

Video Article

Peptide:MHC Tetramer-based Enrichment of Epitope-specific T cells

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Abstract

A basic necessity for researchers studying adaptive immunity with *in vivo* experimental models is an ability to identify T cells based on their T cell antigen receptor (TCR) specificity. Many indirect methods are available in which a bulk population of T cells is stimulated *in vitro* with a specific antigen and epitope-specific T cells are identified through the measurement of a functional response such as proliferation, cytokine production, or expression of activation markers¹. However, these methods only identify epitope-specific T cells exhibiting one of many possible functions, and they are not sensitive enough to detect epitope-specific T cells at naive precursor frequencies. A popular alternative is the TCR transgenic adoptive transfer model, in which monoclonal T cells from a TCR transgenic mouse are seeded into histocompatible hosts to create a large precursor population of epitope-specific T cells that can be easily tracked with the use of a congenic marker antibody^{2,3}. While powerful, this method suffers from experimental artifacts associated with the unphysiological frequency of T cells with specificity for a single epitope^{4,5}. Moreover, this system cannot be used to investigate the functional heterogeneity of epitope-specific T cell clones within a polyclonal population.

The ideal way to study adaptive immunity should involve the direct detection of epitope-specific T cells from the endogenous T cell repertoire using a method that distinguishes TCR specificity solely by its binding to cognate peptide:MHC (pMHC) complexes. The use of pMHC tetramers and flow cytometry accomplishes this⁶, but is limited to the detection of high frequency populations of epitope-specific T cells only found following antigen-induced clonal expansion. In this protocol, we describe a method that coordinates the use of pMHC tetramers and magnetic cell enrichment technology to enable detection of extremely low frequency epitope-specific T cells from mouse lymphoid tissues^{3,7}. With this technique, one can comprehensively track entire epitope-specific populations of endogenous T cells in mice at all stages of the immune response.

Video Link

The video component of this article can be found at <https://www.jove.com/video/4420/>

Protocol

1. Cell Isolation from Lymphoid Tissue

1. Add 1 ml of ice cold cEHAA (EHAA + 10% FBS, pen/strep, gentamycin, 2 mM L-glutamine, 55 mM 2-mercaptoethanol) or other equivalent T cell medium, to a 60 mm culture dish containing a small square of 100 µm nylon mesh and place on ice.
2. Euthanize mouse.
3. Remove the spleen and as many easily accessible lymph nodes as possible. These should include at least the inguinal, axillary, brachial, cervical, and mesenteric lymph nodes. Place them on top of the nylon mesh in the culture dish.
4. Using the flat top of a closed 1.5 ml microfuge tube, **gently** mash the lymphoid tissue over the nylon mesh to liberate lymphocytes. Add another 1 ml of cEHAA and pipet up and down to work cells into a suspension. Transfer the cells through another piece of nylon mesh placed over the top of a 15 ml polypropylene centrifuge tube. Rinse the dish and mesh with another 1 ml of ice cold cEHAA, pooling the volumes into the same tube. Repeat to achieve a final cell suspension volume of 4 ml.
5. Add cold sorter buffer (PBS + 2% FBS, 0.05% Azide) to a final volume of 15 ml and centrifuge the tube for 5 min at 300 x g, 4 °C.
6. Carefully aspirate the supernatant, making sure no droplets of liquid are left on the sides of the tube. Resuspend the cell pellet in Fc block (sorter buffer + 2.4G2 antibody) to a final volume equal to approximately twice that of the pellet itself. For example, the spleen and lymph nodes of a naive mouse usually produces a cell pellet of about 100 µl. In this case, add 100 µl of Fc block to bring the volume to 200 µl. If a large degree of cell clumping has occurred, carefully remove the cell clump at this point with a pipet tip.

2. Tetramer Staining

1. Add PE- or APC-labeled pMHC tetramer to a final concentration of 10 nM (or empirically optimized concentration).
2. Mix and incubate for 1 hr at room temperature (or empirically optimized time and temperature).

3. Add cold sorter buffer to a volume of 15 ml and centrifuge the tube for 5 min at 300 x g, 4 °C. Keep samples on ice or at 4 °C from now on.
4. Carefully aspirate the supernatant, making sure no droplets of liquid are left on the sides of the tube. Resuspend the cell pellet in sorter buffer to a final volume of 200 µl. For double tetramer enrichment, resuspend to a final volume of 150 µl.

3. Magnetic Enrichment

1. Add 50 µl of Miltenyi anti-PE or anti-APC microbeads. For double tetramer enrichment, add 50 µl of both.
2. Mix and incubate for 20 min at 4 °C.
3. Add cold sorter buffer to a volume of 15 ml and centrifuge the tube for 5 min at 300 x g, 4 °C.
4. While waiting, set up a Miltenyi LS magnetic column on a MidiMACS or QuadroMACS magnet. Position an open 15 ml polypropylene centrifuge tube on a rack directly underneath the column.
5. Add 3 ml of sorter buffer to the top of the column, allowing it to drain into the 15 ml tube.
6. Place a 100 µm nylon mesh square on top of the column.
7. When cells have finished spinning, carefully aspirate the supernatant and resuspend the pellet in 3 ml of sorter buffer.
8. Transfer the cell suspension through the nylon mesh onto the top of the column.
9. When the cell suspension has completely drained into the column, rinse the original tube with another 3 ml of sorter buffer and transfer the buffer through the nylon mesh into the column, rinsing the mesh in the process. Discard the nylon mesh.
10. When the buffer has completely drained into the column, add another 3 ml of sorter buffer to the column.
11. Repeat step 10 for a total of 3 x 3 ml washes.
12. Remove the column from the magnet and place over a new 15 ml polypropylene centrifuge tube.
13. Add 5 ml of sorter buffer to the column.
14. Immediately insert the plunger into the top of the column and in one continuous motion, push the plunger all the way down, forcing the buffer out the column into the tube.
15. Centrifuge the tubes containing the eluted bound fraction and the flow-through unbound fraction for 5 min at 300 x g, 4 °C.
16. Carefully aspirate the supernatant from the bound fraction, making sure no droplets of liquid are left on the sides of the tube. Resuspend the cell pellet in sorter buffer to a final volume of exactly 95 µl. Aspirate and resuspend the unbound fraction to a final volume of 2 ml.

4. Flow Cytometry

1. For each fraction, remove 5 µl and add to 200 µl of counting beads (adjusted to a concentration of 200,000/ml in sorter buffer) in a 5 ml FACS tube. Set aside at 4 °C for analysis later. To save time, beads can be pre-aliquoted to labeled tubes before the start of the experiment.
2. Prepare a master mix of antibodies to stain surface markers on the cells (**Table 1**). To save time, this can be done prior to the start of the experiment.
3. For the bound fraction, add a dose of antibody cocktail directly to the ~90 µl of cells in the tube. If analysis of the unbound fraction is desired, transfer 90 µl of cells to a 5 ml FACS tube and add a dose of antibody cocktail.
4. Set up a panel of single-color compensation controls for the flow cytometer. For each fluorochrome to be used, mix 50 µl of leftover unbound fraction cells in a 5 ml FACS tube with 1 µl of anti-CD4 antibody conjugated to the corresponding fluorochrome. Remember to set up an unstained control as well.
5. Vortex and incubate all samples for 30 min at 4 °C.
6. Add 5 ml of sorter buffer to each tube and centrifuge for 5 min at 300 x g, 4 °C.
7. For the bound fraction, carefully aspirate supernatant and resuspend the cells in 200 µl of sorter buffer. Transfer the cells into a 1.2 ml FACS microtube. Rinse the tube with another 200 µl of sorter buffer and pool into the same microtube. If cell clumps are apparent, pass the cells through a 50 µm filter.
8. For the unbound fraction and compensation controls, decant or aspirate the supernatant and resuspend in 2 ml sorter buffer.
9. Set up the flow cytometer using the single-color compensation controls. Select side scatter-width (SSC-W) as an additional parameter to be recorded.
10. Analyze the stained samples using a sequence of successive inclusion gates to identify CD4+ or CD8+ T cells as illustrated in **Figures 1 and 2**. For bound fractions, collect as many cells as possible up to a maximum of 2,000,000 total events. For unbound fractions, collect 1,000,000 total events. Keep the acquisition rate at or below 3,000 events per second.
11. Using the same machine settings, analyze the counting bead samples. Collect 10,000 total events.
12. Save all data as FCS files.

5. Data Analysis

1. Analyze FCS data files for the counting bead samples using FlowJo software. Plot forward scatter by FITC and set a gate around the counting beads (see **Figures 1 and 2**). Determine the total number of counting beads detected in each sample. Subtract from the total number of collected events to determine the number of cell events collected.
2. Calculate the total number of all cells in each sample using the equations outlined in Box 1.
3. Analyze FCS data files for the stained bound and unbound fraction samples. Set up a sequence of successive inclusion gates to identify lymphoid+, side-scatter-width^{lo}, dump-, CD3+, CD4+ or CD8+, tetramer+ T cells in each sample as illustrated in **Figures 1 and 2**.
4. Multiply the total number of cells in the sample by the frequencies of each of the inclusion gates used to define CD4+tetramer+ or CD8+tetramer+ cells to calculate the total number of epitope-specific T cells (Box 1).

Representative Results

Figure 1 depicts representative flow cytometry plots of pMHCII tetramer enriched spleen and lymph node samples from naive mice, while **Figure 2** depicts representative data for mice previously immunized with the relevant peptide+CFA. Serial gating removes autofluorescent and

other unwanted events from the analysis of CD4+ T cell populations. The CD8+ T cell population serves as a useful internal negative control for pMHCII tetramer staining of CD4+ T cells. Note that bound fractions from the enrichment usually contain a significantly higher proportion of autofluorescent cells than the unbound fractions, making gating more challenging.

Absolute numbers of epitope-specific T cells in a sample are calculated by multiplying the total number of all cells in the bound fraction of the enriched sample, as determined from the bead count analysis, with the proportion of these cells that are tetramer+, as determined from the cell staining analysis (Box 1).

For the naive mouse in **Figure 1**, the concentration of all cells in the bound fraction of the sample is (4411/5589) (200,000) (0.200/0.005) = 6.31×10^6 /ml. The total number of all cells in the sample is (6.31×10^6 /ml) (0.095) = 6.00×10^5 . Finally, the total number of epitope-specific CD4+ T cells is (6.00×10^5) (41.5%) (96.6%) (10.2%) (62.3%) (0.64%) = **98**.

For the immunized mouse in **Figure 2**, the concentration of all cells in the bound fraction of the sample is (6410/3590) (200,000) (0.200/0.005) = 1.43×10^7 /ml. The total number of all cells in the sample is (1.43×10^7 /ml) (0.095) = 1.36×10^6 . Finally, the total number of epitope-specific CD4+ T cells is (1.36×10^6) (40.9%) (93.9%) (9.54%) (72.0%) (42.7%) = **1.53×10^4** .

The efficiency of enrichment declines as the number of epitope-specific T cells increases⁸, so tetramer+ cells may be seen in the unbound fraction of samples containing very high frequencies of epitope-specific T cells. In such cases, the number of epitope-specific T cells present in the unbound fraction can be calculated separately and added to the number found in the bound fraction. Therefore, in **Figure 2**, the concentration of all cells in the unbound fraction of the sample is (9031/969) (200,000) (0.200/0.005) = 7.46×10^7 /ml, the total number of all cells is (7.46×10^7 /ml) (2.0) = 1.49×10^8 , and the total number of epitope-specific CD4+ T cells is (1.49×10^8) (62.7%) (96.4%) (44.5%) (54.7%) (0.0409%) = **8.97×10^3** . Adding the numbers in the bound and unbound fractions, there are $1.53 \times 10^4 + 8.97 \times 10^3 = \mathbf{2.43 \times 10^4}$ total epitope-specific CD4+ T cells in the whole sample. Indeed, if epitope-specific cell expansion is sufficiently robust, the enrichment process can be skipped.

Fluorochrome	Antibody
Pacific Blue	dump (B220, CD11b, CD11c, F4/80)
Pacific Orange	CD8
FITC	CD3
PE	pMHC tetramer or phenotypic marker
PerCP	CD4
APC	pMHC tetramer or phenotypic marker
AlexaFluor 700	CD44

Table 1. Suggested antibody staining strategy

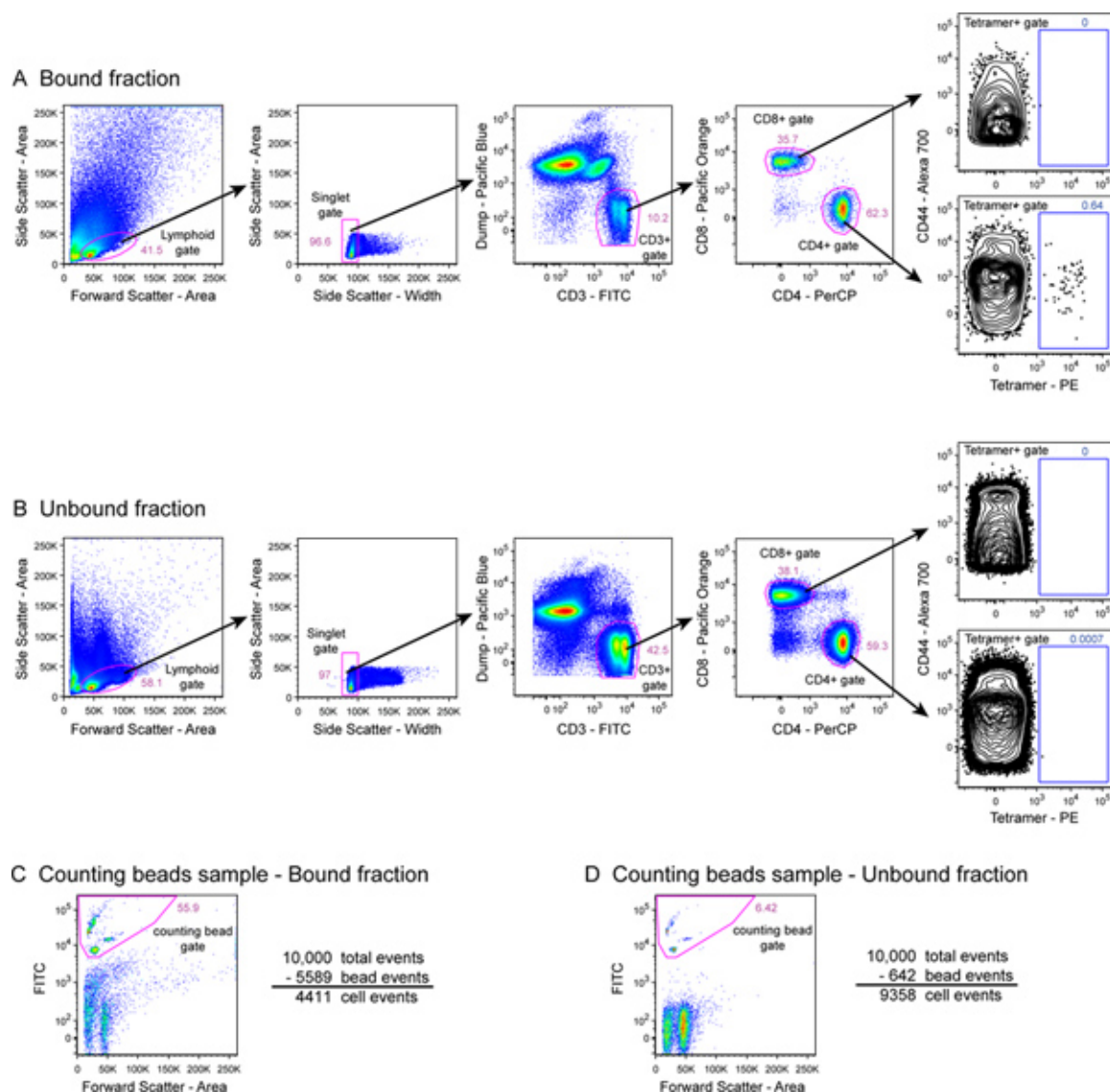


Figure 1. Flow cytometric analysis of epitope-specific CD4+ T cells in naive mice following pMHCII tetramer-based enrichment. Representative plots are shown for the bound (A) and unbound (B) fractions. A succession of gates are set to select lymphoid-scatter+, side-scatter-widthlo, dump-, CD3+ events. Of these, CD4+ or CD8+ events are gated for the analysis of epitope-specific T cells or background staining. Aliquots of unstained cells from the bound (C) and unbound (D) fraction were mixed with fluorescent counting beads and analyzed separately. [Click here to view larger figure.](#)

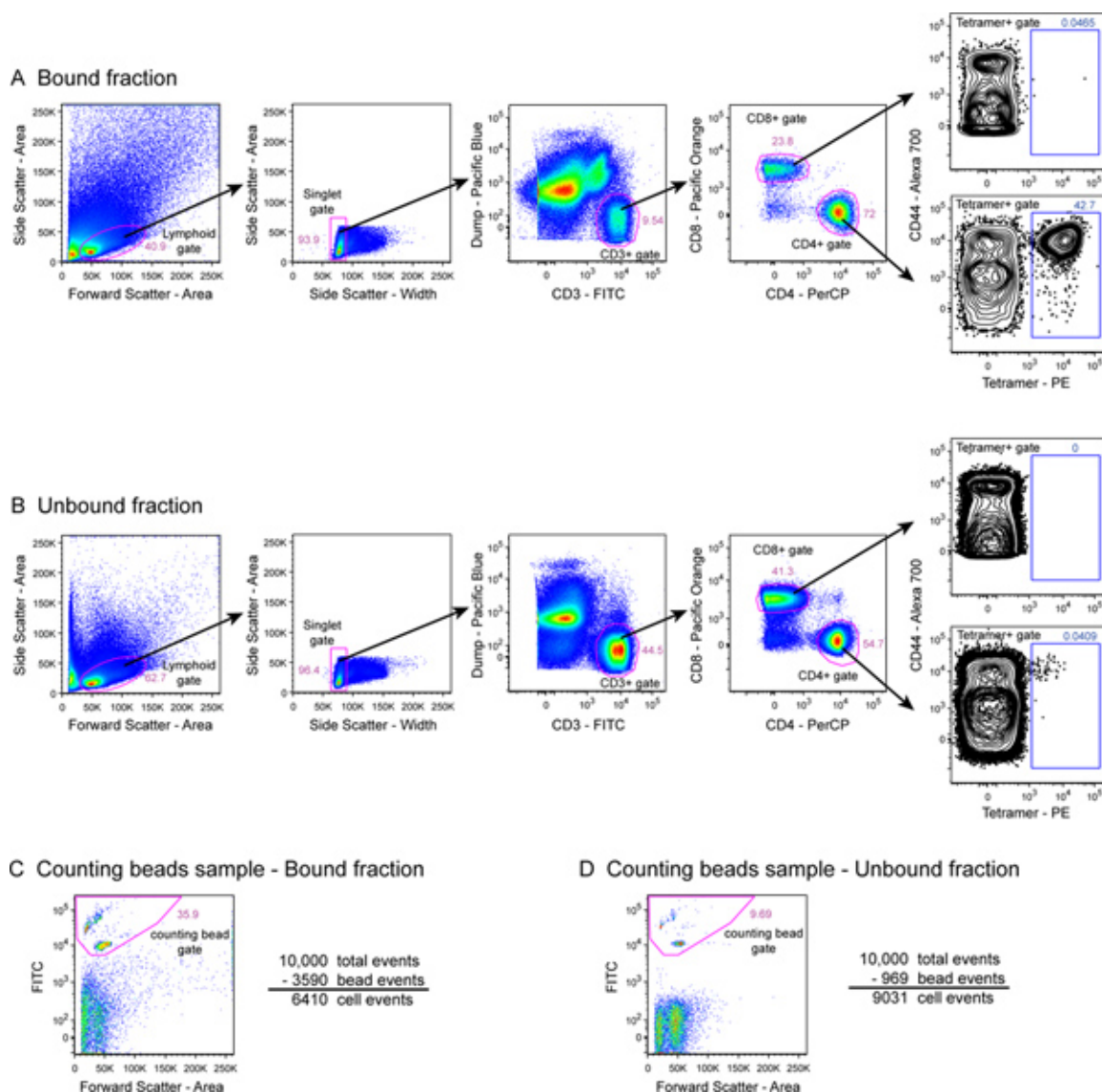


Figure 2. Flow cytometric analysis of epitope-specific CD4+ T cells in peptide-immunized mice following pMHCII tetramer-based enrichment. Representative plots are shown for the bound (A) and unbound (B) fractions. A succession of gates are set to select lymphoid-scatter+, side-scatter-widthlo, dump-, CD3+ events. Of these, CD4+ or CD8+ events are gated for the analysis of epitope-specific T cells or background staining. Aliquots of unstained cells from the bound (C) and unbound (D) fraction were mixed with fluorescent counting beads and analyzed separately. [Click here to view larger figure.](#)

Box 1. Calculation of epitope-specific T cell numbers

Absolute numbers of epitope-specific T cells are best calculated with the aid of fluorescent counting beads. An aliquot of unstained cells from each sample is mixed with a defined volume of counting beads set at a known concentration and then analyzed by flow cytometry. The concentration of cells in the sample can be inferred from a comparison of their frequency with the known concentration of fluorescent counting beads.

$$\text{cell concentration} = \left(\frac{\text{cell count}}{\text{bead count}} \right) \left(\frac{\text{bead stock}}{\text{conc}} \right) \left(\frac{\text{bead vol}}{\text{cell vol}} \right)$$

The total number of cells in the sample is then calculated by multiplying the cell concentration with the total sample volume.

$$\text{total number cells in sample} = \left(\frac{\text{cell}}{\text{concentration}} \right) \left(\frac{\text{total sample}}{\text{vol}} \right)$$

The total number of epitope-specific T cells in the sample is simply the total number of all cells in the sample multiplied by the percentage of cells that are tetramer-positive.

$$\text{total number epitope-specific T cells in sample} = \left(\frac{\text{total number cells in sample}}{\text{cells in sample}} \right) \left(\frac{\% \text{ tetramer-positive events}}{\text{events}} \right)$$

Discussion

The pMHC tetramer based cell enrichment method presented by this protocol is a powerful tool for studying epitope-specific T cells from endogenous T cell repertoires. The use of pMHC tetramers enables detection of epitope-specific T cells based directly on the ability of their TCRs to bind cognate pMHC ligands. The enrichment provides a level of sensitivity such that extremely rare populations of antigen-specific T cells can be detected from endogenous repertoires of T cells without any manipulation of their genetic makeup or precursor frequency. As a result, this technique allows the investigator to directly track endogenous antigen-specific T cell populations from *in vivo* experimental systems from their naive levels through all stages of the immune response.

This protocol has been optimized for the use of pMHC class II (pMHCII) tetramers to enrich epitope-specific CD4+ T cells from the secondary lymphoid organs of mice. However, the technique is also applicable to pMHC class I (pMHCI) tetramers and CD8+ T cells⁹. Unlike CD4, the CD8 coreceptor plays a significant role in stabilizing TCR-MHC interactions, and this can have practical implications for pMHCI tetramer staining¹⁰. Most notably, the use of CD8 antibodies should be restricted to clones that do not impair CD8-tetramer binding, and they should be added to cells after tetramer staining. Indeed, some pMHCI tetramers have been engineered with mutated MHCI-CD8 binding sites to mitigate nonspecific binding to CD8+ T cells^{11,12}.

Tetramer concentration, incubation time, and incubation temperature can greatly affect the efficiency of tetramer staining, and conditions should be optimized to achieve the best combination of high tetramer signal, low background signal, low tetramer internalization, and minimal changes to cell physiology. Ideally, these conditions should be empirically determined for each unique reagent. In our hands, however, a final concentration of 10 nM and an incubation of 1 hr at room temperature provides good generic conditions for most pMHCI or pMHCII tetramers. In general, pMHCI tetramers seem to stain more easily than pMHCII tetramers, and staining can often be performed at 4 °C for as little as 30 min.

The scale of this procedure is suited for the analysis of nearly all the secondary lymphoid organs of a mouse in a single sample. Therefore, each sample represents a fairly comprehensive analysis of the entire circulating peripheral T cell repertoire of a mouse. Epitope-specific T cells can also be enriched from other relevant tissues, including the thymus^{13,14}. When thymii from 4-5 week old mice are analyzed, epitope-specific single positive thymocytes can be detected at numbers similar to those of peripheral naive T cells. Epitope-specific double-positive thymocytes, however, are very difficult to detect due to their low levels of TCR expression.

This protocol can also be adapted to detect epitope-specific human CD4+ or CD8+ T cells in blood or other tissues¹⁵⁻¹⁷. The frequency of epitope-specific T cells is roughly the same between mice and humans¹⁷, so the analysis of 50 - 100 ml of blood would yield comparable numbers of epitope-specific T cells as the pooled spleen and lymph nodes of a mouse¹⁸.

A major challenge in the flow cytometric analysis of cells following tetramer-based enrichment is distinguishing true cell events from background. This is largely due to the fact that many autofluorescent cells are also non-specifically enriched during the process. If not carefully gated out, these autofluorescent cells can appear as false tetramer-positive cells and throw off the accuracy of the analysis, particularly in the case of rare naive T cell populations. Our protocol employs a two-step gating strategy in which CD3+ events are first gated away from dump lineage+ events, and then CD4+ events are gated away from CD8+ events. In the process, autofluorescent cells, which tend to lie along the center diagonal of any FACS plot, are gated out of the analysis in two iterative steps. The effective removal of autofluorescent events comes at the cost of many fluorescent colors, so we highly recommend the use of flow cytometers capable of at least 6 fluorescent parameters.

Most pMHC tetramers are conjugated to PE and APC due to the brightness of these fluorochromes, and anti-PE and anti-APC magnetic microbeads are readily available to enable enrichment with them. However, other fluorochromes can also be used so long as corresponding magnetic microbeads are available. Indeed, multiple tetramers with different fluorochrome labels can be used together with the corresponding antibody-conjugated microbeads to simultaneously enrich multiple epitope-specific T cell populations from the same sample. We have outlined a very basic antibody-fluorochrome setup that is optimized for the use of PE- and APC-labeled tetramers (Table 1), but many other effective combinations are possible to increase flexibility in the study of phenotypic markers.

CD8+ T cell populations can be used as an internal negative control, since they should not bind to pMHCII ligands (and vice versa for epitope-specific CD8+ T cell enrichment). The frequency of tetramer+ CD8+ cells in a sample provides a good assessment of the level of background tetramer staining, although *bona fide* tetramer-positive CD8+ T cells with cross-restricted specificity to pMHCII epitopes may exist at very small frequencies¹⁹. Occasionally during very strong immune responses, these cells may exist at expanded frequencies. If desired, TCR transgenic T cells with or without relevant epitope specificity can be used as additional positive and negative controls. Note that for unknown reasons, some TCR transgenic cells and hybridomas do not stain well with their relevant tetramers.

Our protocol involves the use of fluorescent counting beads to assist in the calculation of cell numbers. While cell counts can also be achieved manually with a hemacytometer, we find that the use of counting beads results in much greater experimental precision, especially when multiple investigators are involved. Due to the small numbers and volumes of cells that are handled in this protocol, the minimization of experimental error should be a high priority.

Tetramer staining is compatible with cell fixation and permeabilization, and several studies have successfully analyzed intracellular cytokine and transcription factor expression in cells following tetramer-based cell enrichment^{20,21}. However, the extra steps involved contribute to additional cell losses in the samples.

Disclosures

No conflicts of interest declared.

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