen, cat# tlr1-pic)

Beckman tabletop centrifuge

Vortex-genie 2

Magnets (Stemcell Technologies, cat# 18000)

Flowjo (Tree Star)

Prepare a single cell suspension from lymphoid tissue

1. We normally use three 6–10 week old male or female C57BL/6 mice (purchased from The Jackson Laboratory) for each experimental and control group in a typical experiment and repeat the experiment once. This approach is normally sufficient to detect statistically significant differences between groups on the order of two-fold. As an example, we describe here a hypothetical experiment to assess the effect of the adjuvant Poly I:C on the number, and optionally, T cell lineage-defining transcription factor expression of CD4⁺ T cells specific for an I-A^b-binding peptide called 2W (Moon et al., 2007). The 2W peptide is very immunogenic in C57BL/6 mice (Moon et al., 2007) and is therefore a useful peptide for peptide:MHCII tetramer-based cell enrichment experiments. Of course, there are many variations on this theme involving other peptides and immune responses.
2. Inject groups of C57BL/6 mice intraperitoneally with 0.1 ml of PBS, or 0.1 ml of PBS containing 10 µg of 2W peptide, 20 µg of Poly I:C, or 10 µg of 2W peptide and 20 µg of Poly I:C (Kotov, Kotov, Goldberg, & Jenkins, 2018).

3. Seven days after the injections, add 1.5 mL of cold sorter buffer to each well of a 6-well plate (one well per mouse) along with a two cm² piece of nylon mesh. Place the plate on ice.
4. Euthanize mice with CO₂ and harvest spleen and lymph nodes (inguinal, axillary, brachial, submandibular, cervical, mesenteric, para-aortic) (Reeves & Reeves, 1992). Place the harvested tissue in the six well plate on top of the nylon mesh.
5. Place a piece of nylon mesh on the tissue in each well of the plate so that the tissue is sandwiched between the two pieces of nylon mesh.
6. Use flat end of a syringe plunger to press the tissue in a circular motion between the two pieces of nylon mesh until only white fragments remain (approximately 30 seconds of mashing).
7. Place a piece of nylon mesh on top of a 15 ml polypropylene conical tube with an individual tube for each sample. Filter the supernatant from the plate through the nylon mesh on the tube. Keep the tubes on ice.
8. Rinse each well with two mL of cold sorter buffer and filter it through nylon mesh into the corresponding tube.

Stain cells with antibodies and p:MHCII tetramer

9. Spin the tubes in a centrifuge for five minutes at 1600 rpm (600 rcf) and 4°C.
10. Pour out the supernatant, suspend the pellet by vigorous vortexing, and add sorter buffer to adjust the volume to 200 µL.
11. Add 2 µL of 1 µM 2W:I-A^b/streptavidin-PE tetramer and 2 µL of Fc block to each tube, mix well, and incubate for one hour at room temperature in the dark.
12. Wash the tubes by adding three mL of cold sorter buffer to each tube and then centrifuge the samples for five minutes at 1600 rpm (600 rcf) and 4°C.
13. Pour out the supernatant and add cold sorter buffer to the pellet of each tube for a final volume of 500 µL.

For naïve mice, spleen and lymph samples can be stained with 2W:I-A^b(PE) and 2W:I-A^b(APC) tetramers, 10 nM each, and then enriched in Steps 14–19 with PE and APC antibodies. Dual tetramer enrichment enhances the specificity of the assay by allowing gating on cells that bind both tetramers as expected for TCR binding and exclusion of rare cells that bind only one of the tetramers that therefore must be artifacts. Exclusion of these artifactual cells, which occur at the level of 10–20 cells per mouse is helpful when the naive population of interest consists of less than 100 cells per mouse. Dual tetramer enrichment is less helpful in cases where the tetramer-binding cells are present in larger amounts than this.

Enrich epitope-specific CD4⁺ T cells

14. Steps 14–19 describe how to use EasySep Positive Selection kits to enrich PE-labeled p:MHCII tetramer-bound cells. Due to the rarity of the epitope-specific CD4⁺ T cells, volumes of the EasySep reagents used in this protocol are lower

than what is specified by the manufacturer. Vortex the samples and add 6.25 μ L EasySep PE antibody cocktail to each tube. The EasySep antibody cocktail is a reagent provided in the EasySep Positive Selection kits. Vortex again and incubate at room temperature in the dark for 15 minutes.

15. When using the EasySep Positive Selection Kit, mix EasySep magnetic particles with a pipette (do not vortex). For the EasySep Positive Selection Kit II, vortex the EasySep magnetic particles for 30 seconds. After resuspending the magnetic particles, add 25 μ L of particles per sample tube. Vortex the tubes and incubate at room temperature in the dark for 10 minutes.
16. Resuspend the samples to a final volume of 2.5 mL using cold sorter buffer. Filter each sample through nylon mesh into a five mL polystyrene round-bottom tube.
17. Vortex samples on a low setting to prevent spilling and place them in EasySep magnets for five minutes. During this step, set up collection tubes for the unbound cells (use round bottom 13 mL 100 X 16 mm polypropylene tubes when using the EasySep magnets in an EasySep multistand). Decant sample tubes into the collection tubes while the tubes containing the bound cells are still in the magnet.
18. Wash the bound-cell samples by removing the tubes from their magnets and adding 2.5 mL cold sorter buffer to each tube. Vortex samples on a low setting (2–3 on a Vortex-genie 2) and place them back in the EasySep magnet for five minutes. Decant as described in step 17. Repeat two more times for a total of three washes (four times on the magnet total).
19. Remove the tubes containing tetramer-bound cells from the magnets. Add three mL of cold sorter buffer and centrifuge for five minutes at 1600 rpm (600 rcf) and 4°C.

Stain the enriched cells with antibodies

20. Pour out the supernatant of the bound-cell samples and resuspend the pellets by vigorous vortexing. The unbound-cell samples should be vortexed and then 100 μ L should be transferred into five mL Polystyrene round-bottom tubes. Prepare a master mix for surface staining with the expectation of a 100 μ L volume for each sample tube (refer to Table 1 and 2 for staining panels).

It is critical that the staining panel contains antibodies that are specific for non-T lineage cells such as B cells, macrophages, and dendritic cells. These antibodies can be labeled with the same fluorophore because they will be used to exclude (some say dump) non-T cell lineage cells.

21. Apply the master mix prepared in step 20 to each five mL Polystyrene round-bottom tube. The volume of master mix added to each tube will vary depending on the staining panel, but is typically in the range of 5 to 10 μ L. Vortex the tubes, and incubate the samples for 25 minutes on ice or in a 4°C refrigerator in the dark.

- 22.** During the incubation step, prepare single color controls using Ultracomp eBeads by placing one drop, approximately 50 to 75 μ L, of eBeads into individual five mL tubes labeled, one for each of the fluorophores used in the experiment plus an additional tube to serve as the negative control. Add 50 μ L of sorter buffer to each tube and then add 0.5 μ L of the relevant fluorophore-labeled antibody except for the negative control tube. Incubate for 15 minutes on ice or in a 4°C refrigerator in the dark.

Single color controls need to be prepared for the entire staining panel including any intracellular stains, the p:MHCII tetramer, and any antibodies that were also added during the tetramer staining step. For the p:MHCII tetramer control, use CD4 antibody conjugated to the fluorophore of the tetramer, for example, use PE-labeled CD4 antibody in an experiment with PE-labeled tetramer.

- 23.** Wash the Ultracomp eBead-containing tubes by adding three mL of cold sorter buffer to each tube and then centrifuging the samples for five minutes at 1600 rpm (600 rcf) and 4°C. Decant the supernatant, add 300 μ L of sorter buffer to the pellets, and store the tubes in the dark at 4°C until use.

At this point either proceed to fixing the cells for analysis of T cell lineage-defining transcription factors (Basic Protocol 1 steps 24 and 25) or immediately proceed to preparing the samples for flow cytometric analysis (Basic Protocol step 26). Storing samples for an extended period of time after harvesting (12 hours) the mice will lead to significant cell death.

- 24.** Optional: When staining for transcription factors in cells expressing a fluorescent protein such as green fluorescent protein, fix the samples after surface staining with antibodies by adding 200 μ L BD Fixation/Permeabilization solution to each sample tube, vortexing the tube, and incubating on ice or in a 4°C refrigerator in the dark for 10 minutes. Wash the tubes by adding three mL of cold sorter buffer to each tube and then centrifuging the samples for five minutes at 2100 rpm (1026 rcf) and 4°C.

This fixation step preserves the majority of the fluorescent protein signal, which would otherwise be quenched upon fixation with the eBioscience Foxp3 transcription factor fixation/permeabilization kit, a required step for transcription factor staining. Please note that fixed cells should be centrifuged at 2100 rpm (1026 rcf) rather than 1600 rpm (600 rcf) to reduce cell loss that occurs after every centrifugation step.

- 25.** Optional: To stain for transcription factors, vortex the samples after surface staining with antibodies, add 400 μ L diluted eBioscience Foxp3 transcription factor fixation solution to each sample tube, vortex the samples again, and incubate for 45 minutes at room temperature in the dark. Perform two wash steps with three mL of diluted permeabilization solution to each tube and then centrifuging the samples for five minutes at 2100 rpm (1026 rcf) and 4°C. Prepare a master mix for transcription factor staining with a 1:100 final dilution for each antibody unless the manufacturer specifies a certain amount per test

(refer to table 1 and 2 for staining panels). Add the master mix to each sample tube, vortex, and incubate for one hour at room temperature or overnight in a 4°C refrigerator in the dark. Wash the tubes by adding three mL of cold sorter buffer to each tube and then centrifuging the samples for five minutes at 2100 rpm (1026 ref) and 4°C.

Some antibodies are provided in more dilute concentrations than the standard 0.2 mg/ml or provided with a defined test volume. In these cases, use the antibody at the manufacture-specified dilution or perform a titration experiment to determine the optimal antibody concentration for staining.

26. Add 100 μ L of sorter buffer and 100 μ L of AccuCheck counting beads (200,000 beads/mL) to each sample tube for a final amount of 20,000 beads per sample. Vortex and then filter each sample through a 100 μ m filter into a five mL Polystyrene round-bottom tube.
27. Analyze the samples on a flow cytometer by generating a compensation matrix with the single color compensation controls and then acquiring each sample. Flow cytometry training is typically provided by a flow cytometry core facility and will include a detailed explanation of how to perform this step. A refresher on flow cytometry use and performing compensation can be found here (Cossarizza et al., 2017; Szaloki & Goda, 2015).

Acquire the entire sample to maximize detection of the rare epitope-specific CD4⁺ T cells.

28. Load the flow cytometry data files for each sample into Flowjo for analysis. Check the previously generated compensation matrix by examining lymphocytes using forward and side scatter area and then identifying single cells based on forward scatter-area and forward scatter-width. For this population of single cells, compare every fluorescent channel against every other fluorescent channel, like FITC against APC, to check for proper compensation. Optimal compensation is indicated by two populations having the same mean fluorescence intensity in a given channel if they equally express the marker that is detected within that channel, for example, an AF700⁺ APC⁻ population and a AF700⁻ APC⁻ population should have a similar mean fluorescence intensity for APC (refer to Figure 1). Manually edit the compensation matrix as needed using Flowjo.

For the staining panels described in table 1 and 2, the most common combinations that have signal spillover are APC and AF700, AF700 and APC-ef780, and AF700 and APC-ef780.

29. Enumerate epitope-specific CD4⁺ T cells in Flowjo by gating on single cells that are dead/dump⁻ CD90.2⁺ CD4⁺ p:MHCII tetramer⁺ (refer to Figure 2A for a gating scheme for identifying epitope-specific CD4⁺ T cells in naïve mice and mice infected with attenuated *Listeria monocytogenes* bacteria expressing the 2W peptide). Identify the counting beads by gating on events with a very high side scatter area but a side scatter width similar to lymphocytes, then gating on

the four populations expressing FITC, because the count beads naturally fluoresce in this channel. Finally, exclude doublets based on the side scatter width (refer to Figure 2B for a gating strategy to identify beads). The number of epitope-specific CD4⁺ T cells in the sample is calculated by the following equation:

$(20,000 / \text{count bead events}) * \text{epitope-specific CD4}^+ \text{ T cell events.}$

Further phenotyping of the epitope-specific CD4⁺ T cells can be performed by including additional markers in the staining panel, like intracellular markers indicative of various CD4⁺ T cell lineages (refer to the intracellular stains in table 2). To set the gates for these additional markers, it is often helpful to initially gate on naïve CD4⁺ T cells (dead/dump⁻ CD90.2⁺ CD4⁺ CD44^{low} p:MHCII tetramer⁻), which will have a defined expression pattern for the additional markers and then apply those gates to the epitope-specific CD4⁺ T cells (Malhotra et al., 2016). For example, naïve CD4⁺ T cells should not have adopted the Th1 cell fate and will therefore be negative for TBET, the lineage defining transcription factor for this subset. Therefore, any epitope-specific CD4⁺ T cells with a higher fluorescence intensity for TBET than the naïve CD4⁺ T cells have become Th1 cells.

Reagents and Solutions

Sorter buffer

Dilute 10X phosphate buffered saline (PBS; Corning, cat# 20–031-CV) to a 1X concentration with distilled H₂O and supplement it with 2% (v/v) Fetal bovine serum (FBS; Life technologies, cat# 16010–159) and 0.1% (v/v) NaN₃ (RICCA, cat# 7144.8–16). Store at 4°C.

Commentary

Background Information

This protocol relies on the discovery that fluorophore-labeled genetically engineered p:MHC tetramers can bind stably to specific TCRs on T cells (Altman et al., 1996). This was a breakthrough in immunology because it allowed detection of relevant T cells based solely on their TCR specificity without assumptions about their functions (Doherty, 2011). The protocol also relies on studies showing that rare p:MHC tetramer-bound cells can be enriched with magnetic methods (Jang, Seth, & Wucherpfennig, 2003; Luxembourg et al., 1998), and the application of these methods to the problem of detection of naïve T cells (Moon et al., 2007).

Critical parameters

The most critical parameter for successful magnetic-bead enrichment using p:MHCII tetramers is the quality of the tetramer. High quality p:MHCII tetramers can be obtained from the NIH Tetramer Core Facility (<http://tetramer.yerkes.emory.edu>). The concentration of tetramer used to stain the single cell suspension is also a critical parameter. We have

found that a final concentration of 10 nM allows maximal detection of specific T cells while minimizing background staining. Exclusion of dead cells and non-T cell lineage cells is also critical for reducing detection of cells that are autofluorescent or bind the tetramer by some means other than the TCR. Users should avoid the urge to eliminate the exclusion gating aspect of the protocol because it is critical for background reduction. An additional concern is the temperature at which the tetramer staining step is conducted. Contrary to staining with p:MHCI tetramers, staining with p:MHCII tetramers at 4°C often results in poor labeling and identification of the epitope-specific CD4⁺ T cells. Therefore, p:MHCII tetramer staining should always be performed at room temperature or 37°C.

Troubleshooting

p:MHCII tetramers can aggregate over time causing a loss of potency. A pellet in the tube containing the stock tetramer solution is a warning sign that aggregation has occurred. In this event, centrifuge the tube containing the stock tetramer solution for 30 seconds at 2800 rcf to pellet aggregates and transfer the supernatant to a new tube. Calculate the new concentration of the tetramer based on the concentration of its fluorophore. The concentration of the fluorophore can be calculated based on its extinction coefficient using a spectrophotometer. The extinction coefficients for PE and APC are $1.96 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ and $7 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

If naïve CD4⁺ T cells are not detected when using the p:MHCII tetramer-based enrichment protocol, then it is advisable to immunize a mouse with the peptide of interest in Complete Freund's Adjuvant and then repeat the enrichment procedure one week after immunization. Priming should increase the number of p:MHCII-specific T cells by 100-fold. If no tetramer-binding cells are detected under this best-case situation, then the peptide of interest may not really bind MHCII, or there are problems with the tetramer or the flow cytometer set up.

If too many naïve CD4⁺ T cells are detected when using the p:MHCII tetramer-based enrichment protocol, then it is advisable to assess the gating strategy used for the flow cytometry analysis. As described below, although p:MHCII epitope-specific naïve T cell populations vary in size depending on the peptide, the largest populations consist of less than 1,000 cells per mouse. Thus, an enrichment experiment that yields more than 1,000 tetramer-binding naïve T cells should be viewed with skepticism. A back-gating analysis would be warranted in this case to ensure that autofluorescent, dead, or non-T cell lineage cells are not creeping into the gates used to identify the tetramer-binding cells.

Anticipated Results

The limit of detection for magnetic-bead enriched populations in secondary lymphoid organs is approximately 3 cells per mouse. The approximately 20 p:MHCII epitope-specific naïve T cell populations that have been studied to date vary in size with of range of ~10–700 cells per mouse. There are about 300 naïve CD4⁺ T cells specific for the I-A^b-bound model 2W peptide used as an example here (Moon et al., 2007). Thus, in our hypothetical experiment it is anticipated that about 300 2W:I-A^b tetramer-binding CD4⁺ T cells would be detected in the mice injected with PBS or Poly I:C alone since adjuvants do not usually cause proliferation of T cells that do not also receive TCR signals. About 3,000 2W:I-A^b tetramer-

binding CD4⁺ T cells would be detected in mice injected with 2W peptide alone (Kotov et al., 2018) since T cells that receive TCR signals under non-inflammatory conditions proliferate but sub optimally. About 30,000 2W:I-A^b tetramer-binding CD4⁺ T cells would be detected in mice injected with 2W peptide and Poly I:C (Kotov et al., 2018) since adjuvants enhance the proliferation of T cells that receive TCR signals. Poly I:C is good inducer of Th1 cells so it is expected that most of the 2W:I-A^b tetramer-binding CD4⁺ T cells will express TBET.

Time Considerations

The time required for this protocol is highly dependent on the number of samples and whether the optional intracellular staining steps are performed. For a skilled operator, processing six mice and staining for intracellular markers requires six hours, while 20 mice demands 10 or more hours. An additional one to three hours are required for flow cytometric analysis of the samples. Larger experiments involving intracellular staining can be split into two days by storing samples after the initial fixation step at 4°C in the dark overnight.

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Literature Cited

- Altman JD, & Davis MM (2016). MHC-peptide tetramers to visualize antigen-specific T cells. *Curr Protoc Immunol*, 115, 17.13.11–17.13.44. doi:10.1002/cpim.14
- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JL, ... Davis MM (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science*, 274(5284), 94–96. [PubMed: 8810254]
- Cossarizza A, Chang HD, Radbruch A, Akdis M, Andra I, Annunziato F, ... Zimmermann J (2017). Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur J Immunol*, 47(10), 1584–1797. doi:10.1002/eji.201646632 [PubMed: 29023707]
- Davis MM (1990). T cell receptor gene diversity and selection. *Annu Rev Biochem*, 59, 475–496. doi: 10.1146/annurev.bi.59.070190.002355 [PubMed: 2197981]
- Doherty PC (2011). The tetramer transformation. *J Immunol*, 187(1), 5–6. doi:10.4049/jimmunol.1101297 [PubMed: 21690330]
- Jang MH, Seth NP, & Wucherpfennig KW (2003). Ex vivo analysis of thymic CD4 T cells in nonobese diabetic mice with tetramers generated from I-A(g7)/class II-associated invariant chain peptide precursors. *J Immunol*, 171(8), 4175–4186. [PubMed: 14530340]
- Jenkins MK, Chu HH, McLachlan JB, & Moon JJ (2010). On the composition of the preimmune repertoire of T cells specific for Peptide-major histocompatibility complex ligands. *Annu Rev Immunol*, 28, 275–294. doi:10.1146/annurev-immunol-030409-101253 [PubMed: 20307209]
- Kotov DI, Kotov JA, Goldberg MF, & Jenkins MK (2018). Many Th cell subsets have Fas ligand-dependent cytotoxic potential. *J Immunol*, 200(6), 2004–2012. doi:10.4049/jimmunol.1700420 [PubMed: 29436413]
- Luxembourg AT, Borrow P, Teyton L, Brunmark AB, Peterson PA, & Jackson MR (1998). Biomagnetic isolation of antigen-specific CD8⁺ T cells usable in immunotherapy. *Nat Biotechnol*, 16(3), 281–285. doi:10.1038/nbt0398-281 [PubMed: 9528010]
- Malhotra D, Linehan JL, Dileepan T, Lee YJ, Purtha WE, Lu JV, ... Jenkins MK (2016). Tolerance is established in polyclonal CD4(+) T cells by distinct mechanisms, according to self-peptide expression patterns. *Nat Immunol*, 17(2), 187–195. doi:10.1038/ni.3327 [PubMed: 26726812]

- Moon JJ, Chu HH, Pepper M, McSorley SJ, Jameson SC, Kedl RM, & Jenkins MK (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity*, 27(2), 203–213. doi:10.1016/j.immuni.2007.07.007 [PubMed: 17707129]
- Reeves JP, & Reeves PA (1992). Removal of Lymphoid Organs. *Current Protocols in Immunology*, 1(1), 1.9.1–1.9.3. doi:10.1002/0471142735.im0109s01
- Szaloki G, & Goda K (2015). Compensation in multicolor flow cytometry. *Cytometry A*, 87(11), 982–985. doi:10.1002/cyto.a.22736 [PubMed: 26349004]
- Zinkernagel RM, & Doherty PC (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*, 248(5450), 701–702. [PubMed: 4133807]

Significance Statement

CD4⁺ T cells provide immune protection to their vertebrate hosts by using T cell antigen receptors (TCRs) to recognize foreign peptides (p) bound to host major compatibility complex class II (MHCII) molecules. Before the host is exposed to a foreign peptide, the naïve CD4⁺ T cells expressing TCRs specific for its MHCII bound form exist at very low frequencies of 0.1–1 in 10⁶. The rarity of these cells has hindered their detection and created knowledge gaps about early aspects of the CD4⁺ T cell response. Here, we describe protocols involving cell enrichment and flow cytometry that have the sensitivity to detect naïve CD4⁺ T cells expressing relevant TCRs.

Figure 1. Flow cytometry plots demonstrating proper compensation.

CD44 in Alexa Fluor 700 was plotted against 2W:I-A^b in APC for CD4⁺ T lymphocytes to demonstrate overcompensation, under compensation, and correct compensation between the two fluorophores.

Figure 2. Example gating strategies for identifying and enumerating epitope-specific CD4⁺ T cells with p:MHCII tetramers and counting beads.

The figure shows data from spleen and lymph samples from naïve B6 mice or B6 mice infected seven days earlier with *Listeria monocytogenes* bacteria expressing the 2W peptide (*Lm-2W*) after enrichment with 2W:I-A^b tetramer(s). (A) Lymphocytes were identified in the enriched samples by forward scatter area and side scatter area followed by single cell identification using side scatter area versus side scatter width and forward scatter area plotted against forward scatter width. Live T lymphocytes were then distinguished as CD90.2⁺ cells lacking B220, CD11c, CD11b (dump) and viability dye staining. CD4⁺ T cells were detected in the CD90⁺ gate. For naïve mice, spleen and lymph samples were stained with 2W:I-A^b(PE) and 2W:I-A^b(APC) tetramers and enriched with PE and APC antibodies. Epitope-specific cells were identified and as cells that bound 2W:I-A^b(PE) and 2W:I-A^b(APC) tetramers. For *Lm-2W* infected mice, spleen and lymph samples were stained with 2W:I-A^b(APC) tetramer and enriched APC antibodies. Epitope-specific cells were identified and as CD44^{high} cells that bound 2W:I-A^b(APC) tetramer. The CD4⁻ T lymphocytes served as a negative control for p:MHCII tetramer staining. (B) Counting beads were identified based on their side scatter area and width and FITC signal.

Table 1.

An example staining panel for identifying naïve epitope-specific CD4⁺ T cells with p:MHCII tetramers.

Staining Step	Stain	Antibody Clone	Supplier	Fluorophore	Catalog #	Dilution
Pre-Enrichment	2W:I-A ^b tetramer	N/A	Made in-house	PE	N/A	1:100
Pre-Enrichment	2W:I-A ^b tetramer	N/A	Made in-house	APC	N/A	1:100
Surface	CD90.2	53-2.1	ThermoFisher Scientific	FITC	11-0902-82	1:1,000
Surface	CD44	IM7	ThermoFisher Scientific	AF700	56-0441-82	1:200
Surface	CD4	GK1.5	BD Biosciences	BV786	563331	1:200
Surface	Ghost Dye Red 780	N/A	Tonbo Biosciences	780	13-0865-T500	1:1,000
Surface	CD11b	M1-70	ThermoFisher Scientific	APC-eFluor 780	47-0112-82	1:400
Surface	CD11c	N418	ThermoFisher Scientific	APC-eFluor 780	47-0114-82	1:400
Surface	B220	RA3-6B2	ThermoFisher Scientific	APC-eFluor 780	47-0452-82	1:400

Table 2.

An example staining panel for using p:MHCII tetramers to identify epitope-specific CD4⁺ T cells that have responded to their cognate antigen. This panel includes intracellular phenotyping markers that identify various types of the epitope-specific T cells, like T regulatory cells.

Staining Step	Stain	Antibody Clone	Supplier	Fluorophore	Catalog #	Dilution
Pre-Enrichment	2W:I-A ^b tetramer	N/A	Made in-house	APC	N/A	1:100
Pre-Enrichment	CXCR5	L138D7	BioLegend	BV650	145517	1:50
Surface	CD90.2	53-2.1	ThermoFisher Scientific	FITC	11-0902-82	1:1,000
Surface	CD44	IM7	ThermoFisher Scientific	AF700	56-0441-82	1:200
Surface	CD4	GK1.5	BD Biosciences	BV786	563331	1:200
Surface	Ghost Dye Red 780	N/A	Tonbo Biosciences	780	13-0865-T500	1:1,000
Surface	CD11b	M1-70	ThermoFisher Scientific	APC-eFluor 780	47-0112-82	1:400
Surface	CD11c	N418	ThermoFisher Scientific	APC-eFluor 780	47-0114-82	1:400
Surface	B220	RA3-6B2	ThermoFisher Scientific	APC-eFluor 780	47-0452-82	1:400
Intracellular	BCL6	K112-91	BD Biosciences	PE	561522	1:20
Intracellular	TBET	4B10	BioLegend	BV605	644817	1:100
Intracellular	ROR γ t	Q31-378	BD Biosciences	BV421	562894	1:100