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Measurement of the T cell response to pre-erythrocytic vaccination in mice

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Abstract

Whole attenuated parasite vaccines designed to elicit immunity against the clinically silent pre-erythrocytic stage of *Plasmodium* infection represent the most efficacious experimental platforms currently in clinical trial. Studies in rodents and humans show that T cells mediate vaccine-induced protection. Thus, determining the quantitative and qualitative properties of these T cells remains a major research focus. Most rodent models of pre-erythrocytic anti-*Plasmodium* vaccination focus on circumsporozoite-specific CD8 T cell responses in BALB/c mice. However, CD4 T cells and non-circumsporozoite-specific CD8 T cells also significantly contribute to protection. Here we describe alternative approaches that enable detection and functional characterization of total CD8 and CD4 T cell responses induced by pre-erythrocytic vaccination in mice. These flow cytometry-based approaches rely on monitoring the modulation of expressed integrins and co-receptors on the surface of T cells in vaccinated mice. The approaches enable direct determination of the magnitude, kinetics, distribution, phenotype, and functional features of T cell responses induced by infection or whole-parasite vaccination using any mouse-parasite species combination.

Keywords

Radiation attenuated sporozoite; Genetically attenuated parasite; Vaccine; *Plasmodium*; Malaria; CD8 T cell; CD4 T cell; Integrins

1. Introduction

Malaria remains a global health crisis and it has been argued that eradication of the disease cannot be achieved without the development of efficacious vaccines against *Plasmodium* parasites [1]. Although there are several subunit and vectored anti-malarial vaccines under clinical evaluation [2], current experimental vaccines that elicit the most potent and long-lasting protection in humans are those formulated using live attenuated sporozoites incapable of causing disease [3–6]. Radiation- and genetically-attenuated parasites, and the prophylactic use of anti-malarial drugs targeting blood stage parasites, are promising

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strategies currently under evaluation in clinical trials as pre-erythrocytic vaccine platforms [7–10]. Notably, each of these leading pre-erythrocytic vaccine platforms was first predicted and qualified using rodent models [11–13]. Thus, experimental mouse models continue to provide fundamentally important information regarding the mechanisms of immune resistance induced by pre-erythrocytic vaccination.

Studies in rodents have conclusively shown that a central component of protective pre-erythrocytic vaccination involves the induction of parasite-specific CD8 T cells targeting *Plasmodium*-infected hepatocytes [11,14,15]. Data in humans also show strong correlations between the activity of CD8 T cells and protection against experimental mosquito bite challenge [5,6,10,16]. CD8 T cells limit parasite survival by several pathways, including direct cytolysis of infected hepatocytes, and by expressing anti-parasitic cytokines that act to limit parasite survival within infected hepatocytes [14,17–19]. Although data supporting a role for CD4 T cells in limiting liver stage infections is less abundant, several studies demonstrate that CD4+ major histocompatibility (MHC) class II-restricted T cells can exert cytolytic activity against parasite-infected cells [20–23]. Moreover, MHC class I deficient mice, which lack CD8 T cells, are resistant to sporozoite challenge following multiple vaccinations with attenuated *Plasmodium* sporozoites [24]. Protection in the latter studies depended on CD4 T cells and correlated with neutralizing parasite-specific antibody responses. Finally, CD4 T cells also are important regulators of CD8 T cell responses, as *Plasmodium*-specific CD8 T cells fail to form sizable memory populations in the absence of CD4 T cells [25]. Thus, parasite-specific CD4 T cells are also important regulators of vaccine-induced anti-*Plasmodium* immunity via their provision of help for CD8 T cells and B cells, and perhaps through direct cytolysis of parasite-infected cells [20–22]. Collectively, these studies underscore the critical role for T cells in mediating pre-erythrocytic vaccine-induced protection against *Plasmodium*. Therefore, defining the numerical, phenotypic and functional features of parasite-specific CD8 and CD4 T cells activated by pre-erythrocytic vaccination remains an important goal.

High-resolution study of pathogen-specific T cells classically requires knowledge of defined T cell epitopes and MHC restriction elements, or access to immunologic tools such as MHC tetramers or T cell receptor transgenic (TCR Tg) mice. For both *Plasmodium yoelii* and *P. berghei* liver stage parasites several tools exist and at least one dominant CD8 T cell epitope from each parasite has been mapped in inbred BALB/c mice [26]. These CD8 T cell epitopes derive from the sporozoite- and liver stage-expressed circumsporozoite (CS) protein (*P. yoelii*, amino acids 280–288, CS_{280–288}; *P. berghei*, CS_{252–260}, e.g. Fig. 1) [26]. Notably, TCR Tg mice have been generated for the *P. yoelii* CD8 T cell determinant [27].

Plasmodium-specific TCR Tg CD4 T cells have also been generated, but the MHC class II-restricted TCR is specific for a blood stage antigen (MSP-1) and the epitope is only expressed by *P. chabaudi* rodent parasites [28–30]. More recently, Heath and colleagues developed a new TCR Tg mouse line bearing CD8 T cells responsive to an antigen expressed during both blood and liver stage [31]. Strikingly, the epitope from this antigen is conserved in *P. berghei*, *P. yoelii* and *P. chabaudi*, so future applications of this new research tool should reveal important information about cross-stage (blood and liver) specific CD8 T cell immunity. Because the experimental use of TCR Tg CD8 T cells in malaria models was recently reviewed [32], specific protocols are not described herein.

It is well known that CS-specific CD8 T cell populations can be sufficient to limit or stop the progression of liver stage *Plasmodium* parasites [17,26,27,33,34]. Thus, there are clear advantages to studying CS-specific endogenous (polyclonal) or TCR Tg (monoclonal) CD8 T cells. On the other hand, as noted above, CD4 T cells also contribute to resistance and several studies have shown that non-CS-specific CD8 T cells significantly limit *Plasmodium* liver stage infection [35,36]. Indeed, more than 80% of CD8 T cells induced by radiation attenuated sporozoite (RAS) vaccination of BALB/c mice are responding to nonCS antigens [15] (Fig. 1), so studying the biology and behavior of these cells is equally important. However, the lack of additional and validated non-CS-specific CD4 and CD8 T cell epitopes has hampered direct study of these T cell populations of unknown antigenic specificity. To overcome these limitations, cell surface markers of T cell activation have been widely used to monitor *Plasmodium* vaccination-induced T cell responses. For example, modulation of CD62L, CD44, CD122, and CD45RB expression has been used to identify RAS-induced, liver-resident CD8 T cells [37–40]. However, several of these molecules are expressed on both naïve and memory T cells (e.g. CD62L and CD122, [41]) or they exhibit a continuum of expression (e.g. expression of CD44 is not bimodal). Moreover, the expression of several of these markers can be modulated by T cell homeostatic proliferation [42]. These variables add to the difficulty of distinguishing among bona fide naïve, effector and memory T cells through monitoring CD62L, CD44 and CD122.

In an effort to further enhance resolution and more clearly distinguish true naïve and effector and memory T cells, we developed alternative methods to track T cell responses following *Plasmodium* infection or vaccination in both inbred and outbred mice [15,43]. Notably, these methods were first validated using models of virus and bacterial infection [44,45]. We determined that antigen activated CD8 T cells could be distinguished from naïve CD8 T cells based on the coordinate upregulation of the integrin CD11a (the alpha L chain of LFA-1) and downregulation of the CD8 α chain of the co-receptor. Effector and memory CD8 T cells are CD11a^{hi}CD8 α ^{lo} and naïve CD8 T cells are CD11a^{lo}CD8 α ^{hi} (Fig. 2). Unlike CD8 T cells, antigen activated CD4 T cells do not appreciably downregulate the CD4 co-receptor, so monitoring the coordinate upregulation of CD11a and an additional integrin, CD49d (the alpha 4 chain of VLA-4), is used to distinguish naïve CD4 T cells from antigen-activated CD4 T cells [43,45]. Effector and memory CD4 T cells are CD11a^{hi}CD49d^{hi} and naïve CD4 T cells are CD11a^{lo}CD49d^{lo} (Fig. 2). There are three main advantages to using these approaches: (1) modulation of CD8 α , CD11a and CD49d requires TCR cross-linking and does not occur following exposure of T cells to inflammation or homeostatic proliferation [43–45]. Thus, examination of CD11a, CD8 α , and CD49d T cell phenotypes faithfully and durably identifies infection- or vaccination induced, pathogen-specific CD8 and CD4 T cells. (2) endogenous T cell responses can be studied without knowledge of specific *Plasmodium* antigens/epitopes or MHC class I or MHC class II restriction elements; and (3) any combination of inbred or outbred mouse strains and *Plasmodium* parasite species can be studied. Overall, these approaches allow for direct examination of the magnitude, kinetics, distribution, phenotype, and function of T cell responses to *Plasmodium* infection or pre-erythrocytic vaccination. These approaches also allow for longitudinal analyses of T cell responses through analyses of peripheral blood lymphocytes, and thus

permit characterization of T cell responses in individual mice over time prior to parasite challenge studies.

The methods detailed below allow researchers to directly identify and interrogate CD4 and CD8 T cell populations induced by pre-erythrocytic vaccination and recovered from multiple tissues from the vaccinated host. Of note, methods for immunizing rodents with live attenuated *Plasmodium* parasite vaccine platforms have been previously covered [46,47], so our chapter will only describe methods for examining T cell responses induced by pre-erythrocytic vaccination. Such studies will continue to provide fundamentally important insight into the factors that determine the potent sterilizing immunity induced by pre-erythrocytic vaccines. This information can potentially guide improvement of current pre-erythrocytic vaccines as well as enhance the development of subunit vaccines that elicit similar populations of T cells. Defining the specific numerical and functional properties of T cells that determine protection will increase the likelihood of developing readily deployable vaccines in regions of the world where anti-malarial vaccines are needed most.

2. Materials

2.1 Equipment

2.1.1 Isolation of circulating lymphocytes from peripheral blood

1. Heparinized Micro-Hematocrit capillary tubes.
2. 1.5 mL eppendorf tubes.
3. Hemocytometer for cell counting.
4. Trypan Blue (0.1% solution in phosphate buffered saline (PBS)).
5. Light microscope with a 40× objective.
6. 20 µl, 200 µl, 1000 µl pipettors and pipette tips.

2.1.2 Isolation of lymphocytes from spleen or lymph nodes

1. Scissors and forceps.
2. 15 × 60 mm (or 10 × 35 mm) tissue culture dishes.
3. Fine wire screen or 70 µm cell strainer.
4. Rubber-tipped plunger from 3 or 5 mL syringe.
5. 5 and 10 mL pipettes.
6. Drummond Pipette Aid.
7. Hemocytometer for cell counting.
8. Trypan Blue (0.1% solution in PBS).
9. Light microscope with a 40× objective.
10. 20 µl, 200 µl pipettors and pipette tips.

2.1.3 Isolation of lymphocytes from liver

1. Scissors and forceps.
2. 15 × 60 mm tissue culture dishes.
3. Fine wire screen or 70 µm cell strainer.
4. Rubber-tipped plunger from 10 mL syringe.
5. 5 and 10 mL pipettes.
6. Drummond Pipette Aid.
7. Hemocytometer for cell counting.
8. Trypan Blue (0.1% solution in PBS).
9. Light microscope with a 40× objective.
10. 20 µl, 200 µl pipettors and pipette tips.

2.1.4 Flow cytometric staining and analyses of lymphocytes

1. Refrigerated tabletop centrifuges with fixed-angle rotor for 1.5 mL eppendorf tubes and swinging bucket rotor and inserts to accommodate 15 mL tubes and 96-well plates.
2. Flow cytometer capable of exciting and detecting emissions from common fluorochromes such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), Pacific Blue (PacBlue), and allophycocyanin-cyanine 7 (APC-Cy7) (see Note 1).
3. Sterile 96-well flat bottom tissue culture plates or 5 mL round bottom flow cytometry tubes (BD Falcon, Fisher Scientific) (see Note 2).
4. Flow cytometric analysis software (e.g. FlowJo).

2.2 Reagents

2.2.1 Isolation and staining of lymphocytes from peripheral blood, lymphoid tissues and liver

1. Complete RPMI-1640 medium: 500 mL of RMPI-1640, 50 mL heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 200 mg/mL gentamicin sulfate, 2 mM 2-mercaptoethanol, 100 U/mL penicillin, and 50 µg/mL streptomycin. Store sterile at 4°C.

¹The suggested protocol requires access to a flow cytometer capable of simultaneous detection of 6 or more colors. Examination of additional phenotypic or functional parameters will require more sophisticated cytometers with additional lasers and filter sets. Although not recommended, omitting the anti-mouse-CD90 antibody and/or live/dead reagents will permit examination of additional phenotypic or functional parameters using a 6-color cytometer.

²Protocols described here utilize 96-well flat bottom plates for cell staining, which increases work efficiency and decreases reagent use. However, identical results can be obtained by processing, staining and analyzing cells in 5 mL Falcon tubes designed to fit most commercial analytical flow cytometers.

2. FACS staining buffer: 1 L of 1x PBS, 10 mL fetal bovine serum, 1 mL 20% sodium azide. Store at 4°C.
3. Red blood cell lysis buffer: 1 L of sterile water, 8.25 g NH₄Cl, 1 g KHCO₃, 0.037 g Na₂EDTA, adjusted to 7.2–7.4 pH with HCl. Filter sterilized.
4. Cytotfix (BD Biosciences) or other fixation agent. Store at 4°C.
5. Anti-mouse-CD8α antibody (clone 53–6.7) (see Note 3).
6. Anti-mouse-CD4 antibody (clone GK1.5).
7. Anti-mouse-CD11a antibody (clone M17/4).
8. Anti-mouse-CD49d antibody (clone R1-2).
9. Anti-mouse-CD90 antibody (see Note 4)
10. Fixable cell viability dye (eBioscience) or fixable Ghost Dye (Tonbo) to discriminate between live and dead cells (see Note 5).
11. Anti-mouse-CD16/CD32 (clone 2.4G2) (see Note 6).
12. CS₂₅₂₋₂₆₀ or CS₂₈₀₋₂₈₈ MHC class I tetramer (see Note 7)

2.2.2 Isolation of lymphocytes from liver (unique reagents)

1. Ketamine or other drug to euthanize mouse.
2. Hanks' buffered salt solution (HBSS): 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, 5% fetal bovine serum, 12 mM HEPES buffer. Store sterile at 4°C (see Note 8).
3. 35% Percoll/HBSS solution (Percoll from GE Healthcare). Store sterile at 4°C.

³Each antibody reagent should be individually titrated to determine optimal final concentrations. Specific fluorochrome conjugates for each antibody will be dictated by the capabilities of the analytical flow cytometer.

⁴Including the anti-mouse-CD90 antibody will allow resolution between the T cells of interest and non-T cells expressing CD8α or CD4 (e.g. dendritic cells or macrophages). Although these contaminating subsets are relatively low frequency events, including anti-mouse-CD90 can be particularly helpful when examining T cell responses in lymph nodes, spleen or liver. Commonly available mouse lines such as C57BL/6, BALB/c and outbred mice express the CD90.2 allele. Congenic lines are also available that express the CD90.1 allele. Monoclonal antibodies that distinguish between these two polymorphisms are available and highly specific for each molecule, so care should be taken when selecting a CD90 clone. We recommend clone 53-2.1 for anti-mouse-CD90.2 and clone OX-7 for anti-mouse-CD90.1.

⁵Reagents that resolve between live and dead lymphocytes are particularly useful, as dead cells tend to non-specifically bind fluorochrome-conjugated antibodies. These unwanted interactions can significantly increase background staining and non-specific signals during flow cytometry. If fixatives are included in the protocol, care should be taken that the selected dye withstands fixation (e.g. 4% paraformaldehyde).

⁶Non-specific binding of antibodies can be minimized by including an unconjugated anti-mouse-FcR (CD16/CD32) antibody (clone 2.4G2).

⁷Fluorochrome-conjugated MHC class I (H-2K^d) tetramer reagents for detection of CS-specific CD8 T cells in vaccinated BALB/c mice are available from the NIH tetramer core (<http://tetramer.yerkes.emory.edu>) or MBL, Inc. (<http://www.mblintl.com/>).

⁸HBSS is utilized at two different temperatures in the protocol for isolating lymphocytes from the liver. Be sure to adjust an appropriate volume to RT prior to harvesting liver tissues.

3 Methods

3.1.1 Measuring responses in peripheral blood tissue—This section describes the isolation and flow cytometric staining of lymphocytes recovered from the peripheral blood of mice immunized with pre-erythrocytic vaccines.

1. Collect approximately 20–50 μ L blood (see Note 9) from naïve or vaccinated mice (see Note 10) into 1.5 mL eppendorf tubes containing 0.5 mL of RPMI-1640 medium.
2. Centrifuge at $3\text{--}400 \times g$ for 5 minutes at 4°C in a refrigerated tabletop centrifuge.
3. Aspirate supernatant, suspend cells in 0.5 mL of red blood cell lysis buffer, vortex and incubate at room temperature (RT) for ~3 min.
4. Add 0.9 mL of FACS buffer, spin at $3\text{--}400 \times g$ for 5 min at 4°C in a refrigerated centrifuge (see Note 11 if doing functional analyses).
5. Aspirate supernatant, suspend pellet, in 1.2 mL FACS buffer and spin again at $3\text{--}400 \times g$ for 5 min at 4°C .
6. Aspirate supernatant, suspend pellet in appropriate volume of FACS buffer (see Note 12).
7. Add 100 μ L of cell suspension into wells of flat-bottom 96-well plate (see Notes 2 and 13).
8. Centrifuge plate at $3\text{--}400 \times g$ for 5 min at 4°C in a tabletop centrifuge.
9. Decant or aspirate supernatants (see Note 14), lightly vortex plate to disrupt cell pellets.
10. Stain cells with appropriate staining cocktail(s):

⁹The capillary tubes utilized by our laboratory hold approximately 70 μ L of blood. The total volume of blood collected in any specific interval should not exceed that recommended by IACUC guidelines. Particular care and consideration should be given to the execution of longitudinal experiments designed to include multiple serial bleeds from individual mice.

¹⁰If tracking T cell responses longitudinally in peripheral blood, all animals should be assayed prior to vaccination to determine the individual background frequencies of circulating CD8 α^{hi} CD11a $^{\text{hi}}$ CD8 T cells and CD11a $^{\text{hi}}$ CD49d $^{\text{hi}}$ CD4 T cells. Of note, the described approaches are not likely useful for evaluating T cells responses induced by vectored vaccines designed to target liver stage *Plasmodium* parasites, as the surrogate marker of T cell activation approaches will not distinguish between T cell responses directed against the viral vector versus the *Plasmodium* antigen encoded by the vector.

¹¹If performing functional studies prior to T cell staining, substitute RPMI-1640 medium for FACS buffer. FACS buffer contains sodium azide, which poisons electron transport in eukaryotic and prokaryotic cells. Sodium azide is highly toxic so care should be taken when handling any solution containing this compound.

¹²The volume of FACS buffer in which peripheral blood lymphocytes are suspended is ultimately dictated by the number of distinct flow cytometric analyses that will be performed. For example, if two independent staining cocktails will be applied to the cells, suspend the cells in a minimum of 200 μ L of FACS buffer; if six independent stains will be performed, suspend the cells in 600 μ L of FACS buffer, etc. We routinely recover more than 100,000 lymphocytes from 20 μ L of whole blood when the sample is processed using protocols described above.

¹³Be sure to set aside a sufficient number of cells for the compensation staining control reactions. Only 2000–5000 events are generally required for compensation reactions. An explanation of compensation is not discussed here but has been thoroughly described elsewhere [48].

¹⁴A flick-of-the-wrist can be used to invert the plate to decant (eject) supernatants into a suitable waste receptacle. Alternatively, supernatants can be aspirated individually. Do not tap the plate on a solid surface or flick the plate more than once or the cells of interest will also be ejected from the wells.

- a. If examining CS-specific CD8 responses using CS₂₅₂₋₂₆₀ or CS₂₈₀₋₂₈₈ MHC class I tetramer, cell staining should be performed sequentially:
 - i. First, add 50 µl of the optimally titrated tetramer reagent, mix with pipetting and incubate at RT, 4°C or on ice for 1 hour (see Note 15).
 - ii. Second, without washing cells directly add 100 µl of a cocktail of antibodies directed against cell surface antigens. The surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8α, anti-mouse-CD11a, anti-mouse CD90; cell viability dye. If simultaneously examining CD4 T cells, the cocktail should also include anti-mouse-CD4 and anti-mouse-CD49d.
 - iii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.
- b. If examining total CD8 and CD4 T cells responses, all reagents are added simultaneously:
 - i. Add 100 µl of a cocktail of antibodies directed against cell surface antigens. The surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8α, anti-mouse-CD11a, anti-mouse-CD4, anti-mouse-CD49d, anti-mouse CD90 and cell viability dye.
 - ii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.
11. Wash away unbound antibody and/or tetramer from wells by adding 150 µl of FACS buffer directly to wells containing staining cocktail, spin plate at 3–400 × g for 5 min at 4°C.
12. Decant or aspirate supernatants, lightly vortex plate, add 100 µl of Cytofix and incubate at 4°C for 7–10 min (see Note 16).

¹⁵MHC class I tetramer reagents must be titrated for optimal staining. The incubation temperature (e.g. RT, 4°C or on ice) should also be determined empirically. Anti-CD8α antibody can interfere with tetramer binding to TCR on CD8 T cells, so antibodies directed against CD8α (and other surface antigens) should only be added after the initial tetramer incubation.

¹⁶After surface staining, we routinely fix cells with 4% paraformaldehyde fixative solution such as Cytofix (BD Biosciences). This can be particularly important to stabilize the staining reactions when samples will not be immediately run on the analytical flow cytometer.

13. Wash away Cytofix from cells by adding 150 μ L of FACS buffer directly to wells containing 100 μ L of fixative, spin plate at $3\text{--}400 \times g$ for 5 min at 4°C .
14. Decant or aspirate supernatants, lightly vortex, suspend cells in 180–200 μ L of FACS buffer, then store cells at 4°C protected from light until run on the flow cytometer.

3.1.2 Measuring responses in spleen or lymph node tissue—This section describes the isolation and flow cytometric staining of lymphocytes recovered from secondary lymphoid tissues of mice immunized with pre-erythrocytic vaccines.

1. Harvest desired secondary lymphoid tissues using sterile scissors and forceps and place tissue into 15 mL conical containing 3 mL of RPMI-1640 medium.
2. Transfer the tissue and medium onto a fine mesh steel screen in a 15×60 mm (or 10×35 mm) tissue culture dish. Alternatively, transfer tissue and medium to a $70 \mu\text{m}$ cell strainer (see Note 17).
3. Push the tissue through the screen or filter using the head of plunger from a 3 or 5 mL syringe. Rinse the screen or filter with an additional 3–5 mL of RPMI-1640. Repeated pipetting of the suspension through the screen or filter is essential in order to fully disrupt the tissue and create a single cell suspension. Rinsing screen or filter with fresh medium and combining fractions will enhance cell recovery.
4. Transfer the suspension from tissue culture dish to 15 mL conical and centrifuge samples at $3\text{--}400 \times g$ for 5 min in 4°C tabletop centrifuge.
5. Aspirate or decant supernatant. Suspend cells in 3 mL of red blood cell lysis buffer to the 15-mL conical, vortex, and incubate at RT for 30–60 seconds.
6. Add ~11 mL of FACS buffer (fill tube), centrifuge samples at $3\text{--}400 \times g$ for 5 min in 4°C tabletop centrifuge (see Note 10 if performing functional analyses).
7. Aspirate or decant supernatant, suspend pellet, wash residual red blood cell lysis buffer from cells by adding 5 mL of FACS buffer and spin at $3\text{--}400 \times g$ for 5 min at 4°C .
8. Aspirate or decant supernatant, suspend pellet in 10 mL (for spleen cells) or 1–3 mL (for lymph node cells) of FACS buffer.
9. Determine total numbers of viable cells using hemocytometer, microscope with $40\times$ objective and Trypan Blue exclusion dye.

¹⁷The methods used to disrupt tissues to generate single cell lymphocyte suspensions via mechanical means are not critical. Here we have outlined two methods that are routinely used in our laboratory to create single cell suspensions from secondary lymphoid tissues and liver (wire mesh and $70 \mu\text{m}$ cell strainer). Investigators should empirically determine the optimal method for their experiments, choosing methods that yield the largest number of viable cells.

10. Adjust the cell concentration to 10–20 million viable cells/mL (see Note 18).
11. Add 100 μ L of cell suspension into wells of 96-well flat-bottom plate (see Notes 2 and 13).
12. Centrifuge plate at $3\text{--}400 \times g$ for 5 min at 4°C in a tabletop centrifuge.
13. Decant or aspirate supernatants (see Note 14), lightly vortex plate to disrupt cell pellets.
14. Stain cells with appropriate staining cocktail(s):
 - a. If examining CS-specific CD8 responses using CS₂₅₂₋₂₆₀ or CS₂₈₀₋₂₈₈ MHC class I tetramer, cell staining should be performed sequentially:
 - i. First, add 50 μ L of the optimally titrated tetramer reagent, mix with pipetting and incubate at RT, 4°C or on ice for 1 hour (see Note 15).
 - ii. Second, without washing cells directly add 100 μ L of a cocktail of antibodies directed against cell surface antigens. The surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8 α , anti-mouse-CD11a, anti-mouse CD90; cell viability dye. If simultaneously examining CD4 T cells, the cocktail should also include anti-mouse-CD4 and anti-mouse-CD49d.
 - iii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.
 - b. If examining total CD8 and CD4 T cells responses, all reagents are added simultaneously:
 - i. Add 100 μ L of a cocktail of antibodies directed against cell surface antigens. The surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8 α , anti-mouse-CD11a, anti-mouse-CD4, anti-mouse-CD49d, anti-mouse CD90 and cell viability dye.

¹⁸From our experience, we have found it is best to only plate one to two million cells in a well. Using a known number of cells and optimizing antibody concentrations through titration experiments will ensure that tetramer and antibody reagents are not limiting.

- ii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.
15. Wash away unbound antibody and/or tetramer from wells by adding 150 µl of FACS buffer directly to wells containing staining cocktail, spin plate at 3–400 × g for 5 min at 4°C.
16. Decant or aspirate supernatants, lightly vortex plate, add 100 µl of Cytofix and incubate at 4°C for 7–10 min (see Note 16).
17. Wash away Cytofix from cells by adding 150 µl of FACS buffer directly to wells containing 100 µl of fixative, spin plate at 3–400 × g for 5 min at 4°C.
18. Decant or aspirate supernatants, lightly vortex, suspend cells in 180–200 µl of FACS buffer, then store cells at 4°C protected from light until run on the flow cytometer.

3.1.3 Measurement of responses in liver tissue—This section describes the isolation and flow cytometric staining of lymphocytes recovered from the livers of mice immunized with pre-erythrocytic vaccines.

1. Perfuse liver via portal vein with 10 mL of ice-cold HBSS. Remove the gall bladder.
2. Resect the liver and place in 50 mL conical containing 5 mL of ice-cold HBSS.
3. Transfer the tissue and medium onto a fine mesh steel screen in a 15 × 60 mm tissue culture dish. Alternatively, transfer tissue and medium to a 70 µm cell strainer (see Note 17).
4. Push the tissue through the screen or filter using the head of plunger from a 10 mL syringe. Rinse the screen or filter with an additional 10 mL of ice-cold HBSS. Repeated pipetting of the suspension through the screen or filter is essential in order to fully disrupt the tissue and create a single cell suspension. Rinsing screen or filter with fresh medium and combining fractions will enhance cell recovery.
5. Pass the liver cell suspension through a 70 µm cell strainer seated in a 50 mL conical. Rinse the filter with an additional 20 mL of ice-cold HBSS and combine fractions.
6. Centrifuge cells at 3–400 × g for 10 min at 4°C in a tabletop centrifuge.
7. Aspirate or decant supernatant, and suspend cell pellet in 15 mL of RT 35% Percoll/HBSS.
8. Centrifuge cells at 500 × g for 10 min at RT in a tabletop centrifuge. Important: Brakes should not be used on rotor.

9. Aspirate or decant supernatant and suspend cell pellet in 2 mL of red blood cell lysis buffer. Incubate for 3 min at RT. Add 12 mL of RPMI-1640 and transfer the entire cell suspension into a 15 mL conical.
10. Centrifuge cells at $3\text{--}400 \times g$ for 5 min at 4°C in a tabletop centrifuge.
11. Aspirate or decant supernatant, suspend pellet in 1 mL RPMI-1640 medium.
12. Determine total numbers of viable cells using hemocytometer, microscope with $40\times$ objective and Trypan Blue exclusion dye.
13. Adjust the cell concentration to 10–20 million viable cells/mL (see Note 18).
14. Add 100 μL of cell suspension into wells of 96-well flat-bottom plate (see Notes 2 and 13).
15. Centrifuge plate at $3\text{--}400 \times g$ for 5 min at 4°C in a tabletop centrifuge.
16. Decant or aspirate supernatants (see Note 14), lightly vortex plate to disrupt cell pellets.
17. Stain cells with appropriate staining cocktail(s):
 - a. If examining CS-specific CD8 responses using CS₂₅₂₋₂₆₀ or CS₂₈₀₋₂₈₈ MHC class I tetramer, cell staining should be performed sequentially:
 - i. First, add 50 μL of the optimally titrated tetramer reagent, mix with pipetting and incubate at RT, 4°C or on ice for 1 hour (see Note 15).
 - ii. Second, without washing cells directly add 100 μL of a cocktail of antibodies directed against cell surface antigens. The surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8 α , anti-mouse-CD11a, anti-mouse CD90; cell viability dye. If simultaneously examining CD4 T cells, the cocktail should also include anti-mouse-CD4 and anti-mouse-CD49d.
 - iii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.
 - b. If examining total CD8 and CD4 T cells responses, all reagents are added simultaneously:
 - i. Add 100 μL of a cocktail of antibodies directed against cell surface antigens. The

surface antigen antibody cocktail should include anti-mouse-CD16/CD32 (clone 2.4G2), anti-mouse-CD8 α , anti-mouse-CD11a, anti-mouse-CD4, anti-mouse-CD49d, anti-mouse CD90 and cell viability dye.

- ii. Mix with pipetting and incubate the plate at 4°C for 20–30 min.

18. Wash away unbound antibody and/or tetramer from wells by adding 150 μ l of FACS buffer directly to wells containing staining cocktail, spin plate at 3–400 $\times$ g for 5 min at 4°C.
19. Decant or aspirate supernatants, lightly vortex plate, add 100 μ l of Cytofix and incubate at 4°C for 7–10 min (see Note 16).
20. Wash away Cytofix from cells by adding 150 μ l of FACS buffer directly to wells containing 100 μ l of fixative, spin plate at 3–400 $\times$ g for 5 min at 4°C.
21. Decant or aspirate supernatants, lightly vortex, suspend cells in 180–200 μ l of FACS buffer, then store cells at 4°C protected from light until run on the flow cytometer.

3.2 Analysis

This section describes the collection and analysis of flow cytometric data to determine the frequency of antigen-experienced effector and memory CD8 and CD4 T cells following pre-erythrocytic vaccination. This protocol does not describe how to properly compensate samples on a flow cytometer (see Note 13).

1. Using an analytical flow cytometer program, draw a population gate around viable lymphocytes using forward-area (FSC-A) and side-area (SSC-A) scatter parameters (X- and Y-axis, respectively). This population gate excludes cell debris (or small, likely dead cells) from further analysis.
2. Optional—If the flow cytometer is capable of analyzing forward width scatter (FSC-W), draw a population gate around single cell ('singlet') lymphocyte populations using forward width (FSC-W) and side-area scatter (SSC-A); this gate excludes cell doublets from further analysis.
3. Draw a population gate on lymphocytes that are negative for staining with viability dye reagents. This gate further excludes dead cells and enhances the quality and resolution of data for downstream analyses of T cell phenotype and function.
4. For CD8 T cell analyses, draw a population gate around CD8+CD90+ cells by viewing CD8 α staining on the X-axis, and CD90.2 staining on the Y-axis. Next gate on the antigen-experienced (CD8 α ^{lo}CD11a^{hi}) CD8 T

cells by examining CD8 α expression and CD11a expression on the X- and Y-axis, respectively.

5. Optional—the fraction of CD8 α^{lo} CD11a $^{\text{hi}}$ CD8 T cells that are CS-specific can be determined by examining the fraction of antigen-experienced CD8 T cells that bind the MHC class I tetramer by viewing CD8 α on the X-axis and tetramer on the Y-axis (Figs. 1 and 2).
6. For CD4 T cell analyses, draw a population gate around CD4+CD90+ cells by viewing CD4 staining on the X-axis, and CD90.2 staining on the Y-axis. Next gate on the antigen-experienced (CD11a $^{\text{hi}}$ CD49d $^{\text{hi}}$) CD4 T cells by examining CD11a expression and CD49d expression on the X- and Y-axis, respectively (Fig. 2).
7. Additional parameters of T cell phenotype and function and be analyzed by including additional reagents in the cell staining cocktails, assuming the analytical flow cytometer can accommodate the extra channels required (i.e. appropriate lasers and filter sets).

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Figure 1.

Peripheral blood from BALB/c mice was collected before (naïve) and 7 and 61 days after vaccination with 20,000 *P. berghei* radiation attenuated sporozoites (*Pb*-RAS). Flow cytometric analyses were conducted to determine the fraction of vaccine-induced CD8 T cells (CD11a^{hi}CD8α^{lo}) that were specific for the dominant CD8 T cell epitope that derives from the parasite circumsporozoite (CS) protein (amino acids 252–260). Cells were processed and stained with MHC class I tetramer (K^d/CS₂₅₂) followed by anti-CD90.2, anti-CD8α and anti-CD11a as described in the text. Numbers refer to the fraction of cells within each defined population gate. These examples highlight that MHC class I tetramer reagents can identify epitope-specific CD8 T cells induced by pre-erythrocytic vaccination, enabling subsequent examination of measures of phenotype or function. These data also show that the vast majority (>80%) of the CD8 T cells induced by RAS are specific for undefined or unknown epitopes, further underscoring the utility of surrogate approaches to measure the quantity and quality of the total T cell response following pre-erythrocytic vaccination.

Figure 2.

The spleen from a C57BL/6 mouse was collected 7 days after vaccination with 20,000 *P. yoelii* radiation attenuated sporozoites (*Py*-RAS). Flow cytometric analyses were conducted to determine the fraction of CD8 and CD4 T cells that were activated by the whole attenuated parasite vaccine and recruited into the anti-*Plasmodium* immune response. Antigen-experienced CD8 T cells are CD11a^{hi}CD8α^{lo} and antigen-experienced CD4 T cells are CD11a^{hi}CD49d^{lo} and are easily distinguished from naïve CD8 and CD4 T cells. Numbers refer to the fraction of cells within each defined population gate.