Chapter 3

Evaluation of T Cell Function in Allergic Disease

Brianne R. Barker

Abstract

T lymphocytes play positive and negative roles in the pathogenesis of allergic disease. Isolation and functional characterization of T lymphocyte subpopulations is an important aspect of understanding allergy models and allergy therapies. Measurement of the T cell surface proteins and T cell proliferation can provide insight into T cell activation. T cell function and the identities of T cell subsets can be determined by measuring cytokine production, either via intracellular cytokine staining or ELISPOT. This chapter outlines protocols for T cell isolation as well as the evaluation of surface protein expression, proliferation, intracellular cytokine staining, and ELISPOT.

Key words T cells, Allergy, Flow cytometry, Intracellular cytokine staining, ELISPOT, CFSE

1 Introduction

Allergy is classically defined as a misdirected Th2-type response directed against noninfectious environmental stimuli, suggesting that the understanding of Th2 responses in any model of allergic disease is critical [1, 2]. Measurement of the production of Th2 cytokines including IL-4, IL-5, IL-9, IL-13, and GM-CSF is particularly important in assessing allergic disease [2]. These cytokines function to modulate many of the other effector cell populations acting during an allergic response. However, T cell subpopulations other than Th2 cells can also contribute to the development of or protection from allergic disease. IL-9, originally described as a Th2 cytokine, has more recently been shown to be a product of Th9 cells, which may also produce IL-10 [3]. The production of IL-17 by Th17 cells has been shown to contribute to some forms of allergy [4]. The balance between Th1 and Th2 cells is thought to be dysregulated in allergy, thus resulting in a decrease in Th1 cytokines in allergic disease [5]. Based on these data, therapies to induce immune deviation towards the Th1 phenotype are being attempted to ameliorate allergic disease [6]. In addition, regulatory T cells

Irving C. Allen (ed.), *Mouse Models of Allergic Disease: Methods and Protocols*, Methods in Molecular Biology, vol. 1032,
DOI 10.1007/978-1-62703-496-8_3, © Springer Science+Business Media, LLC 2013

may play a role in protection from allergic disease, likely via their production of TGF-β or IL-10 [7, 8]. NK T cells may also play a role in allergy [2]. Thus, the evaluation of T cells and their function are particularly important in models of allergic disease.

Before measuring T cell function, these cells must be isolated from the mouse. Protocols to isolate leukocytes from peripheral blood, spleen, and lymph nodes are presented below. These organs allow the assessment of systemic immune responses and responses in the draining lymph node. As T cell trafficking to effector sites also plays a key role in the progression of allergic disease, many investigators will also want to isolate T cells from unique organs of interest, such as the skin or the lungs [9]. T cells isolated from these organs may be used in a diverse range of assays; however, the number of isolated cells may be limited. Secondary lymphoid organs, particularly the spleen, allow for the isolation of large numbers of cells that can be used for all of the assays described below. The large numbers of cells recovered from these organs reduce the numbers of mice needed for each assay. All of these protocols result in the isolation of total leukocytes from the organs, which should be kept in mind during subsequent assays. The use of T cell-specific stimuli or flow cytometric analysis of T cell-specific surface proteins is often necessary to ensure specificity of the responses measured. Likewise, magnetic bead-based selection protocols may be used to isolate T cells from these bulk cell populations, but these protocols should be based on negative selection to avoid background activation of T cells before analysis. These selection procedures should be tested to ensure that they result in high yields of unactivated T cells.

The simplest way to evaluate T cells in an allergic model is via surface staining and flow cytometric analysis. This technique allows for the simple measurement of the proportion of T cells or specific T cell subsets among the isolated leukocytes possibly from an effector site or measurement of absolute cell numbers when combined with cell counts. The expression of specific trafficking molecules or activation molecules associated with individual disease phenotypes is often of interest as well [2, 5, 9]. This simple method also underlies some of the more complex protocols that follow. T cell activation can also be measured by examining T cell proliferation, here presented as the measurement of carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution using a flow cytometer. Not only is the measurement of T cell proliferation important for understanding T cell activation, but this technique also allows for an understanding of regulatory T cell activity. Regulatory T cells can control the proliferation and responsiveness of conventional T cells as well as regulate high-dose tolerance in allergy treatment models [6, 7]. In this technique, isolated cells are labeled with the cell-permeant dye CFSE, which labels all cellular proteins. These cells are then stimulated with peptide antigen or other T cell-specific stimuli in in vitro culture. At varying time points following stimulation, cells are removed from culture and analyzed by flow cytometry and

CFSE fluorescence is measured. Lower levels of CFSE fluorescence indicate more rounds of cell division. This method has an advantage over other proliferation assays, including ^{3}H-thymidine incorporation or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) metabolism assays in that surface staining for other T cell-specific markers can be used to ensure that the proliferation of T cells is being measured.

The diverse T cell subpopulations described above are most commonly distinguished based on cytokine production. Two common methods to measure cytokine production are presented here: intracellular cytokine staining (using flow cytometry) and ELISPOT. These methods allow for measurement of cytokines from specific cell populations or following specific stimulation, unlike cytokine ELISAs of serum, which only provide information about systemic cytokine levels. Both intracellular cytokine staining and ELISPOT can be performed on cells taken directly ex vivo or on cells that are stimulated with a specific antigen to measure in vitro cytokine production. Intracellular cytokine staining involves the utilization of cell permeabilization techniques and flow cytometry. This technique can be combined with cell surface staining to allow for the determination of cytokine production from specific T cell subsets or for the conservation of experimental animals. These permeabilization techniques can also be modified for the detection of signal transduction molecules and transcription factors like FoxP3 [10]. Intracellular cytokine production evaluation techniques have the advantage of providing cell subpopulation-specific data regarding cytokine production. ELISPOT involves culturing cells in multiscreen plates onto which cytokines may be secreted. Secreted cytokines are then measured via an ELISA-like protocol. ELISPOT has the advantage of providing data on how many cells in a population are producing a cytokine of interest. The user should decide which of these two techniques are more appropriate for measuring the levels of different cytokines in their studies based on each assay's sensitivities and unique advantages.

2 Materials

2.1 Harvesting Leukocytes from Peripheral Blood

1. Mice to be assessed.
2. Blood collection media: RPMI 1640 with 40 units/ml of heparan sulfate (*see* **Note 2**).
3. Lympholyte M or other types of Ficoll (*see* **Note 3**).
4. 15 ml polystyrene conical tubes.
5. Equipment for the collection of mouse blood (*see* **Note 4**).
6. Centrifuge capable of spinning 15 ml conical tubes.
7. Sterile and pyrogen-free PBS with 2 % fetal bovine serum (sterile filtered) (*see* **Notes 1** and **5**).

2.2 Harvesting Leukocytes from Spleen and Lymph Nodes

1. Mice to be assessed.
2. Equipment to euthanize mice (*see* **Note 6**).
3. Sterile surgical instruments: Forceps; scissors; dissection tray; and dissection pins. Jeweler's forceps may be particularly useful to isolate lymph nodes.
4. 70 % ethanol.
5. Spleen/lymph node collection media: Hanks' Balanced Salt Solution with 4 % fetal bovine serum and 10 mM of HEPES (sterile filtered).
6. Lympholyte M or other types of Ficoll (*see* **Note 3**).
7. 15 ml polystyrene conical tubes.
8. Centrifuge capable of spinning 15 ml conical tubes.
9. Sterile and pyrogen-free PBS with 2 % fetal bovine serum (sterile filtered) (*see* **Note 5**).
10. Hemocytometer and trypan blue (optional).
11. Light microscope.
12. 100 μm cell strainer per mouse (*see* **Note 7**).
13. Plunger from a 1 cc syringe per mouse (*see* **Note 7**).
14. Petri dishes or 6-well tissue culture plates (*see* **Note 7**).
15. R10 media: RPMI 1640 plus 10 % fetal bovine serum, 1 % MEM nonessential amino acids, 0.1 % β-mercaptoethanol, 1 % sodium pyruvate, and 1 % penicillin/streptomycin.

2.3 Surface Staining

1. 12 × 75 mm 5 ml test tubes (*see* **Note 8**).
2. Centrifuge capable of spinning 5 ml conical tubes.
3. Sterile and pyrogen-free PBS with 2 % fetal bovine serum (sterile filtered) (*see* **Note 5**).
4. Ca- and Mg-free PBS or Ca- and Mg-free PBS with 2 % formaldehyde (*see* **Note 9**).
5. Flow cytometer and flow cytometry analysis software.
6. Fluorescence-conjugated antibodies against surface molecules of interest.
7. Vortex.

2.4 Proliferation

1. All materials listed in Subheading 2.3 for surface staining.
2. Hanks' Balanced Salt Solution.
3. 15 ml conical tubes.
4. CFSE. Stock solutions should be generated at 1 mM CFSE in DMSO. This solution is frozen at −20 °C (*see* **Note 10**).
5. R10 media: RPMI 1640 plus 10 % fetal bovine serum, 1 % MEM nonessential amino acids, 0.1% β-mercaptoethanol, 1 % sodium pyruvate, and 1 % penicillin/streptomycin.

6. Rat IL-2.
7. 37 °C incubator.
8. Specific antigenic peptide or overlapping peptide pools.
9. Anti-CD3ε (BD Biosciences).
10. Round-bottom 96-well tissue culture plates.

2.5 Intracellular Cytokine Staining

1. All materials listed under Subheading 2.3 for surface staining.
2. Stimulation media (*see* **Note 11**): RPMI 1640 plus 10 % fetal bovine serum, 1 % MEM nonessential amino acids, 0.1 % β-mercaptoethanol, 1 % sodium pyruvate, 1 % penicillin/streptomycin, 2 μg/ml of anti-CD28 (azide free; BD Biosciences), and 2 μg/ml of anti-CD49d (azide free; BD Biosciences).
3. Fluorescence-conjugated antibodies against cytokines or other intracellular molecules of interest (*see* **Note 12**).
4. Phorbol 12-myristate 13-acetate (PMA).
5. Ionomycin.
6. Specific antigenic peptide or overlapping peptide pools.
7. Cytofix/Cytoperm solution (BD Biosciences). This is a fixation/permeabilization buffer containing formaldehyde and saponin for cell permeabilization.
8. Brefeldin A or Monensin (BD Biosciences).
9. 37 °C incubator.
10. Perm/Wash Buffer (BD Biosciences). This is saponin-containing wash buffer to aid in saponin-based permeabilization.

2.6 ELISPOT

1. PMA.
2. Ionomycin.
3. Specific antigenic peptide or overlapping peptide pools.
4. 96 well multiscreen plates (i.e., Millipore Immobilon-P PVDF plates).
5. Ca- and Mg-free PBS (sterile and pyrogen-free).
6. Anti-cytokine antibodies for coating plates (i.e., anti-IL-4).
7. PBS containing 0.25 % Tween 20 (PBS/Tween).
8. PBS containing 10 % fetal bovine serum.
9. Multichannel pipette.
10. 96-well plate washer (optional).
11. 37 °C incubator.
12. Distilled water.
13. Biotinylated anti-cytokine antibody (i.e., biotinylated anti-IL-4).
14. Streptavidin alkaline phosphatase.

15. Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) chromogen solution (Pierce).
16. Automated ELISPOT reader and image processing software. Commonly used readers are from Hitech Instruments or CTL Analyzers LLC. Commonly used software packages are Image-Pro Plus image processing software (Media Cybernetics) or CTL software.

3 Methods

3.1 Harvesting Leukocytes from Peripheral Blood

1. Fill one 15 ml conical tube with 3 ml blood collection media per mouse (*see* **Notes 2** and **4**).
2. Collect peripheral blood and immediately place in a 15 ml tube with blood collection media.
3. Underlay 1 ml Lympholyte M with a 2 ml pipette (*see* **Notes 3** and **13**).
4. Centrifuge at 1,875 × *g* for 20 min without brake.
5. Remove the cell layer from the Lympholyte M and add this layer to 10 ml of PBS/2 % FCS to a new 15 ml conical tube.
6. Centrifuge at 500 × *g* for 10 min.
7. Aspirate and resuspend the resulting cell pellet for downstream applications (*see* **Note 14**).

3.2 Harvesting Leukocytes from Spleen and Lymph Nodes

1. Fill one 15 ml conical with 5 ml of spleen/lymph node collection media per mouse per organ to be isolated.
2. Euthanize mice one at a time and isolate the spleen or the lymph nodes from each mouse immediately after sacrifice (*see* **Note 6**). Immediately place the organ in a 15 ml tube with spleen/lymph node collection media. Collect all organs from all mice and place them on ice before proceeding to the next step.
3. Gently homogenize the spleen or the lymph nodes through the 100 μm cell strainer into a small Petri dish using the plunger from the 1 cc syringe until a single-cell suspension is produced. Pipette the single-cell suspension into a 15 ml conical tube. Wash the strainer, plunger, and dish with 5 ml of mouse R10 and add to the same conical tube (*see* **Note 7**).
4. Centrifuge at 500 × *g* for 5 min.
5. Underlay 1 ml of Lympholyte M with a 2 ml pipette (*see* **Notes 3** and **13**).
6. Centrifuge at 1,875 × *g* for 20 min without brake.
7. Remove the cell layer from the Lympholyte M and add this layer to 10 ml of PBS/2 % FCS tubes in a new 15 ml conical tube.

8. Centrifuge at 500 × *g* for 10 min.
9. Aspirate and resuspend the resulting cell pellet in 10 ml of 2 % PBS/2%FCS and count cells for downstream applications (*see* **Note 14**).

3.3 Surface Staining

1. Transfer at least 1×10^6 cells per sample to 12 × 75 mm 5 ml test tubes. Adjust the volume to 100 µl of PBS/2 % FCS. Also transfer at least 1×10^6 cells to a 12 × 75 mm 5 ml test tube to use for unstained controls, single-color controls, and possibly FMO controls. Adjust the volume to 100 µl with PBS/2 % FCS (*see* **Note 15**).
2. Prepare a cocktail containing the appropriate amounts of all of the surface staining antibodies before staining and add this cocktail to the cells (*see* **Notes 16** and **17**). Add individual diluted antibodies to the single-color control tubes. Vortex all samples and incubate them for 30 min on ice in the dark.
3. To wash, add 3 ml of PBS/2 % FCS to each tube and centrifuge at 500 × *g* for 5 min.
4. Aspirate and resuspend in 500 µl of PBS or PBS/2 % formaldehyde while vortexing to reduce clumping (*see* **Note 9**). Store the cells at 4 °C until analyzing on the flow cytometer.

3.4 Proliferation

1. Add 10×10^6 cells to a 15 ml conical tube. Wash cells twice with 10 ml of HBSS (*see* **Notes 18** and **19**). Resuspend 10×10^6 cells/900 µl in HBSS with no serum. Be sure to set up additional cells for unstained and single-color controls for later flow cytometry analysis.
2. Incubate in HBSS with 1 µM of CFSE for 30 min at 37 °C. Mix by flicking with finger vigorously, but not vortexing.
3. After the 30-min incubation, wash cells twice with R10 media (*see* **Note 18**).
4. Resuspend cells at 1.5×10^6 cells/ml in R10 media. Plate the cells in a round-bottom 96-well plate at 200 µl/well. Add 100 ng/ml of peptide antigen or anti-CD3 as a control (*see* **Note 20**). Be sure to set up additional wells without peptide as a control.
5. Add 25 U/ml rat IL-2 on day 2 of culture (*see* **Note 21**).
6. Take cells for staining as desired (anytime between day 0 and day 8) and utilize the surface staining protocol described under Subheading 3.3.

3.5 Intracellular Cytokine Staining

1. Transfer at least 4×10^6 cells per sample to 12 × 75 mm 5 ml test tubes (*see* **Note 8**). Adjust the volume to 500 µl of stimulation media. Also transfer at least 4×10^6 cells to a 12 × 75 mm 5 ml test tube to utilize as controls for staining per experiment

(unstained controls, single-color controls, and possibly FMO controls) and controls for stimulation per sample (unstimulated, antigen stimulated, and PMA/ionomycin stimulated). Adjust the volume to 500 μl of stimulation media (*see* **Notes 11**, **15**, and **20**).

2. Add 1 μl of Golgi-stop to each sample. Add 1 μg of antigenic peptide or peptide pool to each sample to be stimulated with antigen. Add 0.5 μg of PMA and 2.5 μg of ionomycin to each positive control (PMA/ionomycin sample).
3. Vortex cells, place caps on loosely, and incubate for 6 h at 37 °C (*see* **Note 22**).
4. Add 3 ml of PBS/2 % FCS to each tube and centrifuge at 500 × *g* for 5 min to wash. Adjust the volume to 100 μl of PBS/2 % FCS.
5. Prepare a cocktail containing the appropriate amounts of all of the surface staining antibodies before staining and add this cocktail to the cells (*see* **Note 17**). Add individual diluted antibodies to the single-color control tubes. Vortex all samples and incubate them for 30 min on ice in the dark.
6. Add 3 ml of PBS/2 % FCS to each tube and centrifuge at 500 × *g* for 5 min to wash. **Steps 4–6** are optional and are only necessary if you are interested in staining for surface antigens in addition to intracellular antigens.
7. Vortex each sample. Add 500 μl of Cytofix/Cytoperm to each sample while vortexing. Incubate at room temperature for 45 min (*see* **Note 22**).
8. Vortex each sample. Add 2 ml of perm/wash buffer. To wash, centrifuge the samples at 800 × *g* for 7 min. Aspirate and wash with another 2 ml of perm/wash buffer.
9. Vortex each sample. Prepare a cocktail containing the appropriate amounts of all of the intracellular staining antibodies before staining and add this cocktail to the cells (*see* **Notes 12** and **17**). Vortex all of the samples and incubate them for 30 min on ice in the dark.
10. Add 2 ml of perm/wash buffer. Centrifuge at 800 × *g* for 7 min to wash.
11. Aspirate and resuspend the cells in 500 μl of PBS or PBS/2 % formaldehyde while vortexing to reduce clumping (*see* **Note 9**). Store the cells at 4 °C until ready to analyze using a flow cytometer.

3.6 ELISPOT

1. Coat 96-well multiscreen plates with 100 μl per well of 5 μg/ml anti-cytokine antibody diluted in PBS. Incubate plates overnight.
2. Wash plates three times with PBS containing 0.25 % Tween 20 (PBS/Tween) (*see* **Note 23**).

3. Add 200 μl per well of PBS containing 10 % fetal bovine serum to block. Incubate the plate for 2 h.
4. Add 2×10^5 cells and the appropriate antigenic peptides (1 μg/ml) or other stimuli to each well. It is advisable to set up triplicate wells for each condition. Unstimulated cells should also be included in the assay to allow for an assessment of background cytokine production. Incubate the plate for 18 h at 37 °C (*see* **Note 20**).
5. Wash the plates nine times with PBS/Tween and once with distilled water.
6. Add 2 μg of biotinylated anti-cytokine antibody diluted in PBS to a total volume of 100 μl per well. Incubate the plate for 2 h at room temperature.
7. Wash the plate six times with PBS/Tween.
8. Incubate the plate with a 1:500 dilution (100 μl total volume per well, dilute in PBS) of streptavidin alkaline phosphatase for 2.5 h.
9. Wash the plate five times with PBS/Tween and once with PBS only.
10. Develop the plate by adding NBT/BCIP chromogen solution. Stop the reaction once color has developed with tap water and air-dry the plate.
11. Read the pate with an automated ELISPOT reader and quantitate the number of spots apparent using appropriate software. Data from an ELISPOT assay are usually expressed as spot-forming cells (SFC) per 10^6 cells added to the well and compared to background levels seen in unstimulated cells.

4 Notes

1. Prepare all solutions using ultrapure water and pyrogen-free, tissue culture-grade reagents. Store all reagents at 4 °C unless indicated otherwise. Pay careful attention to waste disposal recommendations of your institution; some prepackaged kits or reagents contain preservatives that may require special collection and disposal. We perform all assays using sterile technique in a laminar flow hood, although terminal assays in which cells will not be cultured may be performed on a bench top. Be sure to wear appropriate personal protective equipment throughout the procedure.
2. Blood should be collected in the presence of anticoagulant; however, we have used anticoagulants other than heparin in our blood collection media with generally good results. Calcium chelators such as EDTA can adversely affect some

functional assays including intracellular cytokine staining and should be tested carefully.

3. We use Lympholyte M as a standard way of separating peripheral blood mononuclear cells from other cell types found in peripheral blood or secondary lymphoid organs. We find that this technique provides the cleanest cell population without debris that can cause problems in cytometry. Instead of using Lympholyte M or another type of Ficoll, it is possible to lyse the red blood cells in a single-cell suspension with NH_4Cl lysis buffer (ACK). Further, some tissues, particularly spleen, contain large numbers of red blood cells and cell preparations from these tissues may be improved by adding an ACK lysis step following Lympholyte treatment. ACK buffer is made as follows: Add nine parts 0.16 M NH_4Cl to 1 part of 0.17 M Tris base pH 7.65 and then adjust the pH of the resulting solution to 7.2 and sterile filter. The ACK lysis protocol is as follows:
   (a) Centrifuge heparinized blood or single-cell suspension generated from spleen or lymph node.
   (b) Add approximately 5 ml of ACK solution to the cell pellet. The specific amount will vary based on the number of cells.
   (c) Invert tubes to mix well and incubate for approximately 3 min. The amount of time will also vary based on the number of cells and tissue. Do not overlyse.
   (d) Centrifuge tubes immediately to remove ACK buffer. Overlysis of cells can cause problems with surface staining and functional assays, or can result in poor cell yield.
4. Appropriate techniques for the collection of mouse blood vary among institutions and IACUC committees. We have successfully used blood obtained via retroorbital (generally disfavored among IACUC committees), submandibular, and cardiac puncture routes. Submandibular blood collection utilizing Goldenrod Animal Lancets can be performed on live mice and allows the investigator to follow the same mice throughout the course of disease but results in smaller volumes of blood for experimentation. Blood volume may be replaced with Ringer's lactate solution. Blood collection via cardiac puncture results in larger volumes of blood for experimentation but does not allow mice to be followed.
5. Throughout this protocol, PBS with 2 % fetal bovine serum can be substituted with PBS with BSA.
6. Appropriate techniques for mouse euthanasia vary among institutions and IACUC committees.
7. There are multiple methods of dissociating spleens and lymph nodes to generate single-cell suspensions. We tend to use syringe plungers and disposable cell strainers in either Petri

dishes or wells of 6-well tissue culture plates. We have also used syringe plungers or autoclavable glass rods with autoclavable mesh screens in a similar fashion to the method described here or homogenized spleens between two frosted glass slides.

8. Flow cytometry staining and analysis are traditionally performed in 5 ml test tubes. However, the use of these tubes may be cumbersome when staining large numbers of samples. Alternatively, we have stained cells in round-bottom 96-well plates or strips of PCR tubes. Either of these methods allows for the use of a multichannel pipette. Both alternatives require additional wash steps as cells cannot be washed with large volumes of liquid. A centrifuge capable of spinning 96-well plates is also necessary. Cells can then be transferred into 5 ml test tubes for analysis or may be analyzed directly in a 96-well plate if appropriate flow cytometer hardware is available.
9. We fix our flow cytometry samples with formaldehyde as a standard procedure. Cells do not need to be fixed and can be resuspended in PBS alone provided there are no biosafety concerns and the samples will be analyzed on a flow cytometer immediately. Formaldehyde fixation can alter some fluorophores, so fixed and unfixed samples should not be compared.
10. When attached to proteins, the emission and excitation peaks of CFSE are 492 and 517 nm and it is typically read in the FITC channel. CFSE should be carefully titrated to ensure that spillover into other channels does not occur. Similar compounds with different emission and excitation peaks (i.e., Molecular Probes, Cat. No. 34557) have been developed and allow good results.
11. Stimulation media is the R10 media listed above with the addition of purified, azide-free anti-CD28 and anti-CD49d antibodies. These antibodies allow for co-stimulation of T cells during antigenic stimulation to result in optimal cytokine production. The specific antibodies used in this stimulation media, particularly with regard to the use of CD49d, vary among investigators. If direct ex vivo cytokine analysis is desired, this media is not necessary.
12. The protocol presented here is specific for staining for intracellular cytokines. Other intracellular molecules, particularly transcription factors like the regulatory T cell transcription factor FoxP3 or other signal transduction molecules, can also be assessed by flow cytometry. The protocols for these techniques are generally similar to those presented here with two changes: in vitro cell stimulation is not used and alcohol-based permeabilization methods are sometimes necessary. Specific protocols vary for individual signaling molecule and often require optimization.

13. Lympholyte M and other Ficolls are sucrose solutions that allow for cell separation based on density. Lympholyte M should be stored at 4 °C to prevent contamination once it has been opened. Lympholyte M should then be at room temperature when used to separate cells to ensure that it is at the correct density.
14. Cells at this stage can be stored for short periods of time at 4 °C or may be cryopreserved for later use. Cryopreservation may influence cell performance in functional assays.
15. Flow cytometry experiments require unstained cell controls and controls stained with each of the antibodies individually for setting voltages and compensation. We often stain compensation control beads instead of cells for our single-color controls (BD Biosciences 552843 or 552845 depending on the antibody isotype). This allows conservation of cells and measurable staining with even those antibodies that stain rare populations. Unstained cells are still required to properly set up the flow cytometer. For complex experiments, we also use fluorescence minus one (FMO) gating controls. In an FMO control, all antibodies in a panel except for one are used to stain cells. This aids in setting negative gates [11].
16. We sometimes include MHC–peptide tetramers to allow for staining of antigen-specific T lymphocytes. If staining with tetramers, the protocol should be modified as follows:
    (a) Add appropriate amount of tetramer to cells to stain. Vortex all samples and incubate them for 30 min on ice in the dark.
    (b) Prepare a cocktail containing appropriate amounts of all of the surface staining antibodies before staining and add this cocktail to cells to stain. Add individual diluted antibodies to single-color control tubes. Vortex all samples and incubate them for 30 min on ice in the dark.

    Tetramers should always be added before antibodies, particularly anti-CD3, to allow tetramer access to TCR without hindrance from other antibodies.
17. The concentrations listed on the data sheets included with antibodies are often a useful place to start with staining. Antibodies can usually be further diluted and careful titration can save on reagents and allow for cleaner staining.
18. CFSE is a cell-permeant dye that labels proteins. Staining must be performed in serum-free media to ensure that cellular proteins and not serum proteins are labeled. Once staining is complete, the cells should be washed with a large volume of media containing serum to quench the staining reaction. We have used different types of media (HBSS, PBS, RPMI) for the staining reaction with no adverse effects as long as the staining

was in a serum-free media and the wash was in a serum-containing media.

19. The CFSE staining protocol listed utilizes large numbers of cells. It is also possible to stain smaller numbers of cells with protocol modifications including adding serum to the staining reaction. This helps the cells survive the toxicity associated with CFSE staining.
20. Stimulation of all T cells in a mixed population can be achieved with azide-free anti-CD3 antibody or a mixture of PMA/ionomycin. PMA/ionomycin is preferred for short-term cytokine production but does result in substantial cell death. Anti-CD3 is preferred for proliferation of cells in culture but must be azide-free to allow cells to proliferate. These reagents are useful positive controls to determine the maximum capacity of your cells to produce cytokine or proliferate as compared to cells stimulated with specific antigen. Unstimulated cells are also important negative controls. Stimulation is not necessary when measuring cytokine production directly ex vivo and stimulation steps may be excluded in that case.
21. Rat IL-2 allows for optimal T cell survival in culture.
22. Intracellular cytokine staining is a lengthy procedure. We have had good luck with the following modifications in order to spread the procedure over two days:

    Protocol 3.5, ***step 3***: Utilize a heat block on a timer to incubate at 37 °C for 6 h, followed by cooling to 4 °C until the next day or manually moving cells to 4 °C following the 6-h incubation.

    Protocol 3.5, ***step 7***: Incubate at 4 °C overnight.
23. While using multichannel plates and plate washers makes the ELISPOT protocol less laborious, they are also the cause of many problems with the protocol. Be sure not to touch the membrane with your pipette tips or the plate washer. Multiscreen plates are generally quite sensitive and should be treated with care.

References

1. Palm NW, Rosenstein RK, Medzhitov R (2012) Allergic host defenses. Nature 484: 465–472
2. Holgate ST (2012) Innate and adaptive immune responses in asthma. Nat Med 18: 673–683
3. Jabeen R, Kaplan MH (2012) The symphony of the ninth: the development and function of Th9 cells. Curr Opin Immunol 24:303–307
4. Wang Y, Wills-Karp M (2011) The potential role of interleukin-17 in severe asthma. Curr Allergy Asthma Rep 11:388–394
5. Galli SJ, Tsai M, Piliponsky AM (2008) The development of allergic inflammation. Nature 454:445–454
6. Maggi E (2010) T cell responses induced by allergen-specific immunotherapy. Clin Exp Immunol 161:10–18

7. Hawrylowicz CM, O'Garra A (2005) Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 5:271–283
8. Lloyd CM, Hawrylowicz CM (2009) Regulatory T cells in asthma. Immunity 31:438–449
9. Islam SA, Luster AD (2012) T cell homing to epithelial barriers in allergic disease. Nat Med 18:705–715
10. Krutzik PO, Irish JM, Nolan GP, Perez OD (2004) Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. Clin Immunol 110:206–221
11. Roederer M (2002) Compensation in flow cytometry. Curr Protoc Cytom Chapter 1 Unit 1.4