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Marion Espéli
Michelle Linterman *Editors*

T Follicular Helper Cells

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Preface

After an infection or after deliberate immunization, high-affinity antibody-secreting plasma cells and memory B cells are generated that are able to provide protection against subsequent infection. The production of these effector cells occurs in a specialized microenvironment called the germinal center. The main cellular constituents of the germinal center are rapidly dividing B cells, which are subjected to somatic hypermutation of their immunoglobulin variable region genes. This process can change the affinity, or alter the specificity, of the B cell receptor for antigen which means that newly mutated B cells must undergo a process of selection to ensure that the germinal center produces effector B cells with an increased affinity for foreign antigen, but which do not bind self-antigens. Evidence has accrued over the last 20 years that has identified a specialized subset of CD4+ T cells called T Follicular helper (Tfh) cells that are crucial for the initiation and regulation of the germinal center response and for affinity-based selection of germinal center B cells.

The aim of this book is to bring together the techniques and protocols that have facilitated breakthroughs in Tfh cell biology and to present them to immunologists in a way that will enable both young and experienced researchers to continue to move this exciting field forward.

Tfh cells were first identified in human tonsil germinal centers in 1983 and since this initial discovery the field has grown rapidly. The discovery that the cell surface proteins CXCR5, CD57 (in humans), and PD-1 could be used to identify Tfh cells by flow cytometry enabled these cells to be phenotyped and isolated for multiple downstream applications such as transcriptomics, cell culture, and adoptive transfer experiments. This research paved the way for the use of genetic and viral tools to dissect the molecules and pathways important for Tfh formation and function.

Because the germinal center is a dynamic structure that currently cannot be recapitulated in vitro, analysis of Tfh cells in vivo has been critical for ascertaining their function. In vivo microscopy has facilitated the visualization of the germinal center response in real time and has illuminated the dynamics of the Tfh response and the interactions of these cells with B cells in situ. Moreover, techniques that allow determination of the quality and quantity of the B cell response from the germinal center have been critical for our understanding of Tfh function.

In the last 5 years, the heterogeneity of Tfh cells has been explored in greater depth and new subsets have been identified and characterized. Natural Killer Tfh and T Follicular regulatory cells have been added to the list of cells contributing to the regulation of the germinal center response, and new protocols to study their frequency and function have been developed.

The essential role played by Tfh cells in response to vaccination and their implication in autoimmune diseases mean that an understanding of these cells is highly relevant to human health. The investigation of Tfh biology in humans has been aided by the identification in the peripheral blood of a population of Tfh-like cells which resemble Tfh in many aspects of their phenotype and function. The analysis of circulating Tfh-like cells, combined with

cell culture and analysis of canonical germinal center Tfh cells from human secondary lymphoid tissue, has revealed a central role for Tfh cells in human immunodeficiency, autoimmunity, and infection.

This volume of MiMB brings together the skills and protocols of numerous laboratories that are at the heart of investigation into the biology of Tfh cells, in both mice and humans. It is the expertise and dedication of the contributing authors that has made this timely book possible, and it is our hope that it will help new investigators to delve further into this fascinating field in the future.

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Part I

Mouse

Chapter 1

Identification of Mouse T Follicular Helper Cells by Flow Cytometry

Alexandre P. Meli and Irah L. King

Abstract

T follicular helper (Tfh) cells are a subset of $CD4^+$ T cells that accumulate in the B cell-rich regions of secondary lymphoid organs and provide activation signals essential for long-lived humoral immunity. Herein, we describe a flow cytometric cell-based approach to identify Tfh cells within the total leukocyte population isolated from the spleen, lymph nodes, and Peyer's patches of mice. This protocol focuses on markers that have established relevance in Tfh cell differentiation and function allowing its use across varied settings of infection and immunity.

Key words T follicular helper cell, Phenotype, Flow cytometry, Germinal center, Antibody, Murine, Lymphocyte, Secondary lymphoid organ

1 Introduction

T and B lymphocytes are integral components of the adaptive immune system capable of directing diverse, but specific responses to foreign and self-antigens in the context of immunization, infection, and autoimmune disease. In cases where antigen-specific antibodies contribute to the response, a subset of CD4+ T cells called T follicular helper (Tfh) cells migrate into the B cell-rich follicles of the secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches (and tonsils in humans) and provide direct co-stimulatory and cytokine signals to antigen-presenting B cells [1]. These signals result in a nucleus of proliferating B cells, an event called the germinal center reaction, from which antibody-secreting plasma cells and memory cells arise [2]. Indeed, selective deletion of the Tfh cell population will abrogate germinal center formation as well as halt the production of high-affinity antibodies [3]. Thus, Tfh cells are required for establishing long-lived humoral immunity.

Tfh cells are generally not found within secondary lymphoid organs at steady state, with the exception of Peyer's patches

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that receive a consistent source of antigen from the intestinal microbiota [4]. However, many different immunization strategies and infections can induce the differentiation of Tfh cells. This chapter will provide an explanation of the cell preparation process for detecting Tfh cells via flow cytometry by combining a panel of extracellular and intracellular markers that have been used across multiple settings to delineate and characterize this $CD4^+$ T cell subset. For example, Tfh cells express high levels of CXCR5, the obligate receptor for CXCL13, a chemokine highly expressed within the B cell follicle [5, 6]. Upregulation of CXCR5 is promoted by Bcl6 and Ascl2, transcription factors required for differentiation of Tfh cells [7–10]. In addition, programmed death (PD)-1, as well as other extracellular molecules such as the inducible co-stimulatory (ICOS) molecule, has also been shown to be upregulated by the Tfh population and required for their function [11, 12]. As no single marker identifies Tfh cells, a combination of the aforementioned molecules must be used to identify this cell subset.

It is important to note that although this protocol uses a variety of markers to identify Tfh cells ex vivo, a cellular phenotype is only as valid as the function that it represents. Thus, in addition to the methods provided here, assays to determine the localization and function of Tfh cells (e.g., immunofluorescence microscopy and cytokine secretion assays, methods which are described in other chapters of this book) compared to other T effector cell subsets should be performed in parallel to fully characterize the Tfh cell population in each experimental setting.

2 Materials

2.1 Reagents

1. Heat inactivated fetal bovine serum (hiFBS): Incubate fetal bovine serum for 30 min at 56 °C for complete inactivation prior to use.
2. Red blood cell (RBC) lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Dilute in distilled water and dilute pH to 7.3.
3. Cell processing buffer: Hank's buffered saline solution (HBSS), 2 % hiFBS, 10 mM Hepes.
4. FACS buffer: Dulbecco's phosphate-buffered saline solution (D-PBS), 2 % hiFBS, 10 mM Hepes.
5. Fixation/permeabilization solution: Mix one part Fixation/Permeabilization Concentrate (eBioscience) with three parts Fixation/Permeabilization Diluent (eBioscience) to the desired final volume. For example, for 16 cell samples, add 4 ml of Fixation/Permeabilization Concentrate to 12 ml of Fixation/Permeabilization Diluent.

Table 1
Antibody summary

Antigen	Fluorophore	Dilution	Clone
CD4	Pacific blue	1:400	GKI.5
B220	Fitc	1:250	RA3-6B2
CXCR5	Biotin	1:25	SPRCL5
PD-1	PE-Cy7	1:100	J43
Bcl6	PE	1:20	K112-91
Streptavidin	APC	1:100	
Viability dye	efluor 780	1:1000	
CD16/32 (Fc block)		1:1,000 from 0.5 μg/ml stock	2.4G2

The dilutions listed for the relevant antibodies and viability dye have been carefully titrated in our laboratory and have given us consistent results. However, the optimal antibody concentration may vary depending on the product lot and experimental setting

6. Permeabilization buffer: Dilute 10x permeabilization buffer (eBioscience) to a final 1× concentration in distilled water to desired volume. For example, add 1 ml of permeabilization buffer to 9 ml of distilled water.
7. 70 μm nylon cell strainers.
8. 5 ml syringes.
9. 1 ml plastic syringes.
10. 25 G needles.
11. 6-well flat-bottom plates.
12. 15 ml polypropylene conical centrifuge tubes.
13. 5 ml polystyrene round bottom tubes.

2.2 Antibodies and Dilutions (See Note 1, Table 1)

1. In order to use the combination of fluorophores indicated above, a flow cytometer equipped with a red, blue, and violet laser is needed. To accommodate instruments with different lasers and/or detectors, alternative fluorophores may be used.

2.3 Fluorescence-Activated Cell Sorting Instrument and Analysis Software

2. FACS data must be analyzed using compatible software platforms such as FACSDiva (BD Biosciences) or FlowJo (Treestar, Inc.).

3 Methods

3.1 Cell Preparation (See Note 2)

1. Prepare 6-well plates containing 4 ml of cell processing buffer and add a 70 μm cell strainer to each well. One organ should be allocated to each well.
2. Isolate your organ of interest, such as lymph nodes or spleen, and place them into a 70 μm cell strainer within a well. Ensure that the organ is submerged in cell processing buffer until the animal dissection is complete.
3. For spleen processing only, hold the organ with a pair of forceps over the cell strainer in the 6-well plate, and infuse 1 ml of cell processing buffer directly into one end of the spleen using a 25 G needle. Successful perfusion of the spleen will result in a blanching of the organ throughout its length. Performing this step prior to crushing the organ into a single cell suspension will increase cell viability and yield.
4. For both spleens and lymph nodes, use the rounded side of a plunger from a 10 ml syringe to thoroughly crush the tissues through the cell strainer to generate single cell suspensions.
5. Remove the 70 μm cell strainer and pipette the cell processing buffer now containing your lymphocytes from each well into a 15 ml conical tube.
6. Rinse each well with an additional 1 ml of cell processing buffer and add to the same conical tube.
7. Centrifuge at 400 × *g* for 5 min (min) at 4 °C to wash.
8. For lymph nodes: Aspirate supernatant and resuspend cell pellet in 2–6 ml of cell processing buffer depending on the size of the tissue. Lymph node cells are ready for counting. As a general rule, cells should be resuspended at a concentration of approximately ten million cells/ml for accurate counting.
9. For spleens: Aspirate supernatant and resuspend cell pellet in 2 ml of RBC lysis buffer.
10. Incubate splenocytes for 5 min on ice while mildly vortexing tubes every minute.
11. Add 2 ml of cell processing buffer to splenocyte suspension and centrifuge at 400 × *g* for 5 min at 4 °C to wash.
12. Aspirate the supernatant and resuspend splenocytes in 8–10 ml of cell processing buffer. Splenocytes are ready for counting.

3.2 Cell Staining (See Note 2)

1. After determining the cell number recovered from each organ, add 2–4 million cells from each organ to a 5 ml polystyrene round bottom tube for antibody labeling.
2. Wash the cells with 2 ml of 1× D-PBS. Centrifuge at 400 × *g* for 5 min at 4 °C. Aspirate supernatant.

3. Wash again with 1 ml of 1× D-PBS. Centrifuge at 400 × *g* for 5 min at 4 °C. Aspirate supernatant.
4. Resuspend cells in 100 µl of fixable viability dye diluted in 1× D-PBS. Incubate for 20–30 min on ice or at 4 °C in the dark.
5. Add 1 ml of D-PBS and centrifuge at 400 × *g* for 5 min at 4 °C to wash.
6. Aspirate supernatant and resuspend cells with 20 µl of Fc block diluted in FACS buffer. Incubate for 10 min on ice or at 4 °C in the dark.
7. Without washing the cell suspension, add 100 µl of anti-CD4, B220, CXCR5, and PD-1 at the appropriate concentration diluted in FACS buffer. Incubate for 30 min on ice or at 4 °C in the dark (*see* **Notes 3–7**).
8. Top up with 1 ml of FACS buffer and centrifuge at 400 × *g* for 5 min at 4 °C to wash.
9. Aspirate supernatant and resuspend cells in 100 µl of streptavidin-APC diluted in FACS buffer. Incubate for 30 min on ice or at 4 °C in the dark (*see* **Note 5**).
10. Add 1 ml of FACS buffer and centrifuge at 400 × *g* for 5 min at 4 °C to wash.
11. Aspirate supernatant. Vortex cells to dissociate cell pellet and add 1 ml of fixation and permeabilization solution. Incubate for 30 min to 18 h (*see* **Note 8**).
12. Top up cells in fix/perm solution with 2 ml of 1× permeabilization buffer and centrifuge at 400 × *g* for 5 min at 4 °C to wash. Aspirate all but approximately 50 µl of supernatant (*see* **Note 9**).
13. Add 1 ml of 1× permeabilization buffer and centrifuge at 400 × *g* for 5 min at 4 °C to wash again. Aspirate all but approximately 50 µl of supernatant.
14. Resuspend cells in 100 µl of anti-Bcl6 antibody diluted in permeabilization buffer (*see* **Note 6**). Incubate for 60 min on ice or at 4 °C in the dark.
15. Top off with 2 ml of 1× permeabilization buffer and centrifuge at 400 × *g* for 5 min at 4 °C. Aspirate all but approximately 50 µl of supernatant.
16. Wash cells again by adding 1 ml of permeabilization buffer and centrifuging at 400 × *g* for 5 min at 4 °C. Aspirate all but approximately 50 µl of supernatant.
17. Resuspend cells in 500 µl of FACS buffer in preparation for flow cytometry.

3.3 Flow Cytometric Gating Strategy Steps (Depicted in Fig. 1; See Notes 10 and 11)

1. Gate on the lymphocyte population.
2. Gate out cell doublets.
3. Gate out nonviable (eFluor780 positive) cells.
4. Gate on $CD4^+$ $B220^-$ cells.
5. Gate to compare $CXCR5^+$ $PD\text{-}1^+$ and $CXCR5^-$ $PD\text{-}1^-$ T cell populations.
6. Confirm Tfh cell phenotype by examining Bcl6 expression.

4 Notes

1. While the phenotyping procedure described above can also be applied to human Tfh cells, only some of the antibodies listed are cross-reactive to human lymphocytes [13].
2. All procedures should be carried out on ice or at 4 °C.
3. In general, using a biotinylated primary antibody followed by a secondary streptavidin conjugated to your fluorophore of choice will amplify the signal compared to a primary antibody directly conjugated to a fluorophore. We particularly recommend using this strategy when assessing CXCR5 expression in murine cells as we find that using an anti-CXCR5 antibody directly conjugated to a fluorophore was indistinguishable from isotype control stained cells.
4. It is important to include B220 staining in the Tfh cell antibody cocktail. As germinal center B cells express high levels of both CXCR5 and Bcl6 [2], this extra step helps to rule out false-positive detection of Tfh cells that are actually T-B cell conjugates.
5. Additional markers that have been used to distinguish Tfh cells from other subsets include CXCR4, PSGL-1, CCR7, SAP, ICOS, IL-21, and IL-4 [6, 11, 14–17]. However, the expression of these markers may vary depending on the experimental context. In our protocol, we have chosen markers determined to be expressed across a wide range of experimental settings.
6. The panel of markers used above focuses on phenotyping the conventional, secondary lymphoid tissue resident Tfh cell without taking into account the various subsets of T follicular helper cell that can also contribute to an immune response. For instance, the intracellular staining of the transcription factor Foxp3 can be added to the procedure previously described to identify T follicular regulatory cells [18, 19]. In addition, Tfh-like cells found in the peripheral blood can also be identified by additionally using the marker CCR7 in your antibody cocktail [20].
7. In certain circumstances, such as in the case of LCMV infection, the marker PD-1 is insufficient to delineate the Tfh population as the majority of antigen-specific $CD4^+$ T cells will be $PD\text{-}1^+$ [21].

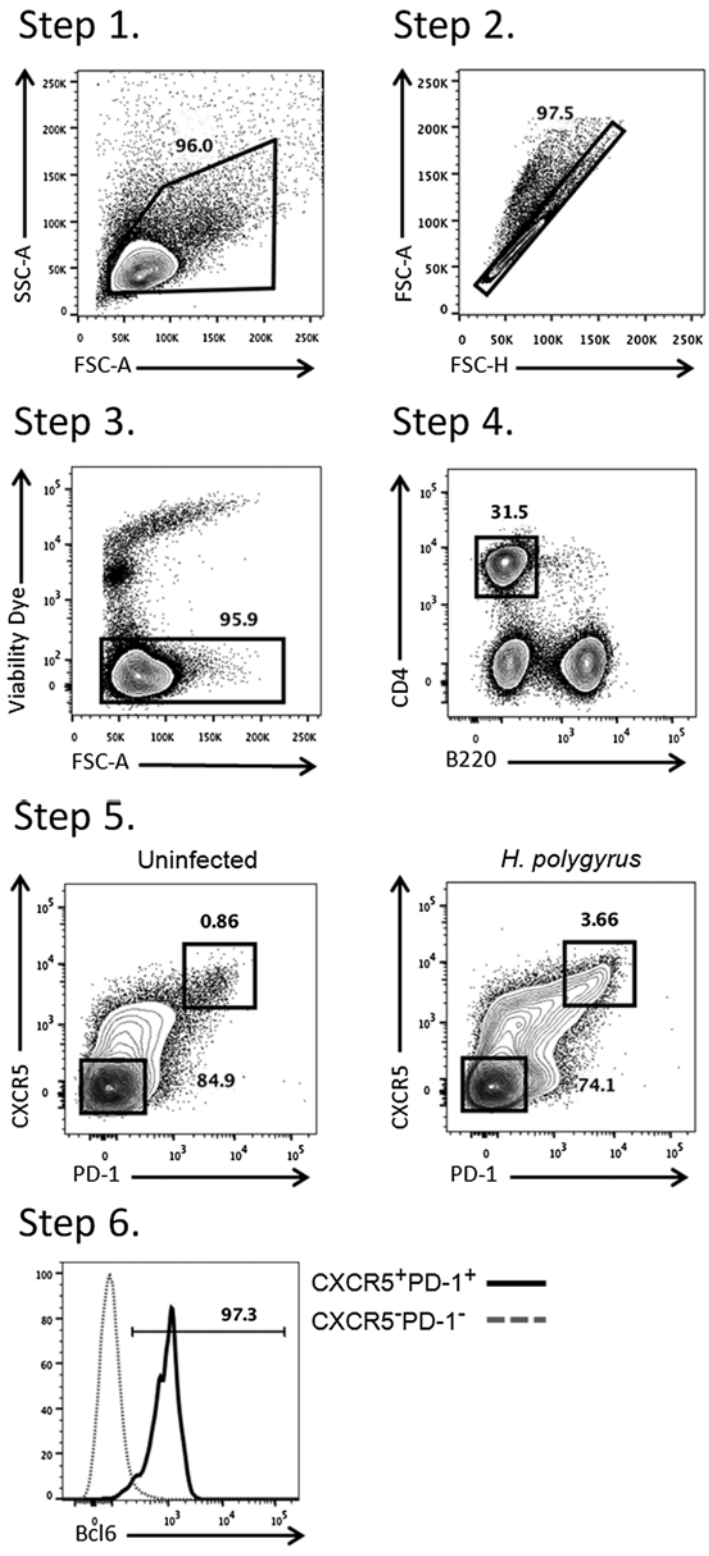

Fig. 1 Gating strategy. The data shown are based on mesenteric lymph node cells isolated from C57BL/6 mice left uninfected or infected with the murine intestinal helminth *Heligmosomoides polygyrus* [14]. The cells were isolated and prepared as described in Subheading 3.1. All figures subsequent to **step 1** are a depiction of the events selected within the previous gate

Thus, we have emphasized the importance of using a cocktail of antibodies to distinguish Tfh cells from other T effector lineages.

8. In order to avoid cell clumping and maximize cell fixation and permeabilization, it is important to vortex the cells prior to adding the fixation and permeabilization solution.
9. Take care not to aspirate the cell pellet after fixation and permeabilization as permeabilized cells are generally not as adherent as live cells.
10. It is always important to use single-stain compensation controls during flow cytometry to ensure proper experimental setup of the flow cytometer and accurate data collection.
11. In addition to using cell samples from uninfected or unimmunized control animals, the use of antibody isotype controls (at the same concentration as test antibodies) is also recommended to ensure proper gating methods.

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Chapter 2

Identification of Follicular T Helper Cells in Tissue Sections

Yanna Ding, John D. Mountz, and Hui-Chen Hsu

Abstract

Follicular T helper (Tfh) cells are a critical population of CD4 T helper cells that are primarily localized in the germinal centers (GCs) to help B cell maturation and antibody production. Tfh cells can be identified in tissue sections based on the expression of a panel of classical Tfh surface makers, transcription marker(s), and effector-function cytokines, as well as by their unique anatomic proximity to other GC cells, including follicular dendritic cells (FDC) and GC B cells. Here, we describe an immunofluorescence staining method for visualization of GC Tfh cells in frozen spleen tissue sections of the autoimmune BXD2 mouse using a confocal imaging strategy. Tfh cells were characterized based on the expression of CD4, CXCR5, Bcl6, IL-21, and IL-17.

Key words Tfh cells, Bcl6, IL-21, Immunohistostaining, Immunostaining, Immunofluorescence staining, Frozen section, Confocal microscopy, Multicolor staining

1 Introduction

Interaction of Tfh cells with B cells has been an important topic in immunology. Tfh markers include a panel of molecules, including CD4, CXCR5, ICOS, PD-1, Bcl6, IL-21, IL-17, and others [1–7]. Although multi-fluorochrome flow cytometry remains an essential method for quantitating and physically separating Tfh cells from other CD4 T subsets, direct visualization of Tfh cells in situ allows investigators to determine their anatomic location and their contact with other cells in the vicinity. Despite this advantage, in situ identification of Tfh cells remains challenging. Immunohistochemistry staining of paraffin-embedded tissue sections, which was first described in 1966 [8], is a traditional method for identifying cells and tissue structures. In this method, tissues are stained for specific markers and are often counterstained with hematoxylin to visualize cell nuclei. There are, however, limitations to this approach, since only a very limited number of markers and their co-localization can be detected in a single section. One way to circumvent this is to stain for different markers in continuous serial

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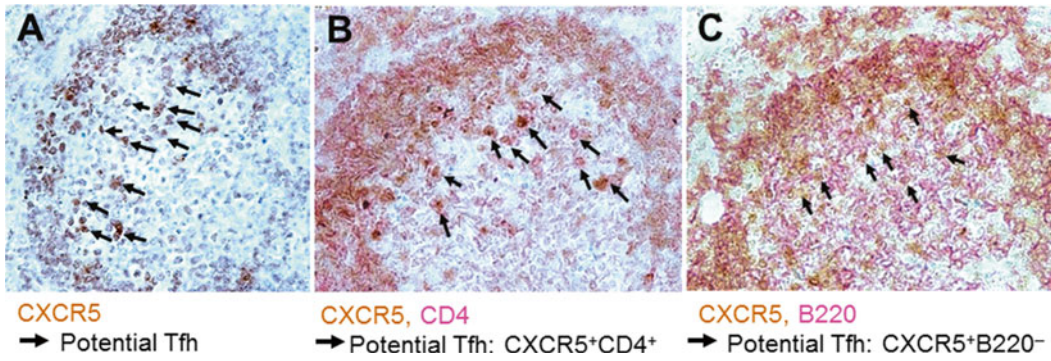

Fig. 1 Immunohistochemistry staining of potential Tfh cells in paraffin-embedded sections of BXD2 mouse spleens. (**a**) Single staining for CXCR5 and double staining for (**b**) CXCR5 and CD4 or (**C**) CXCR5 and B220. Potential Tfh cells are indicated by *arrows*. Magnification of objective lens, 40×

sections of the same tissue block. The co-localization of multiple markers in specific cells can then be determined indirectly under a light microscope. Although a protocol that enables simultaneous staining of triple antigens in paraffin sections has been described [9], due to the limitations of differential excitation/emission of the nonfluorescent dyes used in this method, differentiation between different markers is difficult. For determination of Tfh cells in paraffin-embedded spleen tissue sections, which requires overlapping of CXCR5 and CD4 and distinguishing of these cells from $CXCR5^+B220^+$ B cells, we have carried out double staining of CXCR5/CD4 and CXCR5/B220 in spleen sections (Fig. 1). $CXCR5^+CD4^+$ Tfh cells are polarized toward the region of $CXCR5^+B220^+$ follicular B cells (the mantle zone or corona), yet the current method has its limitations, since specific markers representing GCs or FDCs cannot be included in the same slide. Multicolor immunofluorescence staining can also help identify different markers in the same paraffin-embedded tissue section. However, there is an additional limitation for immunostaining in paraffin-embedded sections, since tissues have gone through paraformaldehyde fixation, alcohol dehydration, and paraffin embedding. Many antigens are masked and some are degenerated or destroyed. Consequently, various antigen-retrieval procedures are needed to recover masked antigens, whereas degenerated or destroyed antigens would be lost permanently. Therefore, immunostaining in paraffin-embedded sections presents challenges to identifying Tfh cells in situ.

Here, we describe an efficient method for detecting Tfh cells in situ using multicolor immunofluorescence staining, followed by confocal microscopic imaging of frozen tissue sections [7]. The snap-frozen tissue strategy helps preserve the antigenic epitopes, and fluorescent labeling enables the separation of multiple markers. Up to four florescence-labeled antibodies or dyes can be simultaneously

applied to a given tissue at the same section level (*see* **Note 1**). Continuous serial sections can also be used to indirectly compare the expression of additional Tfh and GC cell markers in nearby sections.

2 Materials

Unless specified, all solutions and buffers should be stored at 4 °C or freshly diluted; sera should be stored at–20 °C, while antibodies and staining reagents should be stored at 4 °C and diluted in staining buffer or PBS at room temperature before use.

2.1 Equipment

1. Labeled Cryomold plastic molds.
2. –80 °C freezer.
3. Slides and cover slips.
4. Hydrophobic pen used to create liquid-repellent area around the tissue section on slides.
5. Staining dishes, slide racks, and immunostain moisture chambers.
6. Confocal microscope (e.g., Leica DMIRBE inverted Nomarski/epifluorescence microscope outfitted with Leica TCS NT laser confocal optics).

2.2 Chemical Reagents, Solutions, and Buffers

1. 2-Methylbutane: store at room temperature and prechill in a –80 °C freezer to snap-freeze tissue.
2. Frozen tissue matrix (OCT®) for embedding fresh tissue. Store at room temperature.
3. Acetone: prechill on ice before used.
4. PBS: should be clean and stored at room temperature.
5. Staining buffer: 1 % BSA in PBS (*see* **Note 2**).
6. Blocking buffer: 10 % horse serum in PBS (v/v) or 10 % rat serum in PBS or 1 % BSA in PBS.
7. Freshly diluted 3 % H_2O_2 in PBS.
8. Avidin/Biotin Blocking Kit.
9. Tyramide Signal Amplification (TSA™) Biotin Tyramide Reagent Pack.
10. Clean mounting gel (*see* **Note 3**): store at –20 °C, pre-warm to room temperature when used.

2.3 Antibodies and Conjugates (See Note 4)

1. Alexa 647-conjugated anti-mouse CD4 (clone GK1.5).
2. Biotin-peanut agglutinin (PNA, Vector Laboratory) and streptavidin (SA)-Alexa 350.
3. Alexa 555-conjugated anti-mouse IgM.

4. Alexa 488-conjugated anti-mouse CD35/21 (clone 8C12).
5. Purified rat anti-mouse CXCR5 IgG (clone 2G8), goat anti-rat IgG-biotin, and SA-Alexa 488.
6. Polyclonal rabbit anti-Bcl6 IgG (sc-858, Santa Cruz Biotech), goat anti-rabbit IgG-HRP, and SA-Alexa 488.
7. Goat anti-mouse IL-21 (AF594, R&D Systems) and Alexa 555-conjugated donkey anti-goat IgG.
8. Rat anti-mouse IL-17 (TC11-18H10.1, Biolegend) and Alexa 555-conjugated goat anti-rat IgG.

3 Methods

3.1 Frozen Section Preparation

1. Place approximately $0.6 \times 0.6 \times 0.6$ cm^3 of diced fresh spleen tissue in a Cryomold® mold half-filled with OCT; fill the mold with OCT to completely embed the tissue, and immediately immerse the tissue into prechilled 2-Methylbutane in a –80 °C freezer to snap-freeze the tissue into a block (*see* **Note 5**).
2. Cut the frozen tissue block into continuous serial cryosections 7 μm thick.
3. Allow sections to air-dry on slides for approximately 15 min. Sections should be kept in a microscope box, transported on ice, and stored in a –80 °C freezer until use.

3.2 Stain with Different Combinations of Markers

1. Prior to the staining process, remove slides from the freezer and let them thaw.
2. Put the slides on a slide rack and fix the sections in prechilled acetone in a staining dish on ice for 12–15 min.

3.2.1 Common Staining Procedure

3. Remove the slide rack from the acetone-containing staining dish, and put the slide rack with slides on ice to allow the remaining acetone to evaporate.
4. Rehydrate the sections in 1 % BSA in PBS for 10 min (*see* **Note 6**).
5. Rinse slides in PBS five times (five changes of PBS) and circle tissue with a hydrophobic pen to later constrain buffer flow over the tissue. Label the slides, and include a blank control or isotype control slide.
6. Block nonspecific staining with 10 % horse serum in PBS at room temperature for 30 min (*see* **Note 7**).
7. Do not rinse with PBS.
8. Primary antibody staining (diluted in 1 % BSA in PBS): incubate the tissue with fluorochrome-labeled primary antibody or antibody cocktail in the dark at room temperature for 30 min in an immunostain moisture chamber (*see* **Note 8**). Drain the staining solution.

9. Rinse in PBS five times.
10. Apply secondary antibodies or secondary staining reagents in the dark at room temperature for 30 min in an immunostain moisture chamber (*see* **Note 9**). Drain the staining solution.
11. Rinse in PBS five times.
12. If applicable, apply the third antibody or staining reagents, and incubate in the dark at room temperature for 30 min in an immunostain moisture chamber (*see* **Note 10**). Drain the staining solution.
13. Rinse in PBS five times.
14. Allow the slides to completely air-dry, mount the tissue with mounting gel, and then apply a cover slip to each slide. To protect the tissue from drying during long storage, clear nail polish can be used to seal the cover slip. Stained slides can be imaged immediately or stored at 4 °C for future imaging.

3.2.2 Specific Staining Procedure: Staining CD4, FDC, IgM, and PNA in Combination

Unless specified, the staining procedure should follow the basic rules described in Subheading 3.2.1. The dilution factor provided below for each antibody is applicable to the specified clone from the indicated source. Since there may be lot-to-lot variation, the optimum working dilution of each antibody should be further tested to ensure successful staining. The volume of all blocking buffer or staining reagents should be sufficient to completely cover the entire tissue on the slide.

1. Follow **steps 1–7** of Subheading 3.2.1 to prepare slides for subsequent staining.
2. Incubate slides with the primary antibody cocktail containing Alexa 647-anti-mouse CD4 at 1:100, Alexa 488-anti-mouse CD35/21 at 1:100, and Alexa 555-anti-mouse IgM at 1:250 in the dark for 30 min at room temperature (*see* **Note 6**).
3. Rinse in PBS five times.
4. Apply biotin-PNA at 1:50, and incubate slides in the dark at room temperature for 30 min.
5. Rinse in PBS five times.
6. Apply secondary staining reagent SA-Alexa 350 at 1:100 in the dark at room temperature for 30 min.
7. Rinse in PBS five times.
8. Follow **step 14** of Subheading 3.2.1 to complete the staining procedure.
9. A representative image obtained using this procedure is shown in Fig. 2.

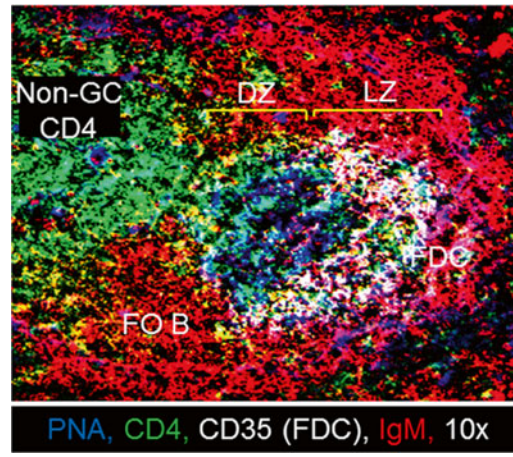

Fig. 2 Confocal imaging analysis of a frozen spleen section from a 4-month-old BXD2 mouse. This representative tissue section was stained with fluorochrome-conjugated reagents or antibodies. Numerous CD4 T cells (*green*) can be seen to colocalize with FDCs (*white*) in the light zone of the PNA^+ GC (*blue*). These are potentially Tfh cells. Magnification of objective lens, 10×. *GC* germinal center, *DZ* dark zone, *LZ* light zone, *FOB* follicular B cell, *FDC* follicular dendritic cell, *PNA* peanut agglutinin

3.2.3 Specific Staining Procedure: Staining CD4, CXCR5, and PNA in Combination

1. Follow **steps 1–7** of Subheading 3.2.1 to prepare slides for subsequent staining.
2. Apply purified rat anti-mouse CXCR5 IgG at 1:50; incubate slides in the dark at room temperature for 30 min (*see* **Note 6**).
3. Rinse in PBS five times.
4. Apply goat anti-rat IgG-biotin at 1:100; incubate slides in the dark at room temperature for 30 min.
5. Rinse in PBS five times.
6. Apply SA-Alexa 488 at 1:200; incubate slides in the dark at room temperature for 30 min.
7. Rinse in PBS five times.
8. Apply 10 % rat serum to resorb any remaining antibody from **step 4**; incubate in the dark at room temperature for 30 min.
9. Apply Alexa 647-anti-mouse CD4 at 1:100 and biotin-PNA at 1:50; incubate in the dark for 30 min at room temperature.
10. Rinse in PBS five times.
11. Apply SA-Alexa 350 at 1:100; incubate slides in the dark at room temperature for 30 min.
12. Rinse in PBS five times.
13. Follow **step 14** of Subheading 3.2.1 to complete the staining procedure.
14. A representative image obtained using this procedure is shown in Fig. 3.

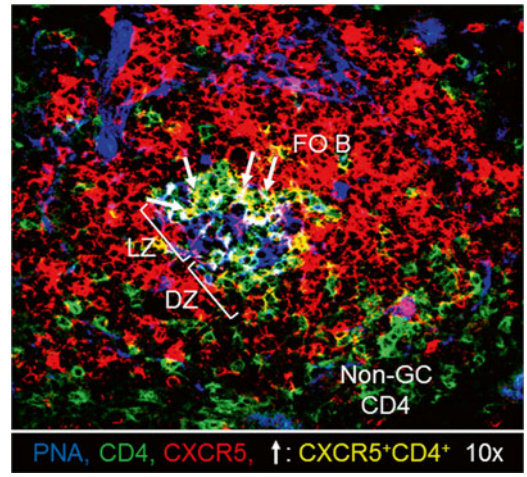

Fig. 3 Confocal imaging analysis of a frozen spleen section from a 2.5-month-old BXD2 mouse. This representative section was stained with the indicated fluorochrome-conjugated reagent or antibodies. *White arrows* indicate representative $CXCR5^+$ CD4 T cells, most of which are located in the light zone of the PNA^+ GC (*blue*). Magnification of objective lens, 10×. *DZ* dark zone, *LZ* light zone, *PNA* peanut agglutinin

3.2.4 Specific Staining Procedure: Staining CD4, Bcl6, and PNA in combination with Tyramide Signal Amplification (TSA™) to amplify Bcl6 signal

Tyramide Signal Amplification (TSA™), sometimes called catalyzed reporter deposition (CARD), is an efficient signal amplification procedure to amplify low-signal markers that cannot be effectively detected by standard immunohistochemistry (IHC) and in situ hybridization (ISH) procedures [10, 11]. In this system, the amplification signal substrate is tyramide, which is a phenolic compound that, when activated by the enzyme horseradish peroxidase (HRP) in the presence of small amounts of hydrogen peroxide, covalently binds to electron-rich moieties on a surface (i.e., predominantly to tyrosine residues in proteins in tissue or cell preparations). This reaction then generates high-density labeling of a target protein or nucleic acid sequence in situ [12]. For detection of intracellular Bcl6, standard staining conditions, including two-step or three-step staining with biotinylated anti-Bcl6, have been carried out but without success. The TSA method was therefore applied to overcome the low signal of intracellular Bcl6.

1. Follow **steps 1–5** of Subheading 3.2.1 to prepare slides for subsequent staining (*see* **Note 6**).
2. Apply 3 % H_2O_2 in PBS; incubate slides at room temperature for 20–45 min to eliminate endogenous HRP.
3. Rinse in PBS five times.
4. Block with 1 % BSA in PBS at room temperature for 1 h.

5. Use an avidin-biotin blocking procedure provided by the Avidin/Biotin Blocking Kit to eliminate endogenous biotin according to the manufacturer's instructions.
6. Rinse in PBS thoroughly.
7. Apply rabbit anti-Bcl6 IgG at 1:1,500; incubate slides in the dark at 4 °C for overnight.
8. Rinse in PBS five times.
9. Apply goat anti-rabbit IgG-HRP at 1:200; incubate in the dark at room temperature for 30 min.
10. Rinse in PBS five times.
11. Amplify the HRP signal by applying the biotinyl TSA reagent at 1:100 (available from the Tyramide Signal Amplification [TSA™] Biotin Tyramide Reagent Pack) for 8 min in the dark at room temperature according to the manufacturer's instructions.
12. Rinse in PBS immediately to stop the TSA reaction five times.
13. Apply SA-Alexa 488 at 1:200; incubate in the dark at room temperature for 30 min.
14. Rinse in PBS five times.
15. Apply Alexa 647-anti-mouse CD4 at 1:100 and biotin-PNA at 1:50; incubate in the dark for 30 min at room temperature.
16. Rinse in PBS five times.
17. Apply SA-Alexa 350 at 1:100; incubate in the dark at room temperature for 30 min.
18. Rinse in PBS five times.
19. Follow **step 14** of Subheading 3.2.1 to complete the staining procedure.
20. A representative image obtained using this procedure is shown in Fig. 4.

*3.2.5 Specific Staining Procedure: Staining CD4, IL-21, and PNA in Combination (See **Note 11**)*

1. Follow **steps 1–7** of Subheading 3.2.1 to prepare slides for subsequent staining.
2. Apply goat anti-mouse IL-21 IgG at 5–15 μg/ml (1:20); incubate in the dark for 3 h at room temperature (*see* **Note 6**).
3. Rinse in PBS five times.
4. Apply Alexa 555-conjugated donkey anti-goat IgG at 1:200; incubate in the dark for 1 h at room temperature.
5. Rinse in PBS five times.
6. Apply Alexa 647-conjugated anti-mouse CD4 at 1:100 and biotin-PNA at 1:50; incubate in the dark for 30 min at room temperature.
7. Rinse in PBS five times.

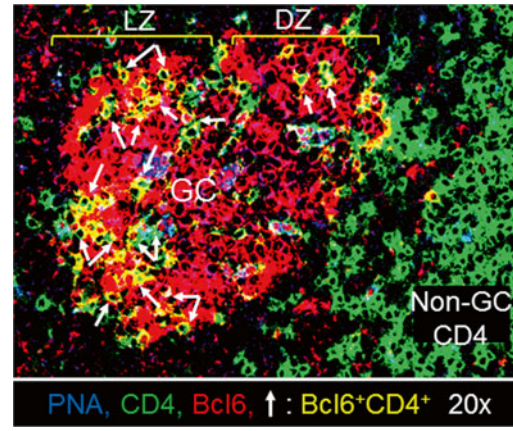

Fig. 4 Confocal imaging analysis of a frozen spleen section from a 4-month-old BXD2 mouse. This representative section was stained with the indicated fluorochrome-conjugated reagents or antibodies. *White arrows* indicate $Bcl6^+CD4^+$ T cells, most of which are located in the light zone of the $Bcl6^+$ GC (*red*). Magnification of objective lens, 20×. *DZ* dark zone, *LZ* light zone, *PNA* peanut agglutinin

8. Apply SA-Alexa 350 at 1:100; incubate in the dark at room temperature for 30 min.
9. Rinse in PBS five times.
10. Follow **step 14** of Subheading 3.2.1 to complete the staining procedure.
11. A representative image obtained using this procedure is shown in Fig. 5 [7].

*3.2.6 Specific Staining Procedure: Staining CD4, IL-17, and PNA in Combination (See **Note 12**)*

1. Follow **steps 1–7** of Subheading 3.2.1 to prepare slides for subsequent staining.
2. Apply rat anti-mouse IL-17A at 1:40; incubate in the dark at room temperature for 1 h (*see* **Note 6**).
3. Rinse in PBS five times.
4. Apply Alexa 555-conjugated goat anti-rat IgG at 1:250; incubate in the dark at room temperature for 30 min.
5. Rinse in PBS five times.
6. Block with 10 % rat serum; incubate in the dark at room temperature for 30 min.
7. Apply Alexa 647-conjugated anti-mouse CD4 at 1:100 and biotin-PNA at 1:50; incubate in the dark for 30 min at room temperature
8. Rinse in PBS five times.
9. Apply SA-Alexa 350 at 1:100; incubate in the dark at room temperature for 30 min.

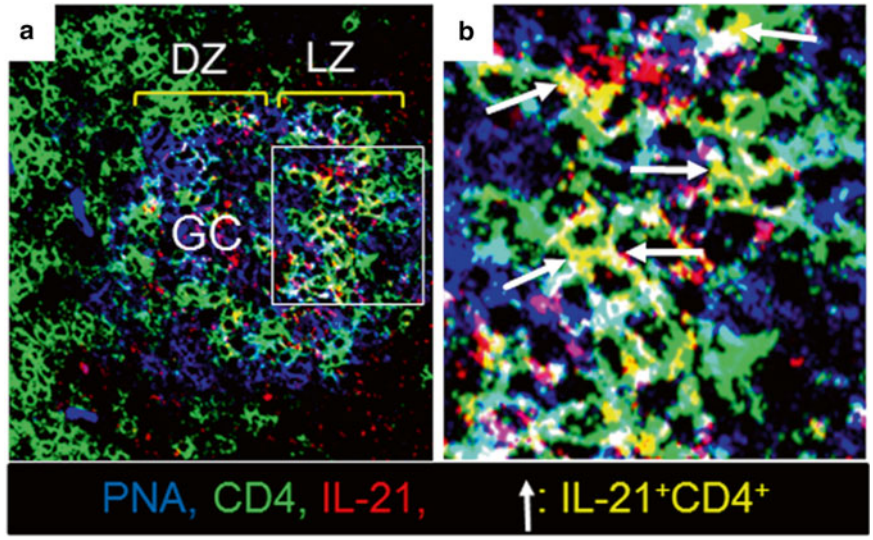

Fig. 5 Confocal imaging analysis of a frozen spleen section from a 3-month-old BXD2 mouse. This representative section was stained with fluorochrome-conjugated reagents or antibodies. A higher-magnification view of the boxed area in the light zone of the PNA^+ GC (*blue*) in (**a**) is shown in (**b**). *White arrows* indicate representative $IL\text{-}21^+CD4^+$ T cells. Magnification of objective lens, 20× in (**a**). *DZ* dark zone, *LZ* light zone, *PNA* peanut agglutinin. Reprint with permission by the Journal of Immunology 2013 Aug 15;191(4):1614–24 [7]

10. Rinse in PBS five times.
11. Follow **step 14** of Subheading 3.2.1 to complete the staining procedure.
12. A representative image obtained using this procedure is shown in Fig. 6 [7].

3.3 Confocal Microscopy and Imaging

Images were captured with a Leica DMIRBE inverted Nomarski/epifluorescence microscope outfitted with Leica TCS NT laser confocal optics. Confocal imaging was analyzed with background intensity subtracted from each image using ImageJ software (version 1.4), developed by the US National Institutes of Health. This software is free to download at http://rsb.info.nih.gov/nih-image/[13].

4 Notes

1. The number of markers that can be detected for each tissue section depends on the number of channels available with the confocal microscope used. Confocal fluorescent images shown in the present protocol were captured by a confocal imaging microscope that is equipped with UV (blue), green, red, and far-red lasers.
2. In case the signal is very weak, PBS can be used as the antibody-diluting buffer and also as the staining buffer.

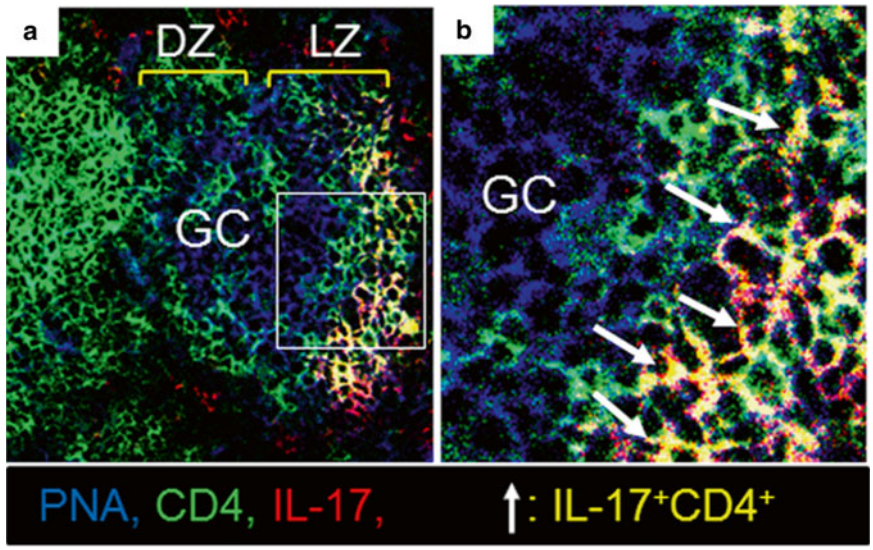

Fig. 6 Confocal imaging analysis of frozen spleen sections from a 3-month-old BXD2 mouse. This representative section was stained with the indicated fluorochrome-conjugated reagents or antibodies. A higher-magnification view of the boxed area in (**a**) is shown in (**b**). *White arrows* indicate representative IL-17$^+$CD4$^+$ T cells (**b**) in the light zone of the PNA$^+$ GC (*blue*). Magnification of objective lens, 20× in (**a**). *DZ* dark zone, *LZ* light zone, *PNA* peanut agglutinin. Reprint with permission by the Journal of Immunology 2013 Aug 15;191(4):1614–24 [7]

3. The mounting gel should be kept clean at all times, which is very important to prevent the presence of debris and microorganisms that could produce autofluorescence on the slides and affect confocal imaging results.
4. Although antibodies can generally be randomly combined with different fluorescence conjugates, allocating the brightest fluorescence to the weakest or the most important markers enhances imaging results.
5. Sample tissues should be immersed in OCT immediately to embed tissue completely. The mold should be snap-frozen in prechilled 2-Methylbutane in a −80 °C freezer. When storing frozen blocks, molds should be completely sealed by an aluminum foil wrap to prevent moisture from the blocks. Slow operation or an irregular shape for the frozen block should both be avoided, as they would affect tissue structure and the subsequent cryosectioning procedure.
6. After rehydration, tissue sections should be kept wet until the entire staining procedure is finished, which is critical to ensure proper antibody and antigen binding and to prevent any background staining. All staining incubation should be carried out in a humid environment, such as in an immunostain moisture chamber. Any fluorescence reagent staining should be protected from light.

7. For blocking buffer, 10 % horse serum in PBS is generally sufficient to block nonspecific background staining. With some exceptions, the serum chosen should be from a different species than the antibody host species. In a multi-marker staining that involves the use of two primary antibodies that are both raised in rat and a secondary anti-rat antibody must be applied in between, the following method was used. Tissues were first stained with the first rat originated primary antibody and an anti-rat secondary antibody. Before staining with the second rat originated primary antibody, 10 % rat serum in PBS was used to resorb the remaining secondary anti-rat antibody from the previous step to reduce nonspecific staining. To avoid introducing extra biotin from the serum, 1 % BSA in PBS can be used.
8. For multi-antibody staining, the most critical marker, the marker with weakest signal, or the marker that requires multistep signal amplification should be stained before staining other easily stained markers or markers with strong signal. Incubation at 4 °C overnight may help to enhance the strength and specificity of the staining signal as opposed to staining at room temperature for 1 h. For overnight staining, the incubation should be in the dark at 4 °C.
9. For secondary antibody or reagent staining, the incubation is generally carried out at room temperature for 30 min. However, the staining time should be adjusted according to the signal strength. To enhance the signal, the staining time can be extended to 1 h or longer. The staining procedure can also be paused at this stage at 4 °C overnight.
10. For third antibody or reagent staining, the incubation is generally carried out at room temperature for 30 min, with the possibility of extending the staining time for the same reason described in **Note 9**.
11. For IL-21 staining, the following procedures were also tested, yet the outcome was less desirable: (a) a biotin TSA kit was used following goat anti-mouse IL-21 IgG and rabbit anti-goat IgG-HRP staining to amplify the IL-21 signal, but this increased the staining in nonspecific cells; (b) a biotin TSA kit was used following rabbit anti-mouse IL-21 IgG-biotin and SA-HRP staining to amplify IL-21, but this also led to increased nonspecific staining in off-target cells. The nonspecific staining in Methods (a) and (b) may be the result of excessive amplification of endogenous biotin that could not be blocked effectively by the biotin blocking kit. **c**. IL-21 was also stained using mouse IL-21R-Fc (human Fc) followed by a PE-conjugated anti-human IgG. Although such methods have been used to detect mouse IL-21 by flow cytometry [14], only a very weak signal was detected using a confocal imaging strategy. PE fluorochrome can quench rapidly under the high-energy laser of the confocal microscope and thus is not a recommended reagent for this purpose.

12. For IL-17 staining, a one-step staining with a PE-conjugated anti-mouse IL-17 antibody has been tested, yet this generally leads to a much weaker IL-17 signal as opposed to the two-step staining procedure.

Acknowledgments

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Chapter 3

Tracking Early T Follicular Helper Cell Differentiation In Vivo

Dirk Baumjohann and K. Mark Ansel

Abstract

T follicular helper (Tfh) cells provide essential help to B cells for the generation of high-affinity antibodies. These mechanisms provide the basis for the success of modern vaccines, but dysregulated Tfh cell responses are also linked to autoimmune diseases. In addition to their established role in driving humoral immunity, Tfh cells are gaining attention for their role in other processes of the adaptive immune system. For example, Tfh cells may serve as transitional differentiation intermediates during effector and memory T-helper cell differentiation and as a reservoir of HIV-infected cells. While B cells are required for the full maturation and maintenance of Tfh cell responses, they are dispensable for the initial induction of the Tfh cell phenotype, which occurs at the priming stage through interaction with dendritic cells. Nevertheless, the precise mechanisms of these early events during Tfh cell differentiation remain relatively unknown. Here, we describe a method for tracking early Tfh cell differentiation by following cell division kinetics and phenotypic changes of recently activated antigen-specific $CD4^+$ T cells in vivo. As an example, we use this method to visualize the requirements for T cell-expressed CD28 for the differentiation of $CXCR5^+Bcl6^+$ Tfh cells.

Key words T follicular helper cells, Follicular helper T cells, Tfh cells, Flow cytometry, FACS, CFSE, CellTrace Violet, CTV, T-dependent antibody response, Bcl6, Bcl-6, CXCR5

1 Introduction

T follicular helper (Tfh) cell differentiation begins at the priming stage when naïve $CD4^+$ T-helper cells interact with antigen-presenting dendritic cells (DCs) in the T zone of secondary lymphoid organs [1, 2]. Activated $CD4^+$ T cells undergo rapid changes in their expression of costimulatory molecules and chemokine receptors. Downregulation of CCR7 expression, which is highly expressed on naïve $CD4^+$ T cells, and concomitant upregulation of the chemokine receptor CXCR5 subsequently allow these activated T cells to migrate to the T-B zone border and interfollicular regions of secondary lymphoid organs, where they interact with antigen-specific B cells [3, 4]. Some of these early Tfh cells, together with a few antigen-specific B cells, enter the follicle to

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establish full-fledged germinal centers in which somatic hypermutation and selection of high-affinity B cells result in the generation of memory B cells and plasma cells that produce high-affinity antibodies [5]. Even though it was initially believed that B cells were essential for the differentiation of Tfh cells, more recent studies have clarified that DCs are able to induce a Tfh cell phenotype in recently activated $CD4^+$ T cells, independent of cognate interactions with B cells [6–10]. Nevertheless, B cells become the major antigen-presenting cell type for Tfh cells at later stages of the immune response, thus being important for the full differentiation and maintenance of germinal center Tfh cells [11, 12].

The introduction of fluorescent dyes for tracking cell divisions of labeled cells has provided important insights into various aspects of T-helper cell biology. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was introduced to immunology labs in the early 1990s [13] and is to date the most widely used of these dyes. More recently, several alternatives to CFSE have provided improved features and additional flexibility in the design of experiments [14]. We have used the division status as a means to track Tfh cell development in adoptively transferred TCR-transgenic (tg) T cells after immunization in wild-type recipient mice. For example, we showed that those T cells in draining lymph nodes that proliferated the most became enriched for $CXCR5^+Bcl6^+$ Tfh cells [6]. In another study, we used this method to show that global microRNA expression in $CD4^+$ T cells was required for the differentiation of these cells into Tfh cells, which was due to an intrinsic defect to induce the Tfh gene expression program, independent of any changes in their proliferative capacity [15]. In this protocol, we describe the methodologic details of these approaches.

2 Materials

2.1 Cell Preparation, Immunization, and Antibody Staining

1. T cell receptor-transgenic (TCR-tg) donor mice, e.g. OT-II mice [16] in which T cells carry a transgenic TCR recognizing ovalbumin $(OVA)_{323–339}$ in the context of MHC class II ($I\text{-}A^b$).
2. Recipient mice: Wild-type C57BL/6 mice or $CD45.1^+$ congenic mice, e.g. B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ mice (The Jackson Laboratory) or B6-LY5.2/Cr mice (National Cancer Institute).
3. Glass slides with frosted ends.
4. 5 cm cell culture dishes.
5. 5 ml polystyrene tubes and 14 ml polypropylene tubes.
6. Nylon mesh (70 μm pore size).
7. Mouse $CD4^+$ T cell isolation kit (e.g. EasySep negative selection kit by StemCell Technologies) and a magnet for pre-enrichment.

8. CellTrace Violet (CTV, Life Technologies): Prepare 5 mM aliquots in PBS and store at −20 °C.
9. NP-OVA (Biosearch Technologies): Prepare 1 mg/ml aliquots in PBS and store at −20 °C. Note: Different conjugation ratios of NP and OVA are available. Here, we used NP_{14}-OVA.
10. Alum (e.g. Imject Alum by Thermo Scientific).
11. 96-well round-bottom plates.
12. Antibodies: Fluorophore-conjugated antibodies against CD4 (clone RM4-5), CD25 (clone PC61.5), CD62L (clone MEL-14), CD44 (clone IM7), Bcl6 (clone K112-91), CD19 (clone 1D3) or B220 (clone RA3-6B2), TCR Vα2 (clone B20.1), CD45.2 (clone 104), CD45.1 (clone A20), and biotinylated anti-CXCR5 (clone 2G8).
13. APC-conjugated streptavidin.
14. Fixable viability dye. For example, eFluor® 780 (eBioscience), which can be detected in the APC-Cy7 channel of a flow cytometer.
15. Insulin syringes for the injection of cells.

2.2 Buffers and Media

1. PBS (Mg^{2+}-/Ca^{2+}-free).
2. Sorting/enrichment buffer: 2 % fetal bovine serum (FBS) and 1 mM EDTA in phosphate-buffered saline (PBS).
3. Complete medium: RPMI 1640 (with L-glutamine) supplemented with 10 % FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM beta-mercaptoethanol, 10 mM HEPES.
4. Staining buffer: 2 % FBS, 1 mM EDTA, and 0.1 % sodium azide (NaN_3) in PBS.
5. Fc-block: 2 % mouse serum, 2 % rat serum, 5 μg/ml anti-CD16/32 in staining buffer.
6. Foxp3/Transcription Factor Staining Buffer Set (eBioscience) for fixation/permeabilization.

3 Methods

3.1 Adoptive Transfers and Immunizations

3.1.1 TCR-Tg T Cell Preparation and Separation

1. Dissect and pool peripheral lymph nodes (inguinal, axillary, and brachial) and spleen of TCR-tg donor mice of desired genotypes (*see* **Note 1**).
2. Prepare single-cell suspensions in PBS by disrupting the tissues between the frosted ends of glass slides in 5 cm plastic dishes.
3. Filter cells through fine mesh into 14 ml tubes that contain at least 1 ml of sorting buffer.

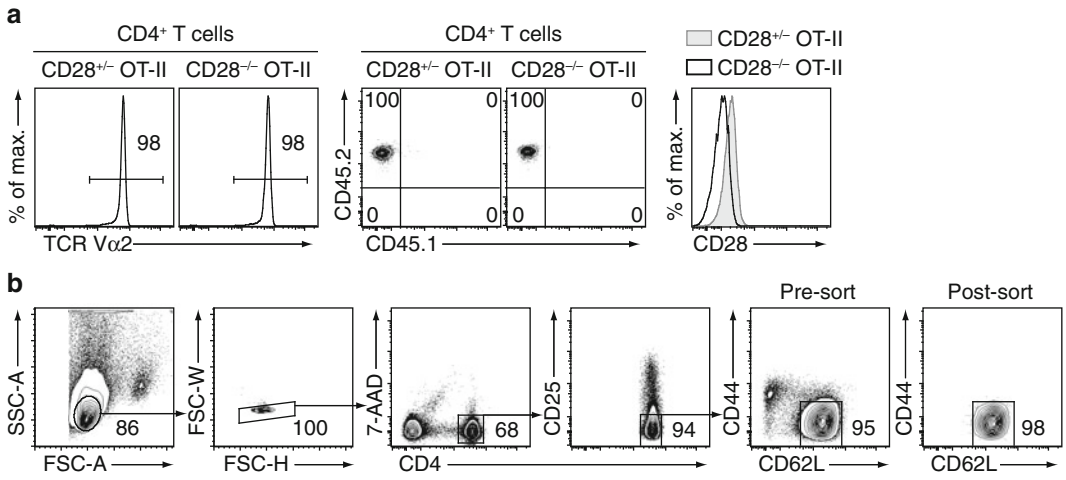

Fig. 1 Isolation of naïve TCR-tg CD4+ T cells for adoptive transfer. (**a**) Single-cell suspensions were prepared from spleen and lymph nodes of CD28$^{+/-}$ or CD28$^{-/-}$ [19] OT-II TCR-tg mice. A small aliquot was stained with PE-conjugated anti-CD4, APC-conjugated TCR Vα2, PerCP-Cy5.5-conjugated anti-CD45.2, eFluor450-conjugated anti-CD45.1, and FITC-conjugated anti-CD28 (clone 37.51) for verification of the phenotype of the donor cells. (**b**) The rest of the cells was pre-enriched for untouched CD4+ T cells by magnetic cell separation, followed by staining with antibodies against CD4 (conjugated to eFluor450), CD25 (APC), CD44 (Alexa Fluor 700), and CD62L (PE-Cy7). To exclude dead cells, 7-AAD was added to the stained cell suspension right before acquisition/sorting on a FACSAria II cell sorter (BD Biosciences). Small lymphocytes were gated by FSC-A vs. SSC-A characteristics. Singlet cells were identified by FSC-H vs. SSC-W gating (note that the use of EDTA in the sorting buffer significantly reduced doublet formation). Treg cells were then excluded by gating out CD25+ cells. Finally, naïve CD4+ T cells were sorted as CD62L^{high} CD44$^{low/int}$ cells. An optional re-sort on a small aliquot of the sorted cells was performed to verify the high purity of the obtained cell population

4. Fill up tubes to 14 ml and spin down. Discard supernatant and resuspend cells in appropriate volumes of sorting buffer (*see* next step).
5. Keep aside a small aliquot of cells for phenotyping: Stain cells with antibodies against CD4, the specific TCR of the TCR-transgene, and congenic markers as well as other relevant markers (compare Fig. 1a and **Note 2**).
6. Prior to sorting by flow cytometry, CD4+ T cells are pre-enriched by negative selection using magnetic beads (*see* **Note 3**). Several commercial kits are available for these purposes, such as column-based products (e.g., MACS by Miltenyi Biotec) or column-free products (e.g., Dynabeads by Life Technologies). Here, we use StemCell Technologies' rapid and column-free EasySep negative selection mouse CD4+ T cell isolation kit according to the manufacturer's instructions. In brief, cells are resuspended at a concentration of 1x10^8 cells/ml sorting buffer, transferred to a 5 ml polystyrene tube, followed by consecutive addition of normal rat serum

(50 µl/ml of cells), primary biotinylated antibody mix (50 µl/ml of cells and incubation for 10 min), and streptavidin-conjugated microbeads (50 µl/ml of beads and incubation for 2.5 min). Tubes are filled up to 2.5 ml with sorting buffer and placed into an EasySep magnet. After 2.5 min, the unbound fraction is poured off in one motion into new 15 ml tubes that contain 10 ml sorting buffer.

7. Pre-enriched $CD4^+$ T cells are spun down and resuspended in 400 µl antibody mix, which includes anti-CD4, anti-CD25, anti-CD62L, and anti-CD44 (compare **Note 4**).
8. Cells are stained for 10 min on ice, washed once, and resuspended again in sorting buffer.
9. Right before sorting on a cell sorter, a viability dye (e.g., 7-AAD) should be added to exclude dead cells (compare Fig. 1b for the sorting gating strategy).
10. Sort cells into cooled 15 ml tubes containing 2–3 ml of medium and keep sorted cells on ice until further processing.

3.1.2 CTV Labeling and Cell Injection

In the next step, sorted naïve TCR-tg $CD4^+$ T cells are labeled with CTV according to the manufacturer's instructions (compare **Note 5**):

1. Dilute the CTV stock solution 1:500 in PBS (10 µM) and pre-warm the tube in a 37 °C water bath.
2. Spin down sorted cells and resuspend them at a concentration of 2×10^6 cells per ml of PBS.
3. Mix equal volumes of cells with the pre-diluted CTV (to a final concentration of 5 µM). *See* **Note 6**.
4. Incubate cells for 20 min in a 37 °C water bath. Invert the tubes to mix at least 2–3 times during the incubation.
5. Fill up each tube with complete medium and incubate for an additional 5 min at 37 °C to quench any surplus CTV.
6. Spin down cells and wash twice with PBS.
7. Count cells and adjust cell numbers to 5×10^5 cells per 200 µl PBS for injection into each recipient mouse. Ideally, injected cells are allowed to equilibrate in the host for several hours before immunization.

3.1.3 Immunization

1. Prepare antigen mixture by combining one part of the NP-OVA stock with one part PBS and two parts of alum.
2. Slowly rotate/invert for 30–45 min at room temperature.
3. Immunize mice with the cognate antigen: Carefully inject 20 µl of antigen mixture (=5 µg antigen) s.c. into each foot pad (*see* **Note 7** for alternative immunization routes).

3.2 Analysis by Flow Cytometry

1. Sacrifice recipient mice according to institutional guidelines and dissect popliteal LNs (pool 2 LNs for each mouse to increase total cell numbers). If desired, dissect non-draining LNs far away from the injection site (e.g. brachial LNs) or from non-immunized control recipients. Popliteal LNs from non-immunized mice are very small and will not yield many CTV^+ cells.
2. Prepare single-cell suspensions in PBS by disrupting LNs between the frosted ends of glass slides.
3. Filter through fine mesh (70 μm).
4. Count cells.
5. Pipet 3×10^6 cells into each well of a 96-well round-bottom plate.
6. If cells are going to be fixed and permeabilized at a later stage, e.g. for transcription factor staining, perform staining with a fixable viability dye at this point (*see* **Note 8**). To this end, wash plate once with PBS and add 200 μl of 1:2,000 diluted eF780 viability dye (in PBS) to each well and incubate for 15 min on ice.
7. Wash plate once by centrifuging the plate at $350 \times g$ for 5 min, remove supernatant, and resuspend cells in 200 μl of staining buffer, then centrifuging the plate again at $350 \times g$ for 5 min. Discard supernatant.
8. Add 20 μl of Fc-block to each well and incubate for 5 min (compare **Note 9**).
9. Without washing, add 20 μl of 2× primary antibody mixture to each well (40 μl final staining volume). *See* **Notes 10** and **11**.
10. Incubate for 30 min at 4 °C.
11. Fill up with 160 μl of staining buffer, spin down, discard supernatant, and wash once more with 200 μl of staining buffer.
12. Add 40 μl of pre-diluted streptavidin-APC (0.2 μg/ml final concentration) to each well and incubate for 15 min at 4 °C.
13. Fill up with 160 μl of staining buffer, spin down, discard supernatant, and wash once more with 200 μl of staining buffer.
14. For immediate cell analysis or sorting with a flow cytometer, resuspend cells in 100 μl flow buffer including 7-AAD (*see* **Note 8**).
15. For transcription factor staining (do not add 7-AAD), resuspend washed cells in 100 μl of freshly prepared fixation buffer and incubate for 15 min at RT, carefully shaking/tilting the plate from time to time (compare **step 6** and **Note 12**).
16. Add 100 μl of the freshly prepared permeabilization buffer and spin down at $400 \times g$ for 5 min. Discard supernatant. Resuspend in 200 μl permeabilization buffer and spin down again.

17. Resuspend cells in 20 µl Fc-block prepared in permeabilization buffer. Incubate for 5 min at RT.
18. Without washing, add 20 µl of 2× transcription factor antibody mixture (prepared in permeabilization buffer) to each well (40 µl final staining volume).
19. Incubate for 30 min at RT.
20. Fill up wells with 160 µl of permeabilization buffer, spin down, and wash once more with 200 µl of permeabilization buffer.
21. Resuspend cells in 100 µl staining buffer and acquire cells on a flow cytometer (*see* **Note 13**). Include single fluorophore stains or fluorescence-minus-one control stains for compensation.

3.3 Analysis of Flow Cytometry Data

1. Load data into your preferred flow cytometry analysis software (e.g. FlowJo).
2. Gate on singlet cells using forward/sideward scatter characteristics.
3. Then, gate on live cells, which are negative for the eF780 viability dye.
4. In the following gates, plot CTV against B220 or CD19 to exclude B cells, against CD4 to identify $CD4^+$ T cells, and against the congenic marker to ultimately identify a pure population of transferred TCR-tg $CD4^+$ T cells that can then be further analyzed for surface marker and/or transcription factor expression (compare Fig. 2 for gating strategy and examples).
5. If desired, individual gates can be drawn for each cell division according to CTV dilution. Usually, a maximum number of up to eight divisions can be reliably detected. These gates can then be used to analyze Tfh marker expression within each division [6, 15].
6. Using the CTV profile of the transferred cells together with the cell division function in FlowJo, further parameters can be determined, including the proliferation index.

4 Notes

1. Adoptively transferred TCR-tg cells can be easily identified within recipient mice if they carry distinct congenic or fluorescent marker genes. For example, different isoforms of the pan-lymphocyte marker CD45 (Ly5) are widely used. Other popular congenic markers include different isoforms of CD90 (Thy1). Crossing TCR-tg strains onto a CD45.1 or CD90.1 (Thy1.1) background provides the advantage that normal C57BL/6 mice, which are $CD45.2^+$ and $CD90.2^+$, can be used as hosts. Usually, heterozygosity in these alleles provides

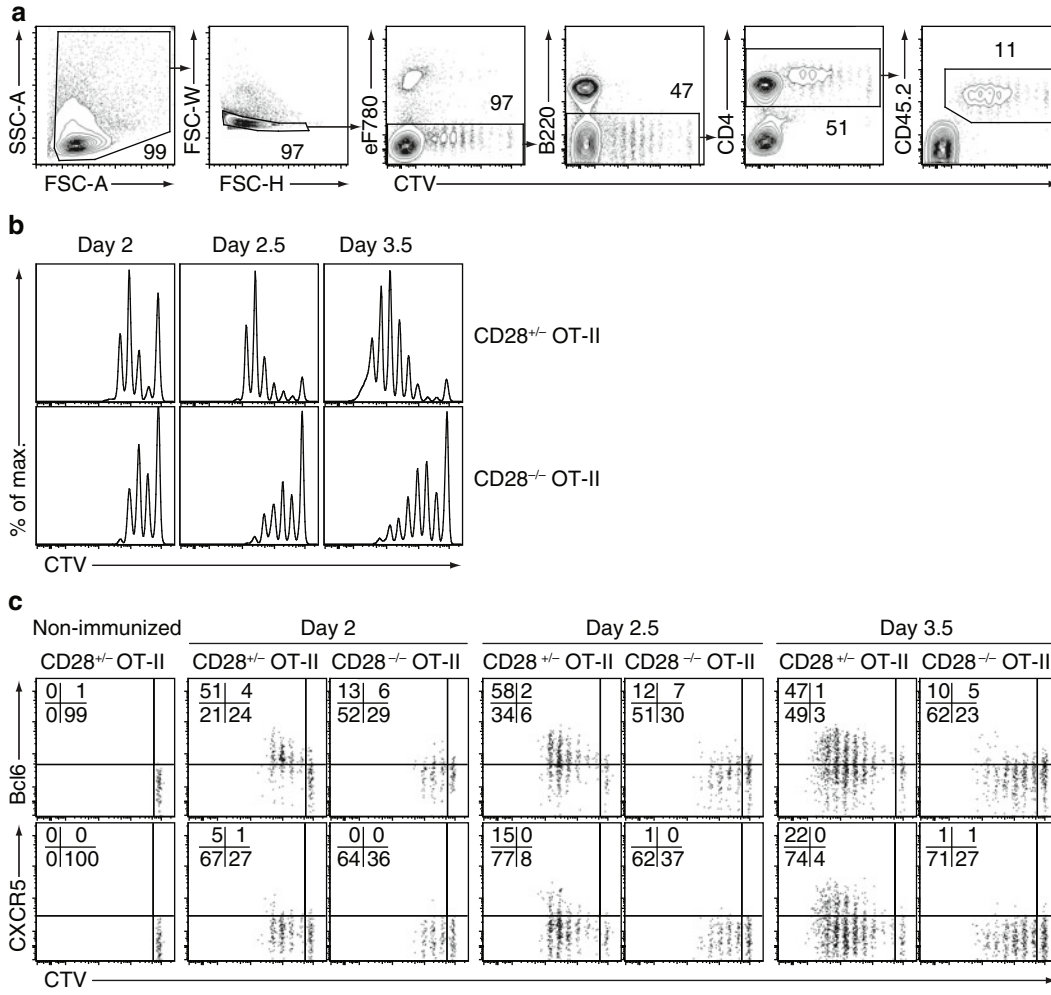

Fig. 2 Example of the described method to dissect early steps during Tfh cell differentiation. The costimulatory molecule CD28 is known to be required for Tfh cell differentiation [20, 21]. Here, we compared CD28-sufficient (heterozygous) control versus CD28-deficient OT-II cells to gain insight into the kinetics of early Tfh cell differentiation. (**a**) Gating strategy for the identification of adoptively transferred OT-II cells. Naïve CD45.2$^+$ OT-II cells (purified as described in Fig. 1) were labeled with CTV and injected into recipient mice. Hosts were immunized with NP_{14}-OVA/alum s.c. in the foot pads at different time points. Draining popliteal lymph nodes from all mice were then analyzed together on the same day. Single-cell suspensions were stained with antibodies as described in the methods section and acquired on a LSR-II cytometer (BD Biosciences). First, 5×10^5 total cells were acquired followed by appended acquisition of CTV$^+$ OT-II cells (*see* **Note 13**). (**b**) Histograms show the differences in the proliferative capacity of control vs. CD28$^{-/-}$ OT-II cells (gated as in **a**). (**c**) CTV profiles of activated OT-II cells at different time points after immunization. Activated CD28-sufficient OT-II cells (gated as in **a**) upregulate Bcl6 within the first few cell divisions. In contrast, induction of the Tfh-defining chemokine receptor CXCR5 occurs at later cell divisions and time points, preferentially in those cells that proliferated the most. CD28 is important for strong Bcl6 upregulation in activated OT-II cells and is absolutely required for the induction of CXCR5. These defects are cell intrinsic and independent of the proliferative capacity of the dividing cells

sufficient expression levels for differentiation between transferred and recipient cells. Choosing heterozygous over homozygous cells might also reduce the risk of rejection of the injected cells, which is an important concern especially in longer-term adoptive transfer experiments. Given the relatively short duration of the described protocol, we have not noticed dramatic changes in cell numbers that might have been caused by potential rejection issues.

2. It is good practice to verify the correct genotype/phenotype of the cells before injection. This can be accomplished by a quick staining of a small aliquot of LN/spleen cells that can be analyzed while at the sorter. Stain for congenic markers and the specific transgenic TCR (e.g. TCR Vα2 in the case of OT-II cells) to verify the correct congenic marker(s) and TCR-tg before injection of the cells into recipients.
3. Pre-enrichment of $CD4^+$ T cells by magnetic bead isolation can help to significantly reduce sorting time on the flow cytometer. Since purity during the pre-enrichment step is not a critical parameter, the volumes of antibodies and beads can be titrated down to spare reagents. We prefer to use a magnetic bead negative depletion approach, which yields "untouched" $CD4^+$ T cells for downstream applications.
4. Naïve cells are preferred over bulk T cell populations as the frequency of activated ($CD44^{high}CD62L^{low}$) T cells might be considerably different between two different genotypes to be analyzed. These memory cells might possess different activation kinetics after adoptive transfer and immunization and could therefore mask the activation characteristics of the naïve cells.
5. We have optimized our protocol for the use of CellTrace Violet (CTV), as it frees the channel that detects FITC, Alexa Fluor 488, GFP, and related flurochromes on the flow cytometer. This has the advantage that GFP/YFP-expressing cells can be easily incorporated into the protocol. CTV also requires much less compensation than CFSE. In our hands, CTV also provides a better resolution than CFSE.
6. The optimal amount of dye for CTV staining should be tailored to the respective TCR-tg cell line as well. OT-II cells tolerate CTV very well [6, 15], whereas SMARTA cells might require lower concentrations of fluorescent dyes to avoid toxicity [7].
7. One major advantage of foot pad immunization with OVA/alum is that the popliteal LN is the only direct draining LN. Furthermore, contralateral injections potentially allow for comparison within the same mouse. One disadvantage is that popliteal LNs of non-immunized mice are very small and thus

harbor few cells. If no comparison is needed, both sides can be used for immunization and both popliteal LNs can be pooled for analysis. In any case, immunization routes need to be performed in accordance with institutional guidelines. In this regard, hock injections, which also drain to the popliteal LNs, represent an ethical alternative to foot pad injections [17].

8. Dead cells may exhibit significant autofluorescence and may bind antibodies nonspecifically. Thus, it is good practice to exclude dead cells during analysis by incorporating a viability dye in the staining procedure. Common dyes such as PI, 7-AAD or DAPI are usually added to samples right before acquisition and thus don't require significant additional hands-on time. It should be noted that these dyes are not fixable. However, staining for intracellular transcription factors or cytokines requires fixation and permeabilization of cells. For these applications, several companies offer special dyes that are fixable and thus allow for the separation of live from dead cells during the acquisition on the flow cytometer and in subsequent analyses of the data.
9. Besides dead cells, other cell populations not necessarily of interest to the question of the experiment might interfere with the analysis of the population of interest, either by high autofluorescence, distinct forward/side scatter characteristics, or due to nonspecific antibody binding. Thus, it is good practice to include Fc-block (unconjugated anti-CD16/32 antibody) and mouse/rat serum incubation steps in each staining protocol. In addition, cells can be excluded in a "dump channel" by staining for multiple lineage-specific markers not expressed on the cells of interest using antibodies conjugated to the same fluorophore. For Tfh cell research, it is highly advisable to at least gate out B cells, as they are $CXCR5^{hi}$ cells, they potentially form T-B cell conjugates, and they are especially "sticky" because of their Fc receptor expression. Addition of EDTA to the sorting and staining buffers reduces cell doublet formation and thereby increases the yield of acquired events as well.
10. Activated cells become blastic, especially during the first few cell divisions. This goes hand in hand with increased FSC/SSC and increased background staining. This should be taken into account when analyzing and drawing conclusions from the data obtained. Antibodies should be carefully titrated by each investigator for optimal staining results. In addition, isotype controls, or, better, cells with genetic deletion of the gene encoding the protein of interest, should be included as negative controls. It should be noted that activated $CD4^+$ T cells from different TCR-tg mouse lines may differ significantly in their FSC/SSC characteristics and fluorescent background, so results for each TCR-tg cell type should be considered

separately. For example, we have noticed that OT-II cells remain relatively small after immunization with OVA/alum, while LCMV-specific TCR-tg SMARTA cells [18] rapidly divide upon viral infection and become highly activated with increased FSC/SSC and autofluorescence.

11. One other issue that might contribute to different results between TCR-tg cell lines might result from the amounts of available antigen and the context of antigenic challenge. Protein immunogens decline in abundance over time, whereas viral infections with replicating virus such as LCMV typically increase rapidly after infection, providing stronger and prolonged stimuli.
12. GFP and other fluorescent proteins are incompatible with the fixation/permeabilization buffers used in the Foxp3 transcription factor staining set. However, they are preserved with PFA/saponin-based fixation/permeabilization procedures and may be compatible with other commercially available buffer combinations as well. This should be kept in mind when using fluorescent reporter mice together with intracellular staining. Investigators are encouraged to determine fixation/permeabilization conditions that fit their own needs.
13. Reducing the size of data files not only saves data storage capacity but also results in much faster software/computer speed during analyses. We usually first acquire 5×10^4 to 1×10^5 events of all cells (to get an overview of background staining and cell types for gating purposes) and then append only gated TCR-tg cells. In this case, gates set up for acquisition should not be as narrow as those used during final analyses of the data.

Acknowledgment

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Chapter 4

Tracking by Flow Cytometry Antigen-Specific Follicular Helper T Cells in Wild-Type Animals After Protein Vaccination

Svetoslav Chakarov and Nicolas Fazilleau

Abstract

Flow cytometry is a valuable technology used in immunology to characterize and enumerate the different cell subpopulations specific for a nonself-antigen in the context of an ongoing immune response. Among them, follicular helper T cells are the cognate regulators of B cells in secondary lymphoid tissues. Thus, tracking them is of high interest especially in the context of protein vaccination. For this purpose, transgenic antigen-receptor mouse models have been largely used. It is now clear that transgenic models are not always the best means to study the dynamics of the immune response since they can modify the response. In this chapter, we describe how to track endogenous antigen-specific follicular helper T cells by flow cytometry after protein vaccination in nonmodified wild-type animals, which ultimately provides a comprehensive way to enumerate, characterize, and isolate these particular cells in vivo.

Key words Flow cytometry, pMHC II tetramer, Monoclonal antibodies, T lymphocytes, B-cell help

1 Introduction

Protein vaccines induce long-term protection through the generation of long-lived plasma cells (PC), which are immunoglobulin (Ig)-secreting cells, and circulating high-affinity memory B cells [1]. Particularly, effectiveness of protein vaccines requires the development of antigen (Ag)-specific helper T cells that regulate the emerging B-cell response. Many reports in the last few years clearly demonstrated that T-cell help involves a distinct lineage of helper T cells called follicular helper T cells (Tfh) [2]. The main feature of Tfh cells is their repositioning in secondary lymphoid organs from the T-cell area to the CXCL13-rich B follicle through CXCR5 expression [3]. There, Tfh cells encounter Ag-primed B cells that express specific peptide MHCII complexes (pMHCII) from the same foreign Ag. This Ag-specific cross talk is finely tuned by a multitude of non-Ag-specific receptor engagements, including co-stimulatory molecules and cytokine delivery, and ultimately, it

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controls the developmental decisions of Ag-primed B cells [4]. It is now clear that Bcl-6, a master transcriptional regulator, drives Tfh-cell differentiation [5–8].

As with all in vivo studies, the choice of the Ag and of the affinities of the specific Ag-receptors (both TCR and Ig) can impact the observations presented in these studies in ways that remained poorly appreciated until recently. It is now well accepted that elevated precursor frequency of Ag-specific T cells impacts the outcome of an immune response [9–11]. Thus, while it has long been thought that transgenic mouse models were helpful to appreciate the dynamics of an immune response, it became obvious that developing new technologies to detect Ag-specific T cells in non-transgenic wild-type mice was needed. In this context, using multiparameter flow cytometry analysis and pMHCII tetramers, we can detect Ag-specific helper T cells in the draining lymph nodes after protein vaccination of wild-type animals.

Here, we will give the example of the immune response of wild-type C57BL/6 mice to the nonself Ag EAWGALANKAVDKA peptide (called 1W1K peptide hereafter) and its specific helper T-cell detection. We propose a protocol to track the 1W1K-specific Tfh cells with a combination of monoclonal antibodies (mAb) and the corresponding pMHCII by flow cytometry analysis. The experiment is usually performed at day 9 corresponding to the peak of the effector response after subcutaneous immunization with Incomplete Freund's Adjuvant (IFA) complemented with type B CpG oligonucleotides (CpG-B), an enhancer of the Tfh-cell response [12], but can also be performed at other time points after immunization such as in the memory phase [13]. Detection of Ag-specific Tfh cells allows the enumeration of the ongoing immune response after protein vaccination but also provides a way to analyze the phenotype and function of these cells as well as to isolate them using a cell sorter in order, for example, to assess their genetic program.

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all local waste disposal regulations when disposing waste materials.

2.1 C57BL/6 Mice

Mice were purchased from Centre d'Elevage Janvier; however, they can also be purchased from other local providers. All experiments were performed in accordance with national and European regulations and institutional guidelines and mouse experimental protocols were approved by the local ethics committee.

2.2 Immunization Components

1. Incomplete Freund's Adjuvant. Store at 4 °C.
2. Sterile Dulbecco's Phosphate-Buffered Saline (PBS).
3. 1W1K peptide (EAWGALANKAVDKA).
4. CpG-B.
5. Double hub microemulsifying needles with reinforcing bar.
6. Glass syringe with metal Luer lock nozzle.
7. Sterile hypodermic needle 26 G.
8. Avertin: 1 g/mL 2, 2, 2-tribromoethanol.
9. Cone-type rodent injection restrainer.

2.3 Lymph Node Collection

1. Operating platform (Styrofoam wrapped in aluminum foil).
2. Appropriate dissecting instruments (forceps, surgical scissors).
3. Glass tissue homogenizer.
4. RPMI 10 %: 10 % fetal bovine serum (FBS) in Roswell Park Memorial Institute 1640 medium (RPMI). Store at 4 °C.
5. 100 μm cell strainer.
6. Cell counter. For example, the Z1 COULTER COUNTER® (Beckman Coulter, CA, USA).

2.4 Cell Staining Components

1. 96-well U-bottom plate.
2. 5 mL FACS tubes.
3. FACS buffer: 5 mM EDTA, 2 % FBS (v/v) in PBS 1×. Store at 4 °C.
4. Blocking buffer: FACS buffer, 2 % naive mouse serum (v/v), and 2 % naive rat serum (v/v). Store at 4 °C.
5. pMHCII tetramer: PE-labeled 1W1K-IA^b tetramer was obtained from the NIH tetramer core facility (Atlanta, GA, USA). Store at 4 °C and protected from light.
6. Labeled monoclonal antibodies (mAb): Alexa Fluor® 488 anti-Bcl-6 (K112-91), allophycocyanin (APC) anti-CXCR5 (2G8); Alexa Fluor® 700 anti-CD8α (53-6.7), Alexa Fluor® 700 anti-B220 (RA3-6B2), PerCP-eFluor® 710 anti-CD4 (RM4-5), phycoerythrin (PE)-Cy7 anti-PD-1 (J43), APC-eFluor® 780 anti-CD44 (IM7). Store at 4 °C and protected from light.
7. Fixable Viability Dye eFluor® 506. Store at −70 °C and protected from light.
8. Foxp3/Transcription Factor Staining Buffer Set (eBioscience).
9. Intracellular blocking buffer: 2 % naive mouse serum and 2 % naive rat serum diluted in permeabilization buffer 1× (eBioscience) prior to use.

2.5 Flow Cytometer

Flow cytometer equipped with three lasers. For example, the BD LSRFortessa™ cell analyzer (BD Biosciences, USA).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Mouse Immunization

3.1.1 Immunization Solution

1. Prepare a stock solution of 1W1K peptide at 10 mg/mL in PBS.
2. Prepare a stock solution of CpG at 1 mg/mL in sterile endotoxin-free physiological water (NaCl 0.9 %). Store at −20 °C.
3. Prepare Ag/CpG-B solution containing 1W1K peptide and CpG-B at 400 μg/mL and 100 μg/mL, respectively, in PBS.
4. Lock two glass syringes onto the double hub needle.
5. Place the Ag/CpG-B solution and an equal volume of IFA into one of the syringe so the final concentration of the emulsion will be 200 μg/mL of 1W1K peptide and 50 μg/mL of CpG-B. Transfer from one syringe to the other until the emulsion is ready (*see* **Note 1**).

3.1.2 Mouse Anesthesia

1. Prepare 100 % Avertin by weighing 10 g of 2, 2, 2-tribomoethanol (Sigma) and dissolving them with 10 mL of 2-methyl-2-butanol (Sigma). Do not vortex but make sure that tribromoethanol is totally dissolved. Store at 4 °C and protect from light.
2. Inject intraperitoneally 2.5 % Avertin at a dose of 0.015 mL/g (*see* **Note 2**).

3.1.3 Subcutaneous Immunization

1. A volume of 100 μL of the immunization solution is injected into each side of the tail (*see* **Note 3**).

3.2 Collection of Draining Lymph Nodes

1. Sacrifice mice 9 days after immunization and put them on the operating platform (*see* **Note 4**).
2. Collect inguinal and periaortic draining lymph nodes (dLN) (*see* **Note 5**).
3. Place dLN in glass tissue homogenizer containing 2 mL RPMI 10 %.

3.3 Cell Suspension and Cell Count

1. Gently crush the dLN using glass tissue homogenizer's piston. Make sure that all the dLN are smashed.
2. Place a 100 μm strainer on the top of 50 mL tube and filter the cell suspension.
3. Wash the cell strainer with 10 mL RPMI 10 % FCS.

4. Measure the total volume and collect 100 μL to count the cell number using a cell counter of your choice.
5. Pellet the cells by centrifugation at 350 × *g* for 5–7 min at 4 °C.
6. Vacuum the supernatant and resuspend the cells in FACS buffer at the volume necessary to yield 8×10^7 cells/mL (*see* **Note 6**). The cells should remain on ice from now on.

3.4 Surface Staining

1. Properly label a 96-well plate and put it on ice. Place 100 μL of sample into each labeled wells.
2. Pellet the cells by centrifugation at 350 × *g* for 5–7 min at 4 °C.
3. Vacuum the supernatant and resuspend the cells with 100 μL blocking buffer and incubate for 15 min at 4 °C.
4. Wash the cells once by adding 100 μL of RPMI 10 % FCS at 350 × *g* for 5–7 min at 4 °C.
5. Remove the supernatant and resuspend the cells in 100 μL FACS buffer containing pMHCII tetramer and APC-labeled anti-CXCR5 mAb (*see* **Note 7**). Incubate for 2 h at room temperature in dark.
6. Add 100 μL of cold FACS buffer and keep the cells at 4 °C for 10 min.
7. Pellet the cells at 350 × *g* for 5–7 min at 4 °C.
8. Remove the supernatant and resuspend the cells in 100 μL FACS buffer containing Alexa Fluor® 700 anti-CD8α, Alexa Fluor® 700 anti-B220, PerCP-eFluor® 710 anti-CD4, PE-Cy7 anti-PD-1, and APC-eFluor® 780 anti-CD44 mAb at optimal concentration and incubate the cells for 45 min on ice protected from light.
9. Wash the cells twice with FACS buffer and once with PBS 1× at 350 × *g* for 5–7 min at 4 °C.
10. After the last wash, vacuum the supernatant and resuspend the cells in PBS 1× containing Fixable Viability Dye eFluor® 506 (*see* **Note 8**) and incubate for 30 min on ice protected from light.
11. Wash the cells three times with PBS 1× at 350 × *g* for 5–7 min at 4 °C.

3.5 Intracellular Staining

1. Prepare a working solution of fixation/permeabilization buffer by diluting Foxp3 fixation/permeabilization concentrate (one part) with Foxp3 fixation/permeabilization diluent (three parts). Store at 4 °C.
2. Remove the supernatant and resuspend the cells in 100 μL fixation/permeabilization working solution and incubate for 30 min at room temperature in the dark.

3. Prepare the permeabilization buffer by diluting the 10× concentrate with deionized/distilled water.
4. Wash the cells three times with permeabilization buffer by centrifugation at 400 × *g* for 5–7 min.
5. After the last wash, remove the supernatant and resuspend cells in 50 μL of intracellular blocking buffer and incubate 15 min at room temperature in the dark.
6. Add 50 μL permeabilization buffer containing Alexa Fluor® 488 anti-Bcl6 mAb at optimal concentration. Homogenize and incubate for 45 min at room temperature in the dark.
7. Wash cells three times with permeabilization buffer at 400 × *g* for 5–7 min.
8. After the last wash, remove the supernatant and resuspend the cells in 100 μL FACS buffer.

3.6 Flow Cytometry Analysis of the Prepared Samples

1. Transfer the cell suspension into its respective FACS tube and add 200 μL of FACS buffer to have a final volume of 300 μL for each sample.
2. Run the samples on a flow cytometry analyzer (*see* **Note 9**).
3. Analyze the data using FlowJo software (Tree Star, CA, USA) or an equivalent software (*see* **Note 10**). As depicted in Fig. 1 (also *see* **Note 11**), 1W1K-specific Tfh cells correspond to the cells that have the following phenotype: eFluor® 506^{-} (CD8α, B220)$^{-}$ CD4^{+} CD44^{hi} 1W1K/IA-^{b} tetramer^{+} CXCR5^{+} PD-1^{+}.

4 Notes

1. Start to pump between the two syringes until a uniform white mixture forms. Carefully remove entrapped air and replace the double hub needle with a sterile hypodermic 26 G needle. Prepare 200 μL mixture in excess because of the dead volume in the double hub needle.
2. Prepare aliquots of 2.5 % Avertin from the 100 % Avertin by diluting in PBS 1×. Be sure to stand 100 % Avertin at room temperature before diluting it. Store 2.5 % Avertin at 4 °C protected from light.
3. Secure mouse restrainer and place the anesthetized animal in restrainer for injection. Whip the area at the base of the tail with 70 % ethanol on a gauze sponge. Hold tautly the skin and insert the needle subcutaneously into the most proximal region of the tail and to one side. Progress needle by angling toward inguinal region for approximately 2/3 of needle's length. Introduce 100 μL of emulsion. Repeat injection to opposite side of the tail toward the other inguinal area.

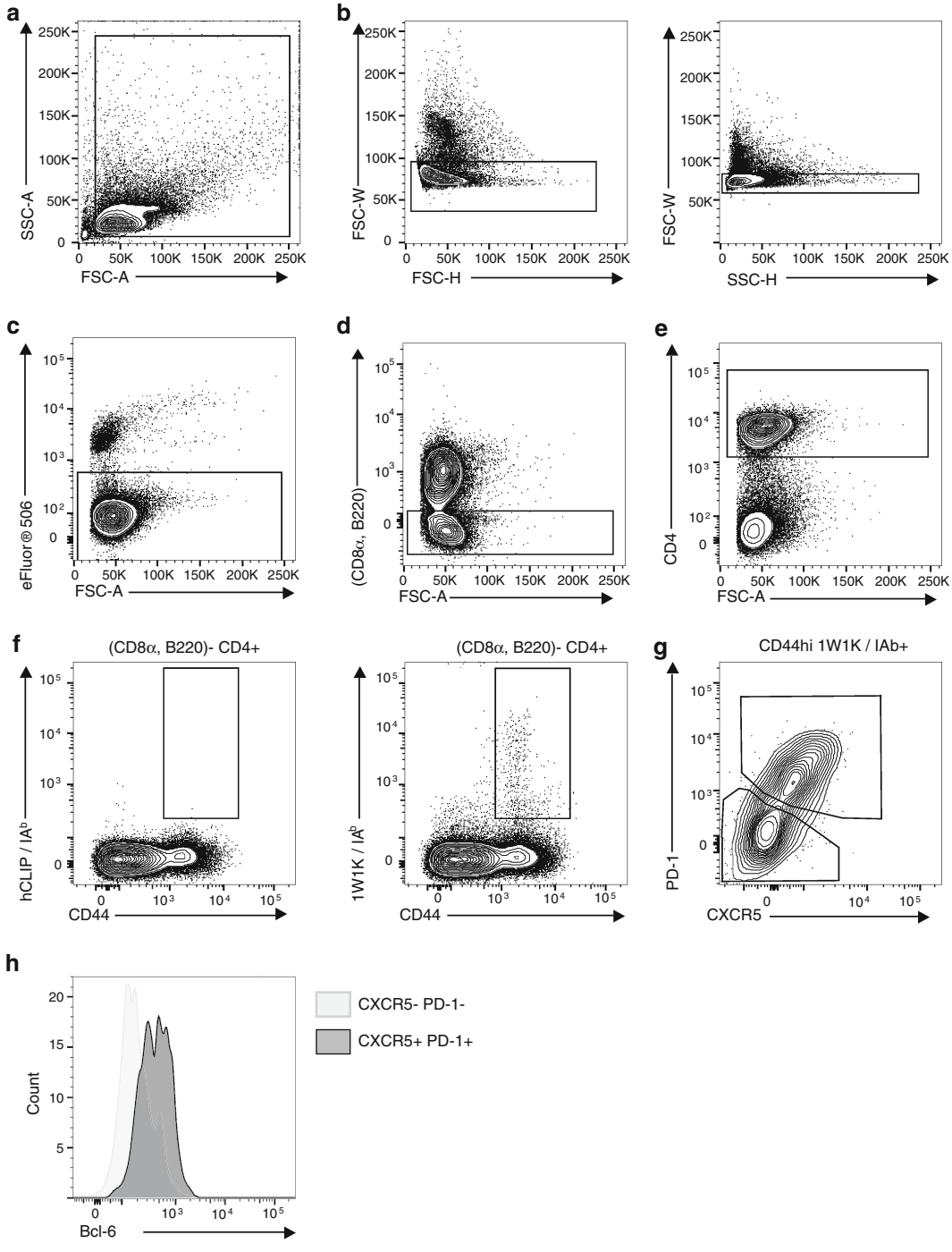

Fig. 1 Gating strategy to get access to 1W1K-specific Tfh cells. Nine days after s.c. immunization with 40 μg of 1W1K in IFA + CpG-B, dLN were analyzed for the detection of 1W1K-specific Tfh cells (**a**-**h**)

4. Prepare the bench where the work is to be done. Place the operating platform on the bench, and disinfect with 70 % ethanol. Lay out the dissecting tool and glass tissue homogenizer containing the appropriate amount of 10 % RPMI.
5. Make an incision in the skin at the midpoint of the abdomen, and using fingers, continue the cut around the mouse's waist. Pull the skin above the first incision up and over the head and secure using a pin. Cut the skin from the abdomen down the midline to the genitals, then from there down each of the hind legs. Pin these flaps open to expose the abdominal region. Make an incision in the abdominal membrane to expose the cavity. Locate and gently remove the inguinal lymph nodes from the skin flaps that were pinned, and the periaortic nodes from around the aorta.
6. Rub the bottom of the tube with your fingers to break up the pellets of sample.
7. Before doing the experiment, each reagent has to be titrated to find optimal concentration of usage. Make a 2× mAb solution containing all the mAb except the mAb to be tested. Pellet 100 µL of cells at 8×10^7 cells/mL. Vacuum the supernatant and resuspend the cells with 50 µL of the 2× mAb solution. Make dilutions of the reagent to be tested and add 50 µL of each dilution to the cell suspension. Stain for 45 min at 4 °C on ice, wash once, and analyze by flow cytometry. The geometric mean fluorescence intensities (gMFI) of the positive and negative signals are estimated for each concentration of the mAb. The dilution to be used corresponds to the one for which the ratio of the gMFI of the positive population/gMFI of the negative population is the greatest. Usually, stainings are performed for 45 min on ice except for pMHCII tetramer and anti-CXCR5 mAb for which stainings are performed for 2 h at room temperature.
8. Fixable Viability Dye eFluor® 506 is supplied as a pre-diluted solution prepared in DMSO. It may be freeze-thawed up to 20 times. Allow vial to equilibrate to room temperature before opening and use it at a dilution of 1:1,000. Other fixable viability dyes can also be used.
9. Before running the samples on the flow cytometer, be sure that you have unstained cells and labeled cells with only one of the mAb so you can set up the BD LSRFortessa™ cell analyzer and make the compensations.
10. In order to objectively place the expression gates, negative controls have to be used. Thus, for each used mAb, a negative control corresponds to the staining of the studied cells with all the mAb except the mAb of interest substituted by an isotype control mAb conjugated with the same fluorophore than the

mAb of interest. The same is done for pMHCII tetramer except that the control reagent is an irrelevant tetramer (human CLIP 87-101 PVSKMRMATPLLMQA-I-A^b, NIH Tetramer Core Facility).

11. To get access to the 1W1K-specific Tfh cells, perform the following gating strategy. Gate on cells (Fig. 1a) and exclude cell doublets (Fig. 1b). Dump out dead cells (eFluor506$^+$ cells) (Fig. 1c) and (CD8α, B220)$^+$ cells (Fig. 1d). Gate on the CD4$^+$ cells (Fig. 1e). Among these latter, focus on the 1W1K-specific helper T cells that are CD44^{hi} tetramer$^+$ (Fig. 1f). Gate of inclusion is placed based on the irrelevant hCLIP/I-A^b tetramer (Fig. 1f). Tfh cells are CXCR5$^+$ PD-1$^+$ cells (Fig. 1g) as proven by their intracellular expression of the master regulator Bcl-6 (Fig. 1h).

Acknowledgments

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Chapter 5

Retroviral Vector Expression in TCR Transgenic CD4+ T Cells

Youn Soo Choi and Shane Crotty

Abstract

The regulation of gene expression is key to understand the function of genes of interest. To explore the biological functions of genes, transgenic knock-in or knockout technologies have served as invaluable tools. While recent advances in molecular biology have introduced new techniques (i.e., CRISPR mediated gene editing) (Cong et al., Science 339(6121):819–823, 2013; Wang et al., Cell 153(4):910–918, 2013) for the generation of transgenic mice in a relatively short period of time, it can still take a long time to test biological hypotheses from scratch to design how to generate knock-in or knockout mice. Here, we describe methods to manipulate gene expression in T cell receptor (TCR) transgenic CD4 T cells, which allow us to investigate gene functions in the study of differentiation pathways of follicular helper T (Tfh) cells.

Key words Tfh cells, Primary CD4 T cells, TCR transgenic CD4 T cells, Retroviral plasmid, Overexpression, Knockdown

1 Introduction

Biological phenomena are outcomes of cellular responses to various external signals. These responses are controlled by cellular strategies to regulate expression of myriads of different genes [3]. Our immune cells are controlled not to overreact to our own cells or tissues in steady state, but to become activated and differentiate into effector cells for elimination of foreign or dangerous substances [4]. Modulation of gene expression is an important mechanism used by immune cells to program their activation and differentiation into effector cells. Therefore, it is very important to understand how effector functions of immune cells are regulated at the level of gene expression. Transgenic mouse technology has made significant contributions to reveal which genes of interest may be critical for regulation of our immune system [5]. Moreover, generation of T cell receptor transgenic (TCRtg) mice [6], whose TCRs are specific for a single antigen, has advanced our understandings on immunobiology of T cells. A technical

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challenge to study naïve T cells, few of which in nature respond to a given antigenic stimulation in vivo, was overcome by the use of TCRtg CD4 T cells and their cognate antigens. These tools enabled researchers to dissect how activation signals are triggered in T cells following recognition of cognate antigens.

Among the subsets of effector CD4 T cells, follicular helper T (Tfh) cells are relatively recently identified [7]. The major function of Tfh cells is to help B cells form germinal center reactions for generation of long lived plasma cells and B cell memory. The transcription factor Bcl-6 has been identified as a major fate regulator for Tfh differentiation [8–10]. However, key information is still missing in our understanding of this process, as forced Bcl-6 expression is not sufficient for activated CD4 T cells to acquire a Tfh differentiation program in vitro [11]. Therefore, more in-depth analysis is needed to reveal the induction and regulation of signaling pathways that control Tfh differentiation [12, 13].

In contrast to other effector CD4 T cells, such as Th1, Th2, Th17, and iTreg cells, there are currently no in vitro stimulation techniques that reproducibly lead to Tfh differentiation of CD4 T cells [7]. As such, we are heavily dependent on in vivo systems to understand how Tfh differentiation is regulated in CD4 T cells. To this end, TCRtg CD4 T cells with known antigen specificity are quite beneficial to track activation and differentiation of CD4 T cells during the early phases of an immune response to in vivo stimulation. In this section, we delineate our protocol to make use of retroviral vector mediated regulation of gene expression in TCRtg CD4 T cells to analyze how CD4 T cells are programmed to differentiate into Tfh cells in vivo.

2 Materials

Prepare all culture media and reagents inside biosafety cabinet under aseptic conditions.

2.1 Cell Culture

1. PLAT-E retroviral packaging cell line.
2. Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and L-glutamine, without sodium pyruvate.
3. Phosphate-buffered saline (PBS) without calcium and magnesium.
4. Trypsin–EDTA (0.25 %).
5. D10 culture media: DMEM, 10 % fetal bovine serum (FBS) (v/v), 2 mM GlutaMAX, 100 U/mL penicillin–streptomycin.
6. 15 cm culture dish: tissue-culture treated.
7. 6-well flat-bottom culture dish.
8. 24-well flat-bottom culture dish.

2.2 Retroviral Vector Preparation

1. Vectors: we recommend using pMIA or pMIG for overexpression and pLMPd or pLMAd for knockdown of your genes of interest (Fig. 1).
2. Taq polymerase (optional).
3. Restriction enzymes with recommended buffers.
4. DNA Ligase.
5. Competent bacterial cells.
6. Luria broth (LB).
7. Selective antibiotic compatible with vector of interest.
8. Luria broth agar plate with antibiotic of choice.
9. Miniprep and maxiprep plasmid purification kits.

2.3 Transfection and Transduction

1. Polyethylenimine (PEI. Linear, MW: 25,000): stock at 1 mg/mL.
2. Polybrene: 10 mg/mL.
3. 0.45 μm syringe filters.
4. 5 mL polystyrene round-bottom tubes.
5. 15 mL or 50 mL conical tubes.
6. 20 mL or 50 mL syringes.
7. Sorting buffer: Sterile PBS, 0.2 % BSA (w/v).
8. Fluorescence activated cell sorter compatible with fluorescent proteins of choice.

2.4 Magnetic CD4 T Cell Isolation

1. MACS buffer: PBS, 0.5 % bovine serum albumin (w/v), 2 mM EDTA.
2. Magnetic mouse CD4 T cell isolation kit.
3. Isolation magnets: As per the manufacturers' instructions.

2.5 In Vitro Activation of CD4 T Cells

1. Antibodies: Anti-CD3 (2C11) and anti-CD28 (37.51).
2. Cytokines: Recombinant human or murine IL-2 and IL-7.
3. 2-mercaptoethanol.

2.6 Mice

1. C57Bl/6J mice.
2. CD4 T cells TCRtg mice that express TCRs specific for LCMV Gp61-80 (SMARTA, SM) or OVA323-339 (OTII).

3 Methods

Perform all procedures inside biosafety cabinet under aseptic conditions. PLAT-E retroviral packaging cell line [14] and primary mouse CD4 T cells are cultured in D10 media unless described otherwise.

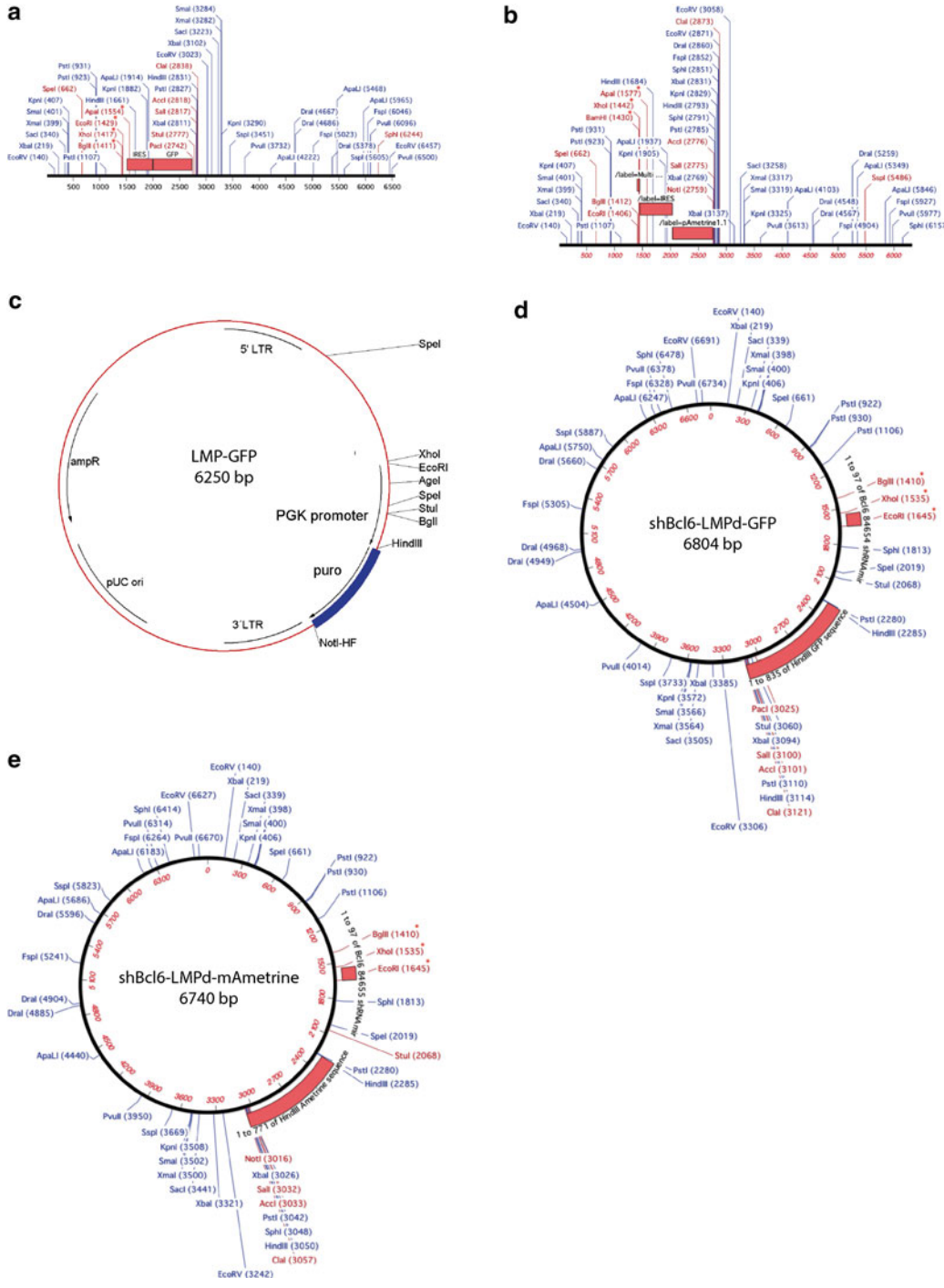

Fig. 1 Maps of retroviral plasmid backbones. (**a**, **b**) Linear maps of retroviral pMIG (**a**) and pMIA (**b**) backbones with restriction enzyme sites (*asterisks*) upstream of IRES-GFP or IRES-mAmetrine. (**c**) Original LMP vector with puromycin resistant gene (highlighted *blue*, adopted from http://www.bioss.uni-freiburg.de). (**d**, **e**) Modified LPMd vectors with shRNAs against Bcl6 upstream of IRES-GFP (**d**) or IRES-mAmetrine (**e**). Restriction enzyme sites for cloning are highlighted with *asterisks*

3.1 Grow and Prepare PLAT-E Cells

1. Grow PLAT-E cells in 15 cm culture dish by seeding 7 and 3.5 million cells for 2 day and 3 day culture, respectively.
2. Split the cells into new flasks by aspirating the old media and wash the plate gently with 5–10 mL of PBS equilibrated at room temperature (RT).
3. Replace PBS with 3 mL of 0.25 % Trypsin–EDTA equilibrated at RT and incubate the plate at 37 °C for 1–2 min.
4. Collect the cells by washing the plate with 12 mL of pre-warmed D10 media, transfer the cells into 50 mL conical tube, and spin the tube at 350 × *g* for 5 min.
5. Discard the supernatant and resuspend the cells in 3 mL of pre-warmed D10 media by gently pipetting up and down culture media.
6. Count cells with a small aliquot of cell suspension and seed the cells at the required concentration for the subsequent procedures. For the method described here we use one million PLAT-E cells per well of 6-well plates in 2 mL of D10 media.

3.2 Retroviral Plasmids Preparation

Depending on the purpose of studies, pMIG (or pMIA) or pLMPd (or pLMAd) backbone vectors (Figs. 1 and 2) can be utilized for ectopic overexpression or knockdown of genes of interest, respectively (*see* **Note 1**). The specifics of the cloning protocol will depend on the gene of interest and the vector used; here a general protocol is outlined for cloning.

1. Prepare DNA. The insert to be cloned can be amplified by PCR (overexpression), generated by annealing of specifically designed palindromic oligonucleotides (shRNA) or subcloned from an existing vector.

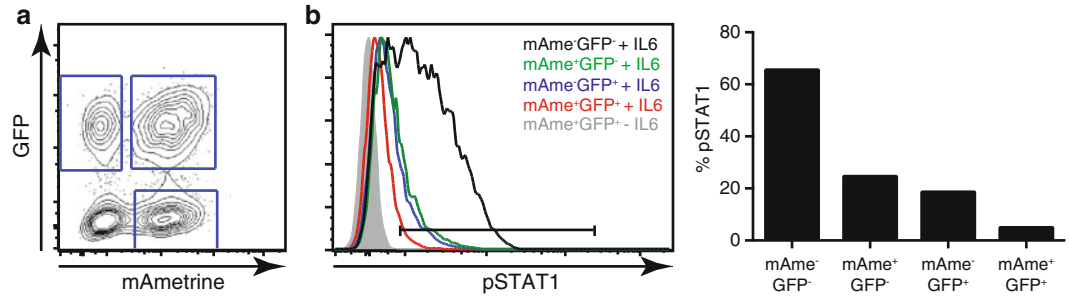

Fig. 2 Double knockdown of STAT1 gene with two shRNAs. TCRtg CD4 T cells were transduced with two shRNAs specific for STAT1 cloned into modified pLMPd-GFP or mAmetrine vectors. (**a**) Representative contour plot of transduced CD4 T cells. Gates indicate single positive (GFP+ or mAmetrine+) and double positive (GFP+mAmetrine+) for reporter proteins. (**b**) Shown here are overlaid histograms of phosphorylated STAT1 of transduced CD4 T cells 30 min after IL-6 stimulation, detected by anti-pY701 STAT1 mAb (BD Biosciences). Quantification made by percent of pSTAT1+ CD4 T cells

2. Select restriction enzymes for your insert and vector and determine the appropriate reaction buffers according to the manufacturer's guidelines.
3. Perform a restriction digest to linearize the vector and prepare the insert for cloning according to the manufacturer's instructions.
4. Ligate the vector and insert using a ligation enzyme according to the manufacturer's instructions. For example DNA T4 ligase is commonly used.
5. Transform the vector into competent bacteria and plate the transformed bacteria on LB agar plates with the relevant antibiotics. Follow instructions specific to the type of competent bacteria you are using.
6. Select positive bacteria by PCR, grow them in Luria broth, and extract the plasmid of interest by miniprep or maxiprep following the manufacturer's guidelines.
7. Check the vectors for correct integration of the insert by restriction digest or sequencing.

3.3 Retrovirus Production

In this protocol the day of seeding PLAT-E cells is designated as Day 0 and retrovirus production is performed in 6-well plates.

3.3.1 Preparation of PEI Solution

1. Add 0.1 g of PEI to 100 mL of room temperature PBS.
2. Adjust the pH to 7.0 by adding a few drops of concentrated HCl with agitation.
3. Hold with agitation for 1 h at room temperature.
4. Sterilize the PEI solution with 0.2 μm filter.
5. Make aliquots and store at −20 °C.

3.3.2 Transfection of PLAT-E Cells

1. At Day 0 (*see* **Note 2**), seed one million PLAT-E cells per well of 6-well plates in 2 mL of D10 media.
2. On the following morning (Day 1), when the PLAT-E cells reach about 60–80 % confluence, replace the old media with 1 mL of pre-warmed (37 °C) D10 media.
3. An hour later, begin to prepare DNA plasmids by adding 5 μL of PEI (Stock: 1 mg/mL) to 100 μL of DMEM (per well) media pre-equilibrated at RT in 5 mL polystyrene round-bottom tubes.
4. Gently vortex and incubate for 10 min at RT.
5. Add 2 μg of plasmid DNA into DMEM/PEI and gently mix the mixture (*see* **Note 3**).
6. Incubate for 15 min at RT.
7. Add ≥100 μL of plasmid DNA/PEI mixture to one well of PLAT-E cells drop wise.

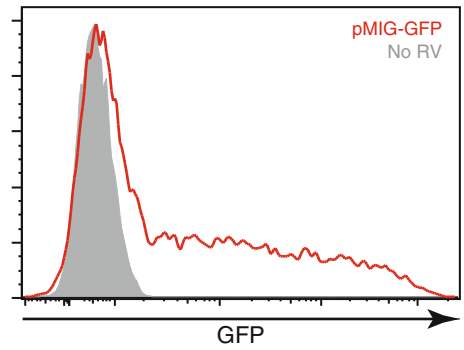

Fig. 3 Transfection efficiency. PLAT-E cells were harvested 2 days after transient transfection with retroviral DNA plasmids with GFP reporter protein. Shown here is a representative histogram of GFP-expressing PLAT-E cells

8. 6 h later, replace old media with 1.5 mL of pre-warmed D10 media.
9. 24 h later (Day 2), collect and filter supernatant through 0.45 μm syringe filters into 15 mL or 50 mL conical tubes (depending on the volume of culture media) and keep at 4 °C.
10. Slowly add 1.5 mL of pre-warmed D10 media to the now empty wells of transfected PLAT-E cells. Incubate at 37 °C.
11. Roughly 24 h later (Day 3), repeat **steps 9** and **10**, except adding 1 mL of PBS instead of pre-warmed D10 media to plates.
12. Optional. Collect the PLAT-E cells by vigorously pipetting PBS up and down to enable checking of the transfection efficiencies with flow cytometry if desired (Fig. 3).

3.4 In Vitro Activation of Primary CD4 T Cells

All the procedures described below are performed at Day 2 relative to retrovirus production. For the convenience of spin-infection (spinoculation) schedule, procedures are recommended to begin between 3 and 4 p.m. (an outline of timing the following steps is shown in Fig. 4).

1. Coat 24-well plates with 8 μg/mL of anti-CD3 and anti-CD28 antibodies diluted in PBS, and incubate for ≥1 h at 37 °C. For example, 330 μL is enough for one well of 24-well plates.
2. Naïve CD4 T cells are obtained through negative isolation from T cell receptor transgenic (TCRtg) mice (*see* **Note 4**). For example use, CD4 T cells specific for LCMV-gp protein (SMARTA, SM) or protein ovalbumin (OTII). Naïve CD4 T cells are isolated using a magnetic CD4 T cell isolation kit of choice according to the manufacturer's instructions (*see* **Note 5**).
3. Inside biosafety cabinet, flick 24-well plates and wash the plates with PBS equilibrated at RT ≥two times to remove uncoated anti-CD3 and anti-CD28 antibodies. PBS remains in the plate

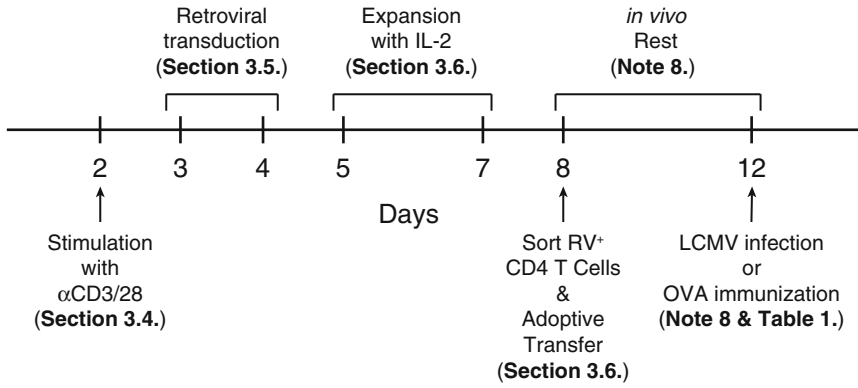

Fig. 4 Time line for ex vivo analysis of Tfh differentiation with RV+ TCRtg CD4 T cells. Schematic representation of time line laying out in vitro stimulation, retroviral transduction, and adoptive transfer of TCRtg CD4 T cells to study Tfh differentiation ex vivo

for the last wash. In the meantime CD4 T cells are being prepared.

4. Resuspend isolated TCRtg CD4 T cells in pre-warmed D10 media including 2 μM 2-mercaptoethanol and 2 ng/mL recombinant human or murine IL-7 at concentration of 2×10^6 cells/mL.
5. Flick the plate to remove PBS and seed 1 mL of the CD4 T cell suspension per well.
6. Spin the plate at $400 \times g$ for 1 min and incubate the plate at 37 °C, proceed with transduction (Subheading 3.5).

3.5 Transduction

This protocol is for double transduction of TCRtg CD4 T cells consisting of two spinoculations at Day 3 (about 22–24 h after stimulation of TCRtg CD4 T cells) and Day 4 (about 40–42 h after stimulation), when T cell mitosis occurs. If a single transduction is required it is recommended to change and optimize this protocol.

1. About 22–24 h after T cell stimulation (Day 3), spin and prewarm centrifuge to 37 °C.
2. Prepare 1 mL of RV supernatant (obtained in Subheading 3.3) per well of 24-well plates and warm in 37 °C water bath or equivalent.
3. Add 2 mM 2-mercaptoethanol and 10 μg of polybrene (Stock: 10 mg/mL) per 1 mL of pre-warmed RV supernatant.
4. Aspirate the old media from 24-well plates with TCRtg CD4 T cells and add 1 mL of RV supernatant.
5. Spin the plate at $400 \times g$ for 1 and ½ to 2 h with temperature set at 37 °C.

6. Aspirate the media containing RV and add 1 mL of pre-warmed D10 media with 2 mM 2-mercaptoethanol and 10 ng/mL recombinant human or murine IL-2.
7. Incubate the 24-well plates at 37 °C overnight.
8. At around 8–9 a.m. on the following morning (Day 4), conduct the second infection by repeating **steps 1–7**.

3.6 Expansion of TCRtg CD4 T Cells and Adoptive Transfer

Following retroviral vector transduction, TCRtg CD4 T cells are cultured in the absence of anti-CD3 and anti-CD28 antibodies for an additional 3 days prior to being adoptively transferred into recipient mice.

1. 66–72 h after in vitro stimulation (Day 5) aspirate old media (*see* **Note 6**) and add 1 mL of pre-warmed D10 media containing 2 mM 2-mercaptoethanol and 10 ng/mL of recombinant IL-2 into each well.
2. Take cells out of the plate by pipetting up and down with additional 1 mL of pre-warmed D10 media containing 2 mM 2-mercaptoethanol and 10 ng/mL of recombinant IL-2 per well and transfer into 6-well plate.
3. Add an additional 1 mL of pre-warmed D10 media containing 2 mM 2-mercaptoethanol and 10 ng/mL of recombinant IL-2 per well (cells should be in ~3 mL of culture media) and culture for 2 days at 37 °C.
4. At Day 7, collect cell suspension into 15 or 50 mL conical tubes (depending on volume of cell suspensions), and spin down the cells at 350 × *g* for 5 min.
5. Remove supernatant and resuspend pellets with pre-warmed D10 media containing 2 mM 2-mercaptoethanol and 2 ng/mL of recombinant human or murine IL-7 (*see* **Note 7**).
6. Plate the resuspended cells into 6-well plates using 3 mL per well, and culture them in an incubator at 37 °C overnight.
7. At Day 8, collect the cells in 15 or 50 mL conical tubes, spin the tubes, and resuspend the cells in sorting buffer.
8. Flow-sort the transduced cells based on positive expression of reporter protein in the expression vector used, such as GFP and/or mAmetrine (Fig. 5).
9. Count reporter protein expressing TCRtg CD4 T cells and prepare the desired number of cells using plain DMEM media for adoptive transfer (*see* **Note 8**).
10. Examine the phenotype of the transferred cells at the timepoint/s of interest (*see* **Note 9**), this will depend on the specific biological question being asked.

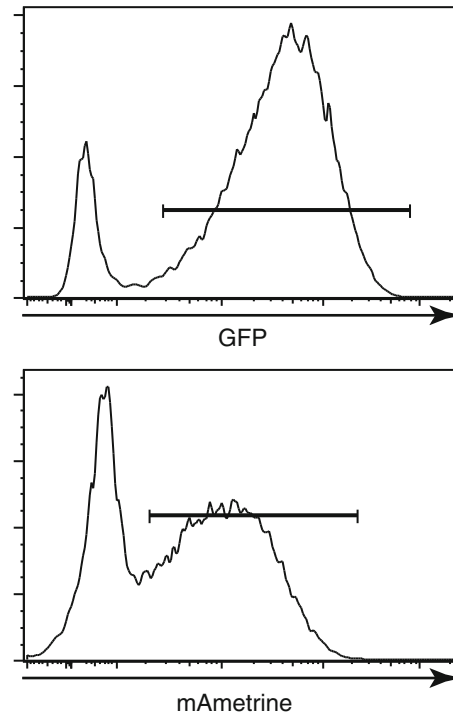

Fig. 5 Sorting infected CD4 T cells for adoptive transfer into mice. Representative histograms of TCRtg CD4 T cells after transduction with GFP (*upper*) or mAmetrine (*lower*)-expressing retroviral vectors after cell separation for adoptive transfer into recipient mice

4 Notes

1. Vectors recommended for use.
   pMIG (MSCV-IRES-GFP) is an MSCV-based mammalian expression vector (Open Biosystems). cDNAs of interest are inserted into the vector using the multiple cloning sites [BglII (1411), XhoI (1417), or EcoRI (1429)] (Fig. 1a), for expression in primary T cells.

   pMIA (MSCV-IRES-mAmetrine) vector was generated by replacing GFP in the pMIG backbone with mAmetrine fluorescent protein (excitation wavelength: 406 nm; emission wavelength: 526 nm) [15] to enhance possible usage of overexpression vector in multicolor flow cytometric analysis by making the FITC channel available [16]. For detection of mAmetrine, 505 LP dichroic filter and 525/50 (530/30 as an alternative) emission filter is used with a multicolor flow cytometry equipped with a 405 nm violet laser. Genes of interest are cloned into the vector using combinations of two restriction enzyme sites among EcoRI (1406), BglII (1412), BamHI (1430), or XhoI (1442) (Fig. 1b).

pLMP [MSCV/LTRmiR30-PIG (Puro^R-IRES-GFP)] is an MSCV-based retroviral vector (Open Biosystems), from which shRNAs are expressed from the LTR promoter (Fig. 1c). Gene specific shRNAs are inserted into the plasmid using XhoI and EcoRI restriction enzyme sites between 5′- and 3′-miR30 sequences. The puromycin resistant gene was deleted in modified version of pLMP, pLMPd (Fig. 1d), due to a rejection problem of pLMP-expressing cells transferred to mice that were subsequently given with in vivo stimulation (i.e., LCMV infection) [17].

pLMAd is a pLMPd derived plasmid encoding mAmetrine (Fig. 1e) instead of the GFP reporter protein. The pLMAd vector is a good alternative to consider for strong inhibition of target gene expression by transducing cells with two different shRNAs [18]. For instance, pLMPd and pLMAd vectors were cloned with two shRNAs that target STAT1 mRNA at different locations, which resulted in the strongest knockdown of the STAT1 gene expression in doubly transduced CD4 T cells (Fig. 2).

2. It is recommended to seed PLAT-E cells at around 5 pm to get 60–80 % confluence in the morning of Day 1. Numbers of PLAT-E cells need to be adjusted to have similar confluence when cells are seeded at different time.
3. To increase virus production, 1 μg of pCL-Eco [19], ecotropic retroviral packaging vector, could be added per 2 μg of plasmid DNA of interest without additional PEI being added.
4. Unless TCRtg mice are on RAG-deficient background, TCRtg mice younger than 12 weeks give good percentage of TCRtg CD4 T cells after isolation. The purity of TCRtg CD4 T cells typically reduces when CD4 T cells are isolated from TCRtg mice on C57BL/6J background older than 12 weeks old.
5. It is recommended to verify the purity of isolated TCRtg CD4 T cells by flow cytometry (Fig. 6).

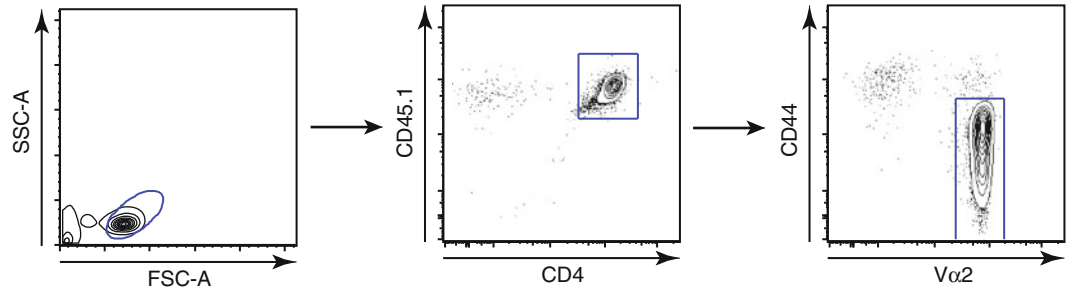

Fig. 6 Purity of TCRtg CD4 T cells after isolation. CD45.1+ LCMV-specific TCRtg CD4 T cells (SMARTA, SM) were negatively isolated for adoptive transfer into CD45.2+ mice. Shown here are representative contour plots of SM CD4 T cells after isolation. ≥90 % of gated lymphocytes (*left*) are Vα2-expressing SMARTA CD4 T cells (*middle* and *right*)

Table 1
Experimental conditions for studies of Tfh differentiation of RV⁺ TCRtg CD4 T cells

TCRtg CD4	Conditions				
SMARTA	Time points of analysis	Day 2	Day 3	Day 4	>Day 7
	Cell number	1.0E+06	5.0E+05	2.5E+05	2.0E+04
	LCMV PFU	1.0E+06	5.0E+05	2.5E+05	2.5E+05
OTII	Time points of analysis	Day 3	>Day 7		
	Cell numbers	1.0E+06	5.0E+05–1.0E+06		
	OVA (μg)	5.0E+01	3.0E+01		

Cell numbers of RV⁺ TCRtg CD4 T cells for adoptive transfers and PFU of LCMV virus for infection and μg of protein OVA for immunization

6. At this stage the color of the media should be yellow. If it is not the case the in vitro stimulation of TCRtg CD4 T cells may not have worked.
7. Cell recovery and transduction efficiencies can be checked by flow cytometry with small aliquots of transduced cells if desired.
8. It is strongly recommended to have an in vivo resting period after adoptive transfer of RV⁺ TCRtg CD4 T cells, prior to administration of stimuli in vivo. Immediate restimulation can cause activation induced cell death. By having a rest period, RV⁺ TCRtg CD4 T cells respond to infection or immunization in similar ways to naïve TCRtg CD4 T cells. Cell numbers of RV⁺ SM and OTII TCRtg CD4 T cells for adoptive transfer and doses of infection or immunization need to be optimized. Common conditions in our laboratory are shown in Table 1 and references [8, 12, 16–18].
9. We have observed that RV⁺ TCRtg CD4 T cells do not migrate into draining lymph nodes as efficiently as spleen. This interpretation is based on much lower abundance of pre-activated RV⁺ TCRtg CD4 T cells in lymph nodes than naive counterparts following immunization, whereas similar frequencies of TCRtg CD4 T cells are found in spleens, regardless of pre-activation steps for RV transduction. We reason this phenomenon occurs due to strong downregulation of CD62L following in vitro stimulation of TCRtg CD4 T cells. There is a strong dependency on CD62L expression to transmigrate into LNs in comparison to migration into spleens.

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Chapter 6

Two-Photon Microscopy for Imaging Germinal Centers and T Follicular Helper Cells

Menna R. Clatworthy

Abstract

One of the principle features of immune cells is their dynamic nature. Lymphocytes circulate in the blood between secondary lymphoid organs and tissues in an effort to maximize the likelihood of a rapid and appropriate immune response to invading pathogens and tissue damage. Conventional experimental techniques such as histology and flow cytometry have greatly increased our understanding of immune cells, but in the last decade, two-photon microscopy has revolutionized our ability to interrogate the dynamic behavior of immune cells, a facet so critical to their function. Two-photon microscopy relies on the excitation of fluorophores by simultaneous application of two photons of longer wavelength light. This allows a greater depth of imaging with minimal photodamage. Thus, living tissues can be imaged, including immune cells in lymph nodes. This technique has been used to interrogate the events occurring in a germinal center response and the interactions between cells in the germinal center, including T follicular helper cells (Tfh), germinal center B cells, and follicular dendritic cells (FDC). Herein, a method is described by which the interactions between Tfh and B cells within a germinal center in a popliteal lymph node can be imaged in a live mouse.

Key words Two-photon, Popliteal lymph node, Tunable laser, Fluorescent reporters

1 Introduction

1.1 Germinal Centers and T Follicular Helper Cells (Tfh)

The generation of high-affinity antibody is the central process in humoral immunity. For T-dependent antigens, this requires a cognate interaction between antigen-specific B and T cells, a process that takes place within secondary lymphoid organs (the lymph node and spleen). Much of our knowledge about this process has been informed by studies in mice. Static imaging via immunofluorescence and confocal microscopy has revealed a highly organized microanatomical structure within lymph nodes in which B cells are constrained within peripheral follicles and T cells reside more centrally in the paracortex (Fig. 1a, b). Upon acquisition of antigen, follicular B cells relocate to the T-B border to seek cognate help from pre-Tfh. If they receive such help, their fate may be twofold;

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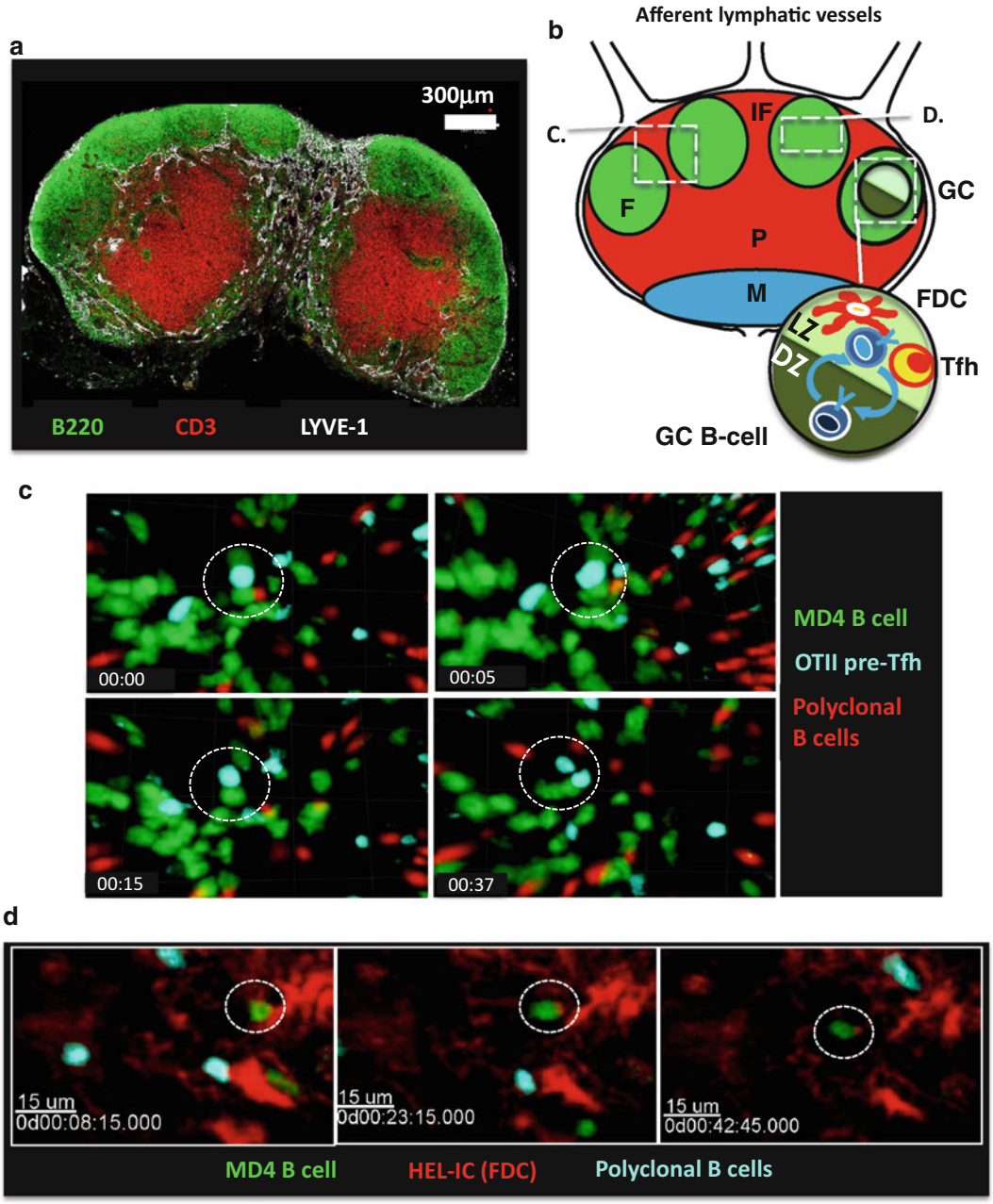

Fig. 1 Lymph node anatomy. (**a**) Confocal micrograph of murine inguinal lymph node demonstrating compartmentalization of B cells and T cells. 30 µm section stained with B220 antibody (*green*), CD3 antibody (*red*), and LYVE-1 antibody (*white*) to label lymphatics. (**b**) Schematic of lymph node showing peripheral B-cell follicles (F) and central paracortex (P) in which T cells are located. B and T cells interact within the interfollicular zone (IF) at the T-B border. Germinal center shown within a B-cell follicle comprised of light zone (LZ) contains follicular dendritic cells (FDC) and T follicular helper cells (Tfh). GC B cells move between light and dark zones and undergo affinity maturation. (**c**) Sequential images obtained by two-photon intravital microscopy of popliteal lymph node of C57BL/6 mouse following transfer of OTII T cells (*cyan*), MD4 B cells (*green*), and CMTMR-labeled polyclonal B cells (*red*) 3 days postimmunization with HEL-OVA conjugate. A prolonged interaction between a B cell and pre-Tfh is circled and results in T-cell division. Time in minutes. (**d**) Sequential images obtained by two-photon intravital microscopy of MD4 B cells (*green*) acquiring immune complexed antigen (*circled*) from FDCs coated in immune complexes (*red*). Time show in minutes and seconds

they either will form short-lived extrafollicular plasmablasts or will move back into the B-cell follicle and form germinal centers (GC). GCs contain two compartments, a light and a dark zone, the former containing FDC and Tfh and the latter containing dividing B cells (Fig. 1b). During a GC reaction, B cells undergo affinity maturation, acquiring mutations in their antigen receptor. Mutations that increase affinity for antigen promote the selection of these cells via competition for Tfh help. Standard microscopic techniques provide information on lymphocyte morphology and anatomical position in x/y/z but do not allow the interrogation of cell behavior in the fourth dimension—time. The development of two-photon microscopy has allowed the investigation of the dynamic behavior of lymphocytes and the nature of environmental cues that impact this behavior within lymph nodes in live animals [1]. The GC response occurs within a time frame that is very amenable to analysis by intravital two-photon microscopy; therefore, much has been learned over the past decade by the application of this technique to GC biology. The methodology delineated in these studies provides a useful adjunct to the methods described in the other chapters of this volume. Thus, two-photon imaging studies have defined factors which determine antigen acquisition by B cells [2, 3], the initial interaction between a B cell and a pre-Tfh at the T-B border [4, 5] (Fig. 1c), B-cell dynamic behavior and B-cell-Tfh interaction within GC [6, 7], B-cell acquisition of antigen from FDCs [8] (Fig. 1d), B-cell movement through light and dark zone [9], and Tfh dynamics in the GC [10].

1.2 Principles of Two-Photon Microscopy

Two-photon excitation microscopy allows three-dimensional (3D) imaging to a depth of 5–600 μm. The resolution of images is sufficient to enable the dynamic behavior of single cells to be captured by time-lapse imaging. The advantages of two-photon microscopy are reduced phototoxicity, increased imaging depth, and the ability to initiate localized photochemistry within 3D samples [11].

Lower wavelength light (400–700 nm) used for single-photon/confocal microscopy is of high energy (Fig. 2a) and therefore has the tendency to damage cells within tissues on persistent or multiple exposures. The concept of two-photon excitation is that two photons of comparably lower energy than needed for one photon excitation can also excite a fluorophore in one quantum event (Fig. 2b). Each photon carries approximately half the energy necessary to excite the molecule. An excitation results in the subsequent emission of a fluorescence photon, typically at a higher energy than either of the two excitatory photons. The probability of the near-simultaneous absorption of two photons is extremely low. Therefore, a high flux of excitation photons is typically required, usually from a femtosecond laser. Since excitation is localized to the focal plane where the two photons simultaneously interact with a fluorescent molecule, there is no out-of-focus

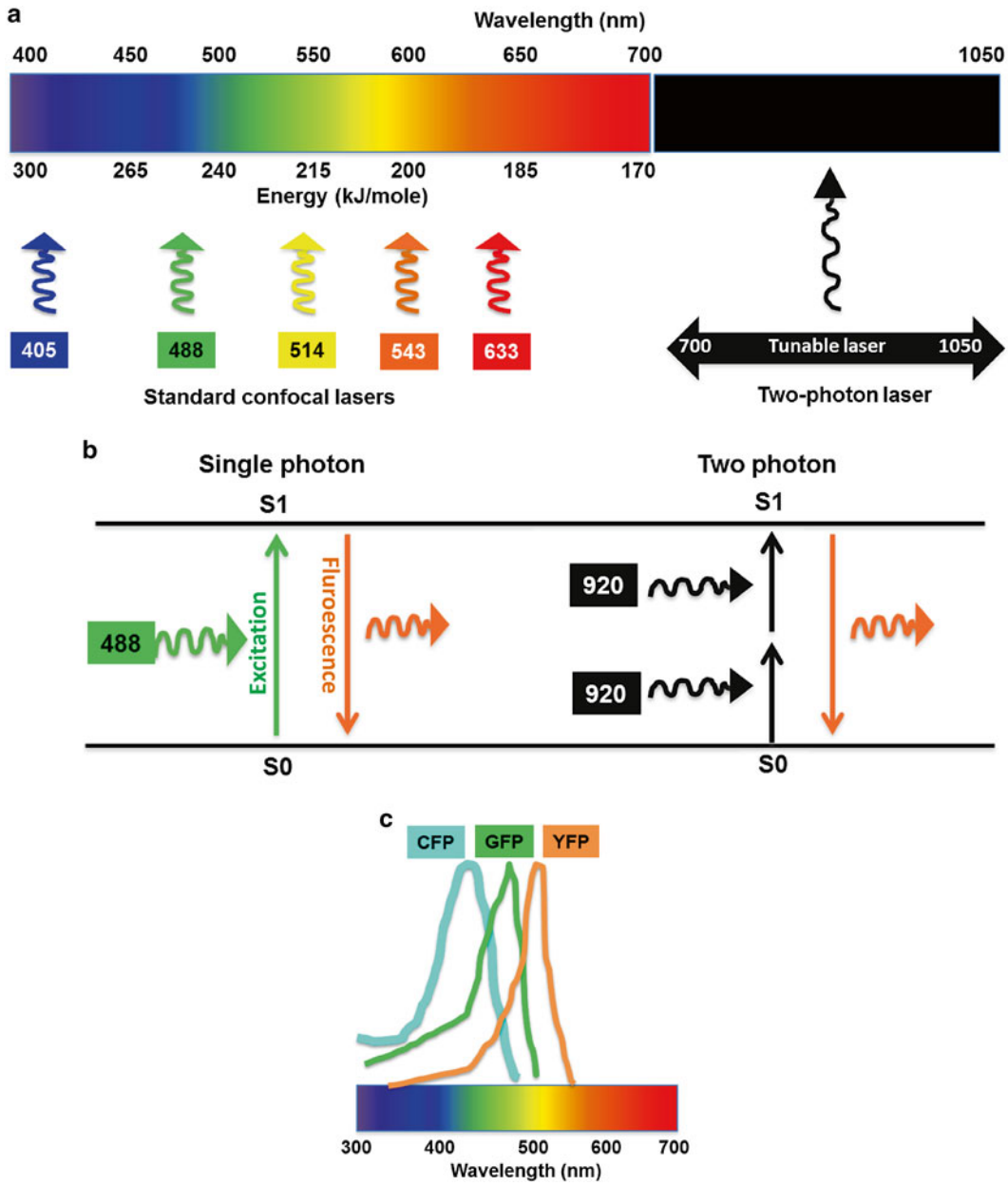

Fig. 2 Principles of two-photon microscopy. (**a**) Schema showing different energies associated with light of increasing wavelength. Standard confocal lasers emit light of a single wavelength and are used on combination. Two-photon microscopy utilizes a tunable laser capable of producing high flux of photons of longer wavelength light between 700 and 1,050 nm. (**b**) Jablonski diagram of single-photon excitation (*left panel*) and two-photon excitation (*right panel*). The singlet ground and first electronic states are labeled S_0, and S_1, respectively. (**c**) Excitation spectra of CFP and GFP lie close together such that they can be simultaneously excited by a femtosecond laser at 850 nm

excitation, thus non-descanned detectors (NDDs) can be used. In contrast to confocal microscopy where combinations of lasers are used to excite different fluorophores, in two-photon microscopy, often a single tunable titanium sapphire laser is used to produce a high flux of photons of longer wavelength light. Thus, the combination of fluorescent proteins that may be visualized in a single field may be limited to those with relatively similar or overlapping excitation spectra, e.g., cyan fluorescent protein (CFP), green fluorescent protein (GFP), and yellow fluorescent protein (YFP) (Fig. 2c).

One of the main limitations to using two-photon microscopy for intravital lymph node imaging is the technical complexity of the preparatory surgery required. This necessitates a familiarity with the relevant anatomy, precise surgical skills, and repetitive training to acquire the appropriate technique. This is critical to minimize tissue damage during lymph node exploration and to optimize tissue stability during imaging. It is also important to ensure the animal/lymph node preparation remains well hydrated, oxygenated, and normothermic. Any perturbation to these factors will result in suboptimal images and misleading results; for example, lymphocyte movement through the lymph node is significantly slowed if the animal or preparation is hypothermic.

As noted above, two-photon intravital microscopy can be used to interrogate a variety of aspects of Tfh and GC biology, and the exact experimental setup required depends on the experimental question. For the purposes of outlining a methodological approach to imaging germinal centers, in the following instructions, I will describe the methodology for an experiment designed to image interactions between GC B cells and Tfh, but the basic technique can be adapted to investigate other aspects of GC biology.

2 Materials

2.1 Mice

1. Fluorescent reporter mice (*see* **Note 1**).
2. Mice with transgenic T-cell receptor (*see* **Note 2**).
3. Mice with transgenic B-cell receptor (*see* **Note 2**).
4. Control mice for imaging and to obtain lymphocytes for cell tracker labeling (*see* **Notes 3** and **4**).

2.2 Preparation of Cells for Transfer

1. Dissection tools: forceps, scissors.
2. 70 % (v/v) ethanol.
3. 5 mL syringe plunger.
4. 70 μm cell strainers.
5. Digestion solution: PBS, 0.2 mg/mL DNAse I, and 1 mg/mL collagenase D.

6. PBS/1 % FCS buffer: PBS, 1 % (v/v) fetal calf serum (FCS).
7. Ice.
8. Refrigerated centrifuge.
9. MACS buffer: PBS, 2 % (v/v) fetal bovine serum (FBS), 2 mM EDTA.
10. CD4 T-cell isolation kit and naive B-cell isolation kit like, for example, those from Miltenyi Biotec.
11. Magnetic separation columns. This protocol describes the use of the LS separation columns from Miltenyi Biotec, but other magnetic isolation methods can also be used.
12. Magnet(s) and stand compatible with magnetic separation method of choice.
13. 15 mL conical tubes.
14. Cell tracker dye.
15. Cell incubator (at 37 °C, 5 % CO_2).
16. Complete RPMI: RPMI1640, 10 % (v/v) FCS, 10,000 units/mL penicillin, 10,000 μg/mL streptomycin solution, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol.
17. CellTracker Orange 5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine (CMTMR, Invitrogen/Molecular Probes).
18. Fetal calf serum.
19. 1 mL syringe with 27G needle for tail vein injection.
20. Cell counter.
21. Trypan blue.

2.3 Immunization to Generate Germinal Centers

1. Ovalbumin.
2. HEL.
3. HydraLink heterobifunctional conjugation kit (SoluLink).
4. Adjuvant for immunization: Alum and LPS.
5. 1 mL syringe with 25G needle for subcutaneous injection.

2.4 Lymph Node Imaging

1. Isoflurane and oxygen for anesthetization.
2. Heating pad on which mouse is placed during surgical preparation.
3. Dissection tools: Watchmaker's forceps × 2, microscissors.
4. Surgical tape.
5. Gauze pads.
6. Warmed PBS.
7. 10 mL syringe with 21G needle for application of PBS.

8. Carrier/stage plate.
9. Small magnets or play dough.
10. Vetbond glue.
11. Glass coverslip and holder.
12. Dissecting microscope for surgical preparation.
13. Two-photon microscope with titanium sapphire laser (Coherent and Spectra Physics are the most commonly used), fitted with light exclusion environmental chamber that can be heated. High numerical aperture and low-magnification water immersion objectives, usually 20× or 25×, are required.

2.5 Image Analysis

1. Analysis software. For example, Imaris (Bitplane) or Volocity (PerkinElmer).

3 Methods

3.1 Preparation of Cells for Transfer

3.1.1 Tissue Extraction

1. Euthanize OTII CFP and MD4 GFP mice, according to institutional/national guidelines (*see* **Note 2**).
2. Place the mouse supine.
3. Sterilize the skin with ethanol, spray forceps and scissors with ethanol, and use to make a left paramedian incision in the abdomen.
4. Identify the spleen and extract it.
5. Identify inguinal and brachial lymph nodes and remove them.
6. Place the organs into in a petri dish and trim away any attached fatty tissue. Place the organs into a 15 mL tube containing 5 mL PBS/1 % FCS on ice.

3.1.2 Cell Preparation

1. Place 70 μm cell strainer into a petri dish.
2. Spray forceps with ethanol, use to remove spleen from falcon, and place onto the cell strainer.
3. Apply 1 mL of cold PBS/1 % FCS to the spleen.
4. Use the plunger of a 5 mL syringe to crush the spleen forcing the cells through the strainer.
5. Wash through any residual cells with a further 2–3 mL of PBS/1 % FCS.
6. Place cell suspension into a 15 mL tube on ice. Repeat this procedure for lymph nodes. A digestion solution may be used to increase yield from lymph nodes. At this step, splenocytes and lymph node cells from a mouse can be combined.
7. Centrifuge the cell suspensions at 350 × *g* for 5 min at 4 °C. Resuspend the cell pellets in 1 mL PBS/1 % FCS and count.

8. Repeat centrifugation and resuspend the cell pellets in cold MACS buffer, and add the required volume of the appropriate Miltenyi magnetic microbeads (CD4 selection kit or CD19 selection kit). Alternatively, follow the manufacturer's instructions of an alternate magnetic separation kit.
9. Incubate the cells with beads at 4 °C. Duration of incubation varies for B and T cells. See the manufacturer's instructions.
10. Add cold MACS buffer up to a volume of 10 mL.
11. Centrifuge the cells $350 \times g$ for 5 min at room temperature (21–24 °C). Resuspend the cell pellet in 3 mL of cold MACS buffer.
12. Place Miltenyi LS column into a magnetic MACS separator. Equilibrate the column by passing 3 mL of MACS buffer.
13. Apply the 3 mL cell suspension labeled with magnetic microbeads onto the equilibrated LS column.
14. Collect the cells of interest into a 15 mL falcon on ice.
15. Count the viable cells (trypan blue staining and a hemocytometer).

3.2 Transfer of Cells to Recipient Mouse and Immunization

1. Cotransfer 5×10^4 CFP-expressing OT-2 T cells with 2×10^5 GFP MD4 B cells into the lateral tail vein of a C57BL/6 mouse (*see* **Note 5**).
2. Following cell transfer, mice are immunized with 20–30 μg OVA-HEL conjugate in alum with 0.2 μg LPS in the footpad, to drain the popliteal lymph node, in accordance with local ethical guidelines.

3.3 Labeling and Transfer of Polyclonal Cells Prior to Imaging

1. Six days after the first transfer and immunization, harvest cells from C57BL/6 mouse (as described in Subheading 3.1 above) to obtain naïve polyclonal B cells.
2. Resuspend the cells in warm complete RPMI at a density of $1–2 \times 10^7$ cells/mL.
3. Add a 1:1,000 dilution of a 20 mM stock of CMTMR in DMSO.
4. Incubate cells with cell tracker at 37 °C for 15 min.
5. Underlay 5 mL of FCS.
6. Centrifuge at $350 \times g$ for 5 min.
7. Wash twice, by adding excess PBS/1 % FCS, centrifuging at $350 \times g$ for 5 min, and then removing the supernatant.
8. Count the cells and transfer 2×10^7 to each recipient by intravenous injection into the lateral tail vein to allow identification of the B-cell follicle.
9. Image the tissues18–30 h later.

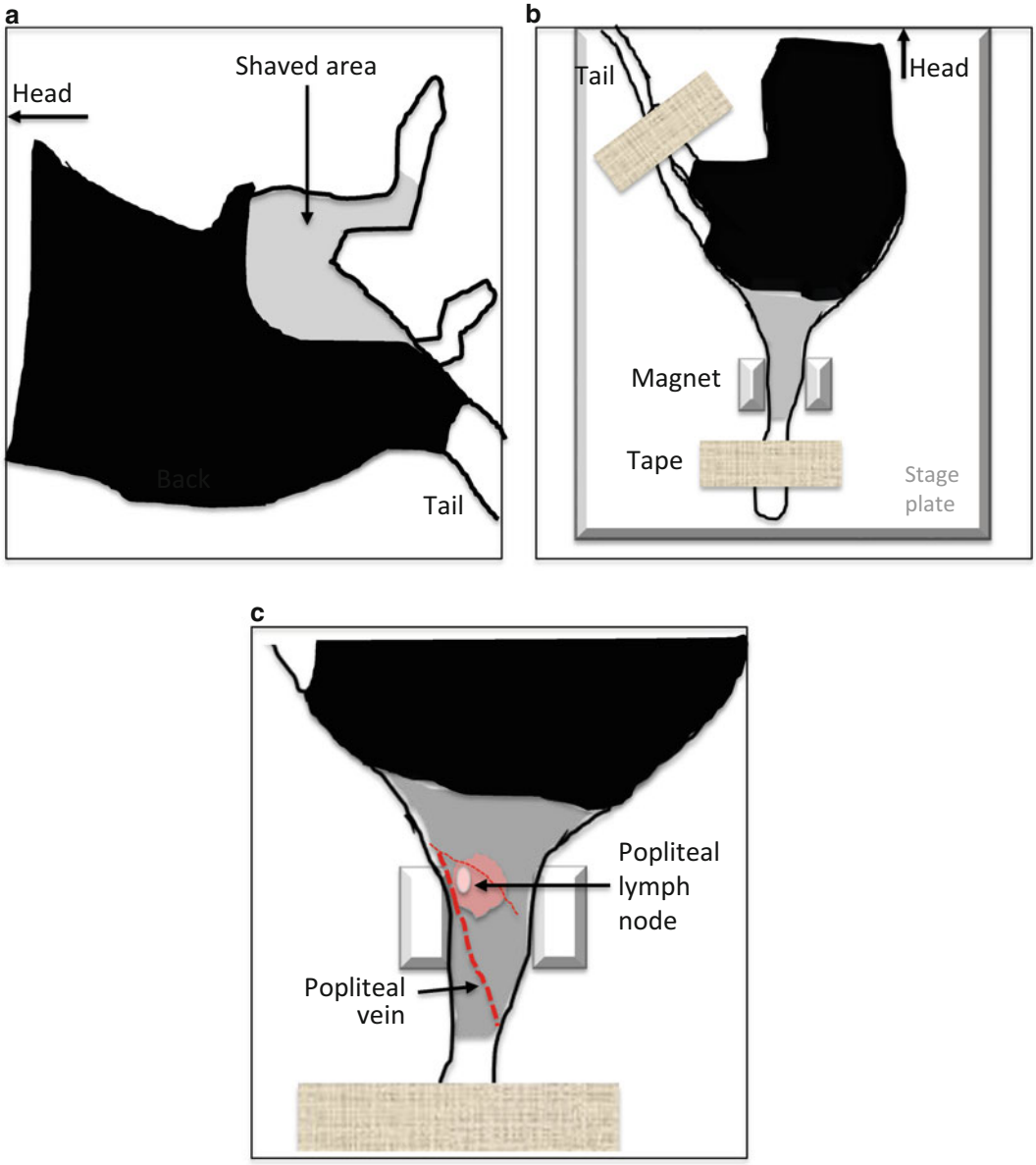

Fig. 3 Popliteal node surgical preparation. (**a**) Schema showing area (*filled gray*) of right leg that should be shaved and remaining hair removed using depilatory cream in preparation for surgery. (**b**) Position in which mouse should be secured on the stage plate with the right leg stretched downwards and taped such that right popliteal fossa is exposed. Left leg and tail should be taped upwards and leg stabilized using Vetbond and by positioning play dough or magnets laterally. (**c**) A small ellipse of skin is removed in the popliteal area, using the popliteal vein as a landmark. The lymph node usually lies inferior to a small branch of the popliteal vein

3.4 Preparation of Mouse for Imaging

1. Anesthetize the mouse with isoflurane (2–2.5 % for induction, 1.5–2 % for surgery/imaging).
2. Use an electric trimmer to remove hair on the right hind leg and inguinal area. Use hair remover (e.g., Nair lotion) to completely clear the area of hair (Fig. 3a).

3. Place mouse on a holder or stage plate and secure the anesthetic mask with tape.
4. Carefully secure the mouse onto the holder, with the right knee down, to expose the right popliteal fossa. Tape the left leg and tail upwards (Fig. 3b). Optimize the stability of the leg using play dough, magnets, or Vetbond glue.
5. Place the holder under the dissection microscope. Ensure the body temperature is maintained by placing the holder on a heating pad or by using a heating lamp.
6. Sterilize the skin with ethanol and use sterile scissors to remove an ellipse of the skin from the right mid-calf/thigh.
7. The LN is located within the popliteal fossa to either the right or left of the popliteal vein (Fig. 3c), depending on the placement of the mouse on the holder. Carefully separate the LN from surrounding adipose tissues and muscles using microdissection forceps. Use blunt dissection to minimize tissue trauma.
8. Once the lymph node is exposed, apply a coverslip lightly to the surface of the lymph node.
9. Throughout the procedure, keep the lymph node preparation moist by applying small volumes of warm PBS to the area under surgical manipulation.

3.5 Two-Photon Image Acquisition

1. Transfer the mouse holder onto the microscope stage (prewarm the microscope environmental chamber to 37 °C).
2. Use the epifluorescent lamp to place the lymph node under the objective.
3. Tune the laser to 840 nm and adjust the power such that cells are visible.
4. Set the upper and lower limits of the *Z* stack (usually around 50 μm) and the number of images in the stack (sample every 3 μm).
5. Acquire image stacks using time intervals appropriate for the cells imaged—for Tfh and B-cell interaction in germinal centers, sample every 30–40 s.
6. Monitor the status of the animal in the microscope chamber frequently.
7. Keep the lymph node prep moist by adding warmed PBS intermittently (*see* **Note 6**).
8. Typical imaging results are shown in Fig. 1c.

3.6 Image Analysis

Image analysis requires software packages that allow 3D visualization and automated tracking of two-photon movies and include Imaris (Bitplane) or Volocity (PerkinElmer). Typical analyses utilize parameters such as average speed, turning angle, directionality,

arrest coefficient, and displacement. Analysis of the duration of interaction between two cell populations is poorly automated, although some methodologies have been described [12], but this often require manual curation.

4 Notes

1. Two-photon imaging requires cells either to express a fluorescent reporter protein or to be labeled with a fluorescent cell tracker dye (*see* Subheadings 3.1 and 3.3) in order to allow imaging. Typical reporter proteins include CFP, GFP, YFP, and red fluorescent protein (RFP). The expression of these proteins may be driven by a ubiquitous protein (e.g., ubiquitin or actin [13, 14]) or may be driven by a cell-specific protein (e.g., lysozyme M [15], CD11c [16]). Reporter mice with photoconvertible proteins have also been generated. These proteins change from green to red fluorescence upon exposure to violet light, for example, Kaede [17–19] and mKikGR [20]. More recently, a photoactivatable GFP reporter has been developed, which allows cells to be labeled during imaging by photoactivation by scanning with a femtosecond-pulsed multiphoton laser tuned to 820–830 nm. This allows GC resident cells (including Tfh) to be labeled and tracked [9, 10]. There are also mouse strains available that can report cellular function, particularly cytokine production, for example, IL-4 [21] reporter mice.
2. In order to assess antigen-specific T- and B-cell responses, these fluorescent reporter mice may be crossed with a strain with a transgenic T-cell receptor (TCR) or B-cell receptor (BCR), e.g., OT2 mouse, in which the TCR on CD4 T cells binds to an ovalbumin (OVA)-derived peptide ($OVA_{323–339}$) or the MD4 mouse, where the BCR binds to hen egg lysozyme (HEL). Thus, the response of these cells to model antigens can be interrogated. Both these strains can be purchased from Jackson.
3. An alternative to using mice with fluorescent reporter proteins is to label splenocytes with a fluorescent cell tracker dye prior to transfer. This technique cannot be used if the expected outcome of the experiment is cell proliferation, as the cell tracker dye is divided between progenies and will therefore be diluted through successive cell divisions, making detection difficult.
4. One of the critical aspects of imaging lymphocyte interactions in lymph nodes is that it is not helpful if all B cells and all T cells in the lymph node are fluorescent, since individual interactions cannot be discerned. Hence, B and T cells are isolated from the spleens of fluorescent reporter mice, and relatively

small numbers transferred to a recipient mouse without a fluorescent reporter, so that the numbers of cells within the imaging field is suitable. Therefore, these studies also required control mice (e.g., C57BL/6 mice) as recipients. The recipient mouse should be of the same background strain as the mouse from which cells are obtained for transfer or else rejection will occur (e.g., lymphocytes from a OTII mouse on a C57BL/6 background should be transferred to a C57BL/6 recipient, not a BALB/c recipient).

5. Recipient animal will need to be warmed to make lateral tail vein more amenable to cannulation.
6. If the lymphocytes are not moving well, then check if the animal is warm, the preparation is adequately hydrated, and that the lymph node is not being compressed by the coverslip.

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Chapter 7

In Vivo Induction of T-Follicular Helper Cells by Modulation of Regulatory T Cell Function

James Badger Wing and Shimon Sakaguchi

Abstract

Regulatory T cells, particularly their follicular resident subset, T-follicular regulatory cells, play a critical role in the maintenance of immune homeostasis and prevention of the expansion of T-follicular helper cells. One of the key mechanisms used by these cells is CTLA-4 dependent suppression of inflammation. In this chapter, we detail methods to increase the formation of T-follicular helper cells following vaccination by either depleting Treg cells, via Foxp3 linked diphtheria toxin receptor, or blocking CTLA-4 function by the use of antibodies.

Key words Regulatory T-cells, CTLA-4, T-follicular helper cells

1 Introduction

Foxp3-expressing regulatory T (Treg) cells make up around 10 % of $CD4^+$ T cells and are critical for the maintenance of immune tolerance [1]. Recently it has become clear that Treg cells contain a follicular resident subset, T-follicular regulatory (Tfr) cells that are particularly important in the control of the development and function of T-follicular helper (Tfh) cells and the resulting germinal centre responses [2–4]. Exploiting the relative resistance of mice to cell death induced by diphtheria toxin (DT), Foxp3-linked expression of human diphtheria toxin receptor has proved extremely useful in allowing the specific depletion of Treg cells [5–7]. Following Treg cell depletion in these systems, antigen presenting cells and effector T cells become highly activated, leading to autoimmunity and inflammation [5].

Treg/Tfr cells have a range of suppressive mechanisms by which they may control humoral immunity [8]. Recently we have found that CTLA-4-mediated immune suppression is a critical mechanism by which Tregs and Tfr cells control the formation of Tfh cells [9].

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In this chapter, we describe methods to dose-dependently deplete Treg cells via a Foxp3 linked diphtheria toxin receptor expression, and antibody-based blockade of CTLA-4. Both of these methods enhance Tfh cell formation with or without concomitant vaccination. We also describe a method for staining of cells for flow cytometry derived from both of these techniques.

2 Materials

1. FACS buffer: Calcium and magnesium free PBS supplemented with 2 % fetal calf serum.
2. RPMI-2: Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 2 % FCS.
3. Diphtheria toxin, unnicked (Caution: highly toxic, appropriate care should be taken).
4. Mice: Foxp3-DTR-GFP mice [5] Strain name: B6.129(Cg)-Foxp3tm3(DTR/GFP)Ayr/J, Wild-type C57BL/6.
5. Antibodies for in vivo experiments {clone}: Anti-CTLA-4 {9D9} Mouse IgG2b isotype control {MPC-11}.
6. Antibodies for flow cytometry {clone}: anti-B220 {RA3-6B2}, anti-CD138 {281-2}, anti-BCL6 {K112-91}, anti-CXCR5 {RF8B2} (BD), anti-PD1 {J43}, antti-Foxp3 {FJK-16 s}, anti-CD11b {M1/70}, anti-F4/80 {BM8}, anti-CD11c {N418}.
7. Fixable dead cell stain kit.
8. Frosted glass slides.
9. 70 μm nylon mesh.
10. 27 G and 29 G 1 ml syringes.
11. 1.5 ml centrifuge tubes.
12. Red blood cell lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Adjust the pH to 7.3.
13. Antigen for vaccination (e.g., OVA, KLH, 2W1S or their NP-conjugated variants as required).
14. Adjuvant—Imject Alum adjuvant.
15. Foxp3 staining buffer kit (eBioscience).

3 Methods

Obtain proper ethical approval from the relevant bodies before conducting experiments. Over the course of the experiment, mice depleted of Treg cells may suffer from significant levels of inflammation [5]. In our hands the highest dosage of DT reported in this paper does not lead to death within the course of this experiment,

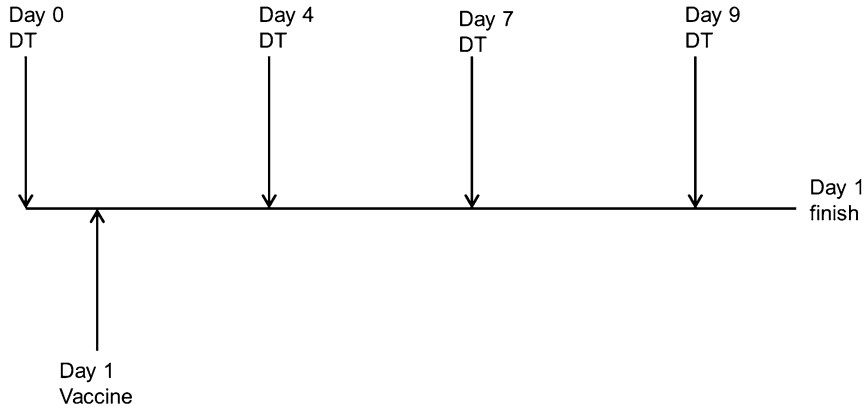

Fig. 1 Expansion of Tfh cells by depletion of Treg cells. Schematic of experimental plan

although splenomegaly/lymphadenopathy may be observed. If death or clear moribund behavior is seen in mice, consider shortening the experiment in line with guidance from the local ethical approval body. Splenomegaly and inflammation in mice treated with anti-CTLA-4 as described in this protocol is mild.

3.1 Treg Depletion (Outlined in Fig. 1)

1. In advance, dilute diphtheria toxin in PBS to 1 mg/ml stock concentration and store aliquots (5–10 μl, depending on anticipated size of experiments and dose) at −20 °C.
2. On day 0 prepare desired concentration of diphtheria toxin by diluting stock in a total volume of 200 μl PBS per Foxp3^{DTR} mouse (*see* **Note 1**). Make fresh each time, defrosted stock can be kept at 4 °C for a week but avoid freeze-thawing.
3. Take up 200 μl DT or PBS into 1 ml 29 G syringe (*see* **Note 2**).
4. Firmly grip mouse by scruff and inject diphtheria toxin intraperitoneally (IP) into lower right quadrant before disposing of needle in sharps bin.
5. On day 1 prepare antigen of choice in adjuvant by mixing 50–100 μg/mouse antigen, diluted to give a final volume of 100 μl/mouse in PBS at a 1:1 ratio with 100 μl alum to give a total final volume of 200 μl/mouse (*see* **Note 3**). Mix gently in an end-over-end tube rotator or suitable alternative for at least 30 min (*see* **Note 4**).
6. Take up 200 μl antigen–alum mixture into a 1 ml 27 G syringe.
7. Firmly grip mouse by scruff and inject antigen IP into lower right quadrant before disposing of needle in sharps bin.
8. Repeat diphtheria toxin IP injection, as described in **steps 1–3**, on days, 4, 7, and 9.
9. Sacrifice mouse at day 11.

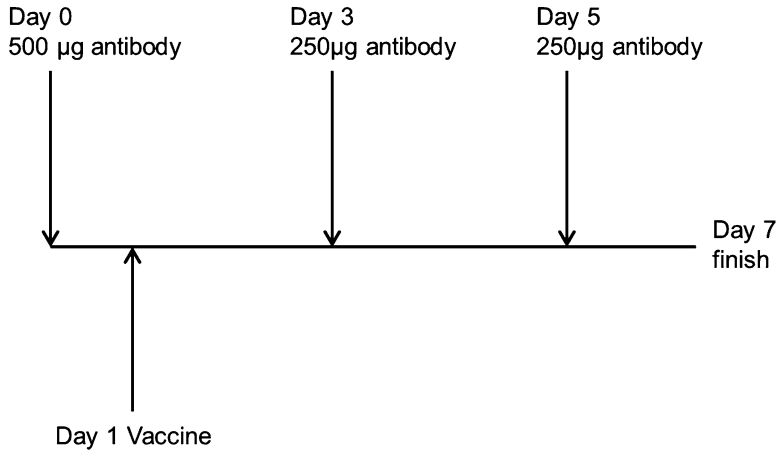

Fig. 2 Expansion of Tfh cells by CTLA-4 blocking. Schematic of experimental plan

3.2 CTLA-4 Blocking (Outlined in Fig. 2)

1. In advance prepare 2 mg/ml working concentration of anti-CTLA-4 or isotype antibody in PBS. Aliquot (500 µl/mouse for complete experiment) and store at −20 °C.
2. On day 0 prepare 500 µg (250 µl at 2 mg/ml) of anti-CTLA-4 antibody (*see* **Note 5**) or isotype control in PBS per C57BL/6 wild-type mouse (*see* **Note 6**). Avoid freeze-thawing antibody, once defrosted antibody can be stored at 4 °C for approximately a month (*see* **Note 7**).
3. Firmly grip mouse by scruff and inject antibody IP into lower right quadrant before disposing of needle in sharps bin.
4. On day 1 prepare antigen of choice in adjuvant by mixing 50–100 µg/mouse antigen, diluted to give a final volume of 100 µl/mouse in PBS at a 1:1 ratio with 100 µl alum to give a total final volume of 200 µl/mouse (*see* **Note 3**). Mix gently in an end-over-end tube rotator or suitable alternative for at least 30 min.
5. Take up 200 µl antigen–alum mixture into 1 ml 27 G needle.
6. Firmly grip mouse by scruff and inject antigen IP into lower right quadrant before disposing of needle in sharps bin.
7. Give further doses of 250 µg (125 µl of 2 mg/ml) anti-CTLA-4 or isotype control IP on days 3 and 5.
8. Sacrifice mouse on day 7.

3.3 Tfh Cell Staining

1. Following the Treg cell depletion or CTLA-4 blocking protocol in Subheadings 3.1 and 3.2, take spleen, and other organs if required (*see* **Note 8**) and place in 60 mm dish containing 5 ml of RPMI-2.

2. Prepare single cell suspension by gently grinding with frosted slides then filtering through a 70 μm nylon mesh into a 15 ml centrifuge tube. Centrifuge for 5 min at 500×*g*. Remove supernatants by pouring/pipette/vacuum.
3. Resuspend pellet in 2 ml red cell lysis buffer and gently mix for 1 min before adding 13 ml RPMI-2.
4. Centrifuge at 500×*g* for 5 min, remove supernatant, and resuspend pellet in 5 ml RPMI-2. Repeat this step twice more.
5. Enumerate cells and place 1×10^6 cells/well into a 96-well U-bottomed plate.
6. Add 1/100 dilution of biotin-conjugated anti-CXCR5 antibody in FACS buffer and incubate for 1 h at 37 °C (*see* **Note 9**). Also prepare a non-biotin treated sample for use as a fluorescence minus one staining control.
7. Centrifuge at 500×*g* for 3 min, remove supernatants, and resuspend pellets in 150 μl FACS buffer. Repeat this step once more.
8. Add 100 μl of 1/100 streptavidin and 1/200 dilutions of anti-B220, CD4, CD11c, CD11b, F4/80, and PD-1, in FACS buffer containing a 1/500 dilution of live/dead fixable stain (*see* **Note 10**). In addition to isotype controls we also recommend using fluorescence minus one controls for the Tfh cell markers, i.e., above staining minus PD-1, above staining in non-biotin treated cells from **step 8**.
9. Incubate at 4 °C for 30 min.
10. Centrifuge at 500×*g* for 3 min, remove supernatants, and resuspend pellets in 150 μl FACS buffer. Repeat this step once more.
11. Resuspend cells in 100 μl Foxp3 fixation buffer.
12. Incubate at 4 °C for 30 min.
13. Centrifuge at 500×*g* for 3 min, remove supernatants, and suspend pellets in 150 μl permeabilization buffer. Repeat this step once more.
14. Add 100 μl of a 1/200 dilution of anti-Foxp3 and anti-BCL-6 antibodies in permeabilization buffer (*see* **Note 11**).
15. Incubate at 4 °C for 30 min.
16. Centrifuge at 500×*g* for 3 min, remove supernatants, and suspend pellets in 150 μl FACS buffer. Repeat this step once more.
17. Filter cells through 70 μm nylon mesh into microcentrifuge tubes and run on appropriate cytometer for analysis (*see* **Note 12**) (*see* Fig. 3).

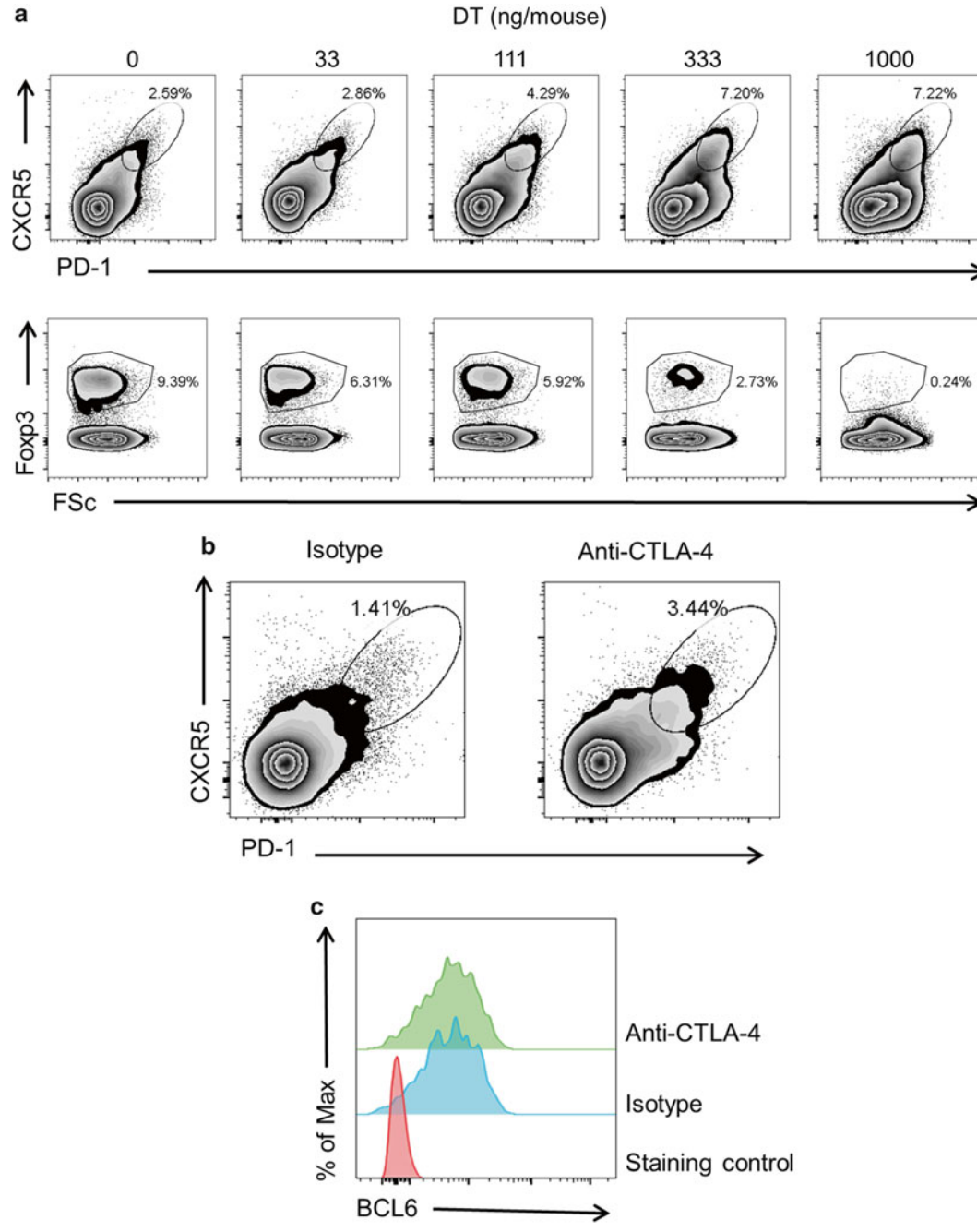

Fig. 3 Tfh formation following Treg cell depletion/CTLA-4 blocking. (**a**) Representative results of Tfh cell formation in spleen of mice treated with the indicated diphtheria toxin doses at days 0, 4, 7, and 9 and 50 μg NP-2WIS-GFP at day 0. Pre-gated on size, singlets, dump$^-$ (B220, CD11c, CD11b, F4/80, LIVE/DEAD), and CD4$^+$Foxp3$^-$. (**b**) Representative Tfh cell formation in spleen of mice treated with 500 μg anti-CTLA-4 or isotype control antibody on day 0, vaccinated with and 50 μg NP-2WIS-GFP on day 1 and then further treated with 250 μg antibody on days 3 and 5. Pre-gated on size, singlets, and dump$^-$ (B220, CD11c, CD11b, F4/80, fixable dead cell stain), CD4$^+$Foxp3$^-$. (**c**) BCL-6 expression in CXCR5$^+$PD1$^+$ Tfh cells gated in (**b**)

4 Notes

1. The Foxp3^{DTR} mice can be depleted of Treg cells in a dose-dependent manner. In some cases, it may be more preferable to only partially deplete Treg cells in order to prevent excessive inflammation. We have carried out similar experiments in both BALB/c and C57BL/6 DEREG mice [9]. These mice give broadly similar results, also enhancing Tfh cell formation, as the Foxp3^{DTR} mice described here with some differences in the kinetics of the depletion and recovery of Tregs.
2. In this example we use Foxp3^{DTR} littermates injected with PBS as the control group. In our hands diphtheria toxin at these doses has no clear effect on wild-type mice; however, in some cases, a weak humoral response to DT might develop [10]. If needed, replace Foxp3^{DTR} littermate controls with Foxp3^{WT} mice injected with DT.
3. Due to its viscosity alum is difficult to pipette, you may find it helpful to cut the end off a 1,000 μl pipette tip to give a wider bore and allow easier pipetting.
4. While we normally perform these experiments in conjunction with vaccination, vaccination is not essential for (although it enhances) the formation of Tfh cells following depletion/inactivation of Treg cells as polyreactive/autoreactive Tfh cells expand. In particular we find that while prolonged Treg depletion may enhance numbers of total Tfh cells, shorter Treg depletion schedules may be more efficient for the generation of antigen-specific Tfh cells following vaccination with a foreign antigen [9]. For these reasons it is important to remember that measuring total Tfh cells may not always be an accurate reflection of antigen-specific cells following vaccination. Determination of antigen-specific Tfh cells can be done via tetramer staining (For more details see the chapter by Chakarov and Fazilleau in this volume of Methods in Molecular Biology) [11].
5. The anti-CTLA-4 antibody used in this method (9D9) blocks the function of CTLA-4 and may increase the percentage of Treg cells due to the resulting inflammatory response [12]. However, in certain microenvironments (such as the tumor microenvironment), antibody-dependent depletion may occur [13].
6. BALB/c mice also show enhanced formation of Tfh cells in similar experiments, but we find that the results are clearer in C57BL/6.

7. It is likely that the antibody is stable for longer periods of time at 4 °C, but we have not tested this.
8. While the example given here is in spleen, enhanced induction of Tfh cells is visible at other sites such as peripheral lymph nodes and Peyer's patches. Even in the absence of a need to specifically assess Tfh cell formation in the Peyer's patches, it may prove a useful positive control for Tfh cell gating.
9. We find that 1 h at 37 °C gives the clearest staining although shorter incubations/lower temperatures also give usable results.
10. B220, CD11c, CD11b, F4/80, and fixable dead cell staining kit may all use fluorochromes with the same excitation and emission spectra to allow use of a dump gate. Since CXCR5 staining in T cells can be dull, CXCR5 is best stained with a bright fluorochrome such as Brilliant violet 421 or Phycoerythrin (PE). For best results titrate all antibodies to find the best concentrations.
11. While the $Foxp3^{DTR}$ mouse contains a GFP reporter, we find that directly staining Foxp3 itself gives clearer results. Even in cases where Treg cells are not of direct interest, we still strongly recommend staining Foxp3 in order to differentiate between $Foxp3^-$ Tfh cells and $Foxp3^+$ Tfr cells within the $CXCR5^+PD1^+$ gate.
12. In this example we use CXCR5 and PD-1 to identify Tfh cells, but we find that other marker combinations such as $CXCR5^+BCL6^+$, $PD1^+BCL6^+$, and $CXCR5^+BTLA^+$ give similar results and contain similar proportions of IL-21 expressing cells. If resolving the Tfh cell population is difficult due to low numbers, it may be advisable to stain and pre-gate on $CD44^+$ cells (Tfh are almost all $CD44^+$) before the Tfh cell gate, since this will raise the percentage of $PD\text{-}1^+CXCR5^+$ cells within the gated population.

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Chapter 8

Assessing T Follicular Helper Cell Function In Vivo: Antigen-Specific B Cell Response to Hapten and Affinity Maturation

Jessica Natt and Marion Espéli

Abstract

The mechanisms controlling affinity maturation have been extensively studied over the last 20 years and the central role of T follicular helper cells (Tfh) in this process has now been clearly established. In order to analyze how Tfh impact on affinity maturation several models have been developed. This chapter aims to present three different techniques to evaluate antigen-specific B cell response and affinity maturation using the NP system: Flow cytometric single cell sorting and sequence analysis, ELISA and ELISpot. They have the advantages of being applicable on all types of mice independently of the presence of a transgenic BCR and to give multiple readout of the antigen-specific immune response and affinity maturation. Although first developed more than 20 years ago, these techniques are still considered to be the gold standard for the analysis of affinity maturation in vivo.

Key words Affinity maturation, NP, Single-cell PCR, ELISpot, ELISA

1 Introduction

Following antigen encounter B cells that receive a second signal provided by T cells enter the germinal center where they proliferate and mutate their antigen receptor (BCR). This process followed by subsequent selection, enables the generation of B cell clones with increased affinity for the immunizing antigen. The mechanisms controlling affinity maturation have been extensively studied over the last 20 years and the central role of follicular helper T cells (T_{FH}) in this process has now been clearly established. By using an anti-DEC205 mediated antigen delivery system Michel Nussenzweig and colleagues demonstrated that competition for T_{FH} help in the germinal center light zone was driving B cell selection and affinity maturation [1, 2]. B cells with the highest affinity for antigen capture and present more antigen to T_{FH} cells which in turn enhance B cell migration to the dark zone, number of cell

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divisions and somatic hypermutation which ultimately culminates in affinity maturation [3]. Moreover, work done by Linterman et al. has shown that follicular regulatory T cells (T_{FR}), a suppressive population of Foxp3$^+$ regulatory T cells in the germinal center, may also play a role in the regulation of affinity maturation following NP-KLH immunization [4].

Measuring the quality as well as the magnitude of the GC is thus central to the functional study of T_{FH} and T_{FR} cells. This can be achieved by using transgenic mouse models like the SW_{HEL} mice for example [5]. Another experimental system allowing the analysis of affinity maturation is based on immunization with the NP hapten conjugated to a protein carrier (e.g., CGG, KLH, OVA). This model antigen first introduced by Imanishi and Makela in 1974 [6] has been studied in great detail in the late 1980s and early 1990s. Following immunization with NP-conjugated to a protein carrier 90 % of the NP specific B cells bear the V186.2 heavy chain and are Igλ^+ [7]. This model antigen was central in determining that affinity maturation is driven by somatic hypermutation followed by selection of high affinity clones [8]. Importantly, a single mutation at amino acid 33, the substitution of a tryptophane by a leucine in the complementary determining region 1 (CDR1) was shown to be associated with ten times greater affinity for NP and was observed in most of the anti-NP antibodies generated following secondary immunization [8–10]. Following this seminal work the development of flow cytometry has allowed the specific analysis, cell sorting and sequence analysis of NP$^+$ B cells [11, 12]. Moreover, ELISA and ELISpot using proteins with different loading of NP molecules for coating have facilitated the analysis of affinity maturation the analysis of affinity maturation in serum antibodies and antibody forming cells, respectively [12, 13].

These techniques, first set-up more than 20 years ago, are still widely used for analyzing affinity maturation during a T-dependent immune response [3, 14]. In the last few years they have been used notably to show that T_{FR} are involved in the control of affinity maturation [4] and that local BLyS production by T_{FH} is important for the maintenance of high affinity germinal center B cells [15].

In this chapter we describe a detailed protocol to immunize mice with NP-KLH and analyze affinity maturation by single cell sort and sequence analysis, ELISA and ELISpot. The single cell sort and sequence analysis of NP$^+$ cells allow the determination of affinity maturation from selected B cell populations (germinal center B cells, memory B cells). NP-specific ELISA allows the determination of affinity maturation from the serum of immunized mice while ELISpot permits the evaluation of the relative affinity for NP of tissue plasma cells. The flexibility of this system allows the determination of affinity maturation and thus T_{FH} function at different stages of the immune response, in different mouse models and in different tissues depending on the user requirement.

2 Materials

2.1 Immunization

1. NP-KLH (4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin). Store at 4 °C.
2. Imject Alum. Store at room temperature.
3. Phosphate Buffered Saline (PBS): 137 M NaCl, 2.7 M KCl, 10 M Na_2HPO_4 and 1.8 M KH_2PO_4. Store at room temperature.
4. Syringe and needle for intraperitoneal injections.
5. 15 mL sterile conical tubes for NP-KLH resuspension.

2.2 Cell Preparation

1. Cell medium culture: DMEM, 10 % of heat inactivated fetal calf serum (FCS), 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin. Store at 4 °C.
2. Phosphate Buffered Saline (PBS): 137 M NaCl, 2.7 M KCl, 10 M Na_2HPO_4, and 1.8 M KH_2PO_4. Store at room temperature.
3. Red blood cells (RBC) lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Adjust the pH to 7.3. Store at room temperature.
4. 15 and 50 mL sterile conical tubes for cell processing.
5. Syringes and needles for bone marrow flushing.
6. 70 μm nylon cell strainers.

2.3 Cell Staining and Single-Cell Sorting

1. Fc block for nonspecific sites saturation.
2. FACS fix buffer: PBS, 1 % of formaldehyde for cell fixation. Store at 4 °C.
3. NP conjugated to PE to stain NP-specific germinal center NP-specific B cells.
4. Antibodies: anti-mouse B220 conjugated to pacific blue, anti-mouse IgG1 conjugated to allophycocyanin (APC), anti-mouse Gr1 and IgM conjugated to fluorescein isothiocyanate (FITC) (DUMP channel).
5. 96-well PCR plates for the single-cell collection.
6. RT-lysis buffer: 2 U/μL RNase inhibitor, 4 mM DTT, 30 ng/μL Random hexamers, 1 % NP40, 0.2× PBS. Prepare fresh and keep on ice until use.
7. PCR adhesive sealing foil.
8. Flow sorter equipped with a plate loader.

2.4 Reverse Transcription and $V_H186.2$ Nested PCR

1. Reverse transcription buffer: 1× reverse transcription buffer, 1 mM dNTPs, 8 mM DTT, 0.2 U/μL Rnase inhibitor, and 100 U SuperScript enzyme.
2. 20 μM of external and internal primer pairs for two-round nested PCRs (*see* Table 1).

Table 1
PCR primers for the two-round nested PCR

	Primer sequence	Molecular weight
1st PCR round		
External 186.2	5' GCT GTA TCA TGC TCT TCT TG	7
External Cγ1	5' GGA TGA CTC ATC CCA GGG TCA CCA TGG AGT	9
2nd PCR round		
Internal 186.2	5' GGT GTC CAC TCC CAG GTC CA	10
Internal Cγ1	5' CCA GGG GCC AGT GGA TAG AC	6

3. Taq polymerase. Store at −20 °C.
4. 10 mM dNTPs. Store at −20 °C.
5. 96-well PCR plates.
6. PCR machine.

2.5 NP-Specific ELISpot

1. ELISpot plates.
2. NP(4)- and NP(15)-conjugated to bovine serum albumin (BSA).
3. ELISpot blocking buffer: DMEM, 10 % of heat inactivated fetal calf serum (FCS), 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin. Keep at 4 °C until use.
4. ELISpot washing buffer: PBS, 0.01 % Tween 20. Store at room temperature.
5. Antibody: goat anti-mouse IgG1 conjugated to horseradish peroxidase (HRP). Keep at 4 °C until use.
6. 3-Amino-9-ethylcarbazole (AEC) tablets. Store at room temperature.
7. *N,N*-dimethylformamide (DMF). Store at room temperature.
8. Acetate buffer: 0.2 M acetic acid and 0.2 M sodium acetate in deionized water, pH 5.0. Store at room temperature.
9. Hydrogen peroxide. Store at 4 °C.
10. ELISpot Reader.

2.6 NP-Specific ELISA

1. 96-well ELISA plates.
2. NP(4)- and NP(15)-conjugated to bovine serum albumin (BSA).
3. ELISA blocking buffer: PBS and 2 % BSA. Store at 4 °C.
4. Standard serum: pooled sera from mice immunized thrice with NP-KLH emulsified in alum. Store at −20 °C.
5. Antibody: goat anti-mouse IgG1-HRP. Keep at 4 °C until use.

6. ELISA washing buffer: PBS, 0.05 % Tween 20. Store at room temperature.
7. 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate for plate revelation. Store at 4 °C.
8. 0.5 M sulphuric acid for stopping the reaction.
9. Spectrophotometer for reading the absorbance at 450 nm.

3 Methods

3.1 Immunization

3.1.1 Primary Immunization

1. Weigh 100 μg per mouse of NP-KLH in a 15 mL sterile conical tube (*see* **Note 1**).
2. Dissolve NP-KLH in PBS 1× to obtain a final concentration of 2 mg/mL.
3. Vigorously shake the Imject Alum bottle for 3–4 min.
4. Add Imject Alum (V/V) dropwise to the PBS-dissolved NP-KLH under constant agitation on a vortex (*see* **Note 2**).
5. Continue vortexing the solution for 30 min.
6. Immunize mice with an intraperitoneal injection with 100 μL of alum-emulsified NP-KLH (Fig. 1a).

3.1.2 Memory Immunization

1. Weigh 100 μg per mouse of NP-KLH in a 15 mL sterile conical tube.
2. Dissolve NP-KLH in PBS 1× to obtain a final concentration at 1 mg/mL.
3. Immunize mice with an intraperitoneal injection of 100 μL of NP-KLH (*see* **Note 3** for secondary immunization) (Fig. 1a).

3.2 Cell Preparation

3.2.1 Isolation of Cells from the Spleen

1. Harvest the spleen in accordance with local ethical guidelines and protocols.
2. Mash the spleen with 5 mL of culture medium and the flat part of the plunger of a 2 mL syringe on a cell strainer placed on top of a 50 mL sterile conical tube.
3. Centrifuge at 400×*g* at 4 °C for 5 min and discard the supernatant.
4. Resuspend the pellet with 1 mL of RBC lysis buffer to lyse the red blood cells. Incubate for 5 min at room temperature then stop the reaction with 10 mL of PBS.
5. Centrifuge at 400×*g* at 4 °C for 5 min, discard the supernatant and resuspend the pellet with 2 mL of culture medium. Count the cells using trypan blue and a counting chamber.
6. Keep the cells at 4 °C.

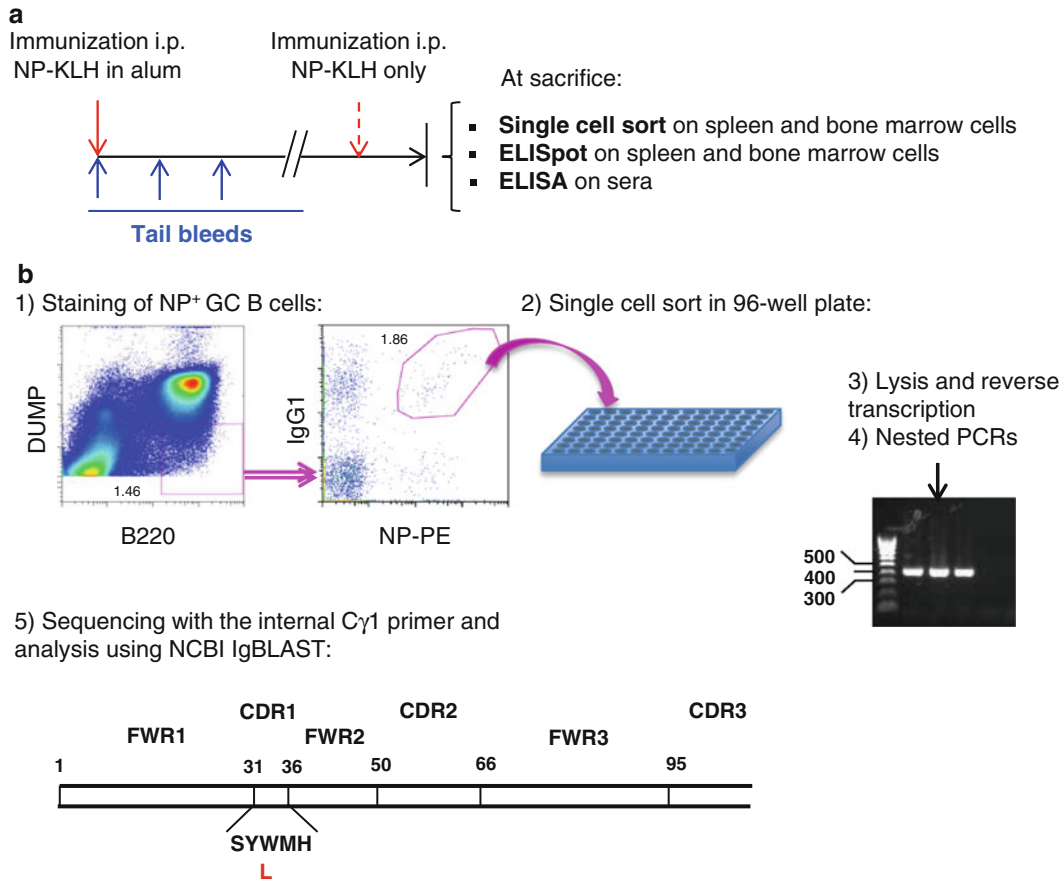

Fig. 1 Determination of affinity maturation by single cell sort and sequence analysis. (**a**) Schematic representation of the immunization strategy with NP-KLH. Mice are immunized a first time with NP-KLH in alum. Serum can be obtained by serial tail bleeds to measure the kinetic of the immune response and affinity maturation. A secondary immunization can be performed to evaluate the memory immune response with NP-KLH only at least 5 weeks after the primary immunization. At sacrifice, single cell sort and sequencing as well as ELIspot can be used to evaluate affinity maturation. (**b**) Schematic representation of Single cell sort and sequence analysis: (1) Spleen or bone marrow cells are stained and processed on a cell sorter. (2) Single NP+ cells are distributed in the wells of a 96-well plate directly in lysis buffer. (3) Cells are lysed and cDNA is prepared by reverse transcription. (4) A 10th of the cDNA is then used for two rounds of nested PCR to amplify the V_H186.2 region. (5) Positive PCRs are then cleaned and processed for sequencing using the internal Cγ1 primer. Sequences are then analyzed for the substitution of a tryptophan for a leucine at position 33 in the CDR1 and for the R/S ratio in the FWR and CDR

3.2.2 Isolation of Cells from the Bone Marrow

1. Harvest the legs in accordance with local ethical guidelines and protocols. Remove all the muscle, separate the tibia from the femur using forceps and scissors, and cut the bone extremities. Keep bones in culture medium on ice.
2. Flush the marrow out in a 50 mL sterile conical tube using a syringe and a 26-gauge needle and 5 mL of culture medium.

3. Centrifuge at 400 × *g* at 4 °C for 5 min. Discard the supernatant.
4. Resuspend the cell pellet with 1 mL of RBC lysis buffer to lyse the red blood cells. Incubate for 5 min at room temperature then stop the reaction with 10 mL of PBS.
5. Centrifuge at 400 × *g* at 4 °C for 5 min, discard the supernatant and resuspend the cell pellet with 2 mL of culture medium. Count the cells using trypan blue and a counting chamber.
6. Keep the cells at 4 °C until further use.

3.3 Cell Staining and Single Cell Sorting

1. Use at least 10×10^6 cells of spleen or bone marrow cell suspension for each staining (*see* **Note 4**).
2. Add 500 µL of the antibody mix to all samples.
3. Incubate for 1 h at 4 °C.
4. Wash with 2 mL of PBS.
5. Centrifuge at 400 × *g* at 4 °C for 5 min and discard the supernatant.
6. Resuspend the cells in 6 mL of PBS 0.5 % FCS. Keep cell suspension at 4 °C in the dark until single-cell sorting.
7. While the cells are incubating with the antibodies, prepare the RT-lysis buffer and add 10 µL of it to each well of a 96-well PCR plate. Keep on ice until further use (*see* **Note 5**).
8. Using a flow sorter equipped with a 96-well plate loader, perform single-cell sort of the $B220^+DUMP^-/NP^+IgG1^+$ cells (*see* **Note 6**) (Fig. 1b).
9. Once the sort is finished, cover the plate with adhesive sealing foil, centrifuge it without delay and freeze it on dry ice. The frozen plates can be kept stored at −80 °C until reverse transcription is performed.

3.4 Reverse Transcription

1. Take the plates directly from the −80 °C freezer and place them in a PCR machine pre-set with the program described in Table 2 (*see* **Note 7**).
2. Heat to 65 °C for 2 min, then to 10 °C for 5 min.
3. Pause the program at 4 min and 30 s of the 10 °C incubation.
4. Add 15 µL of the reverse transcription buffer to each tube.
5. Restart the program and heat to 22 °C for 10 min.
6. Heat to 37 °C for 30 min.
7. Heat to 90 °C for 6 min to inactivate the enzymes.
8. Store the prepared cDNA at −20 °C or use it straight away to perform the first round of the nested PCR.

Table 2
Reverse transcription program

Step	Temperature (°C)	Time (min)
1	65	2
2	10	5[a]
3	22	10
4	37	30
5	90	6

[a]If using a PCR machine, pause the program after 4 min and 30 s of step 2, add 15 μL/well of the reverse transcription buffer containing the SuperScript enzyme and restart the program

3.5 V_H186.2 PCR and Sequencing

1. Use 2.5 μL of the cDNA prepared from a single cell in the first PCR.
2. Use 20 pmol of each of the external primers.
3. Prepare your PCR mix following the instructions from the manufacturer of the Taq polymerase you will be using (*see* **Note 8**).
4. Follow the PCR program PCR1 detailed in Table 3.
5. Dilute the first PCR product 30 times and use 1 μL of this dilution in the second PCR.
6. Use 20 pmol of each of the internal primers.
7. Prepare your PCR mix following the instructions from the manufacturer of the Taq polymerase you will be using (*see* **Note 8**).
8. Follow the PCR program PCR2 detailed in Table 3.
9. Run 5 μL of the second PCR on a 1 % agarose gel to check for positive clones at the appropriate size (350 bp).
10. Purify the remaining 25 μL using the PCR purification kit of your choice.
11. The purified samples are then sequenced using the internal Cγ1 primer (*see* **Note 9**).
12. Analyze the sequence using NCBI IgBlast and confirm sequence read by examination of the chromatogram trace files (Fig. 1b).

3.6 NP-Specific ELISpot

1. The day before the experiment coat ELISpot plates with 5 μg/mL of NP(4)-BSA or NP(15)-BSA diluted in PBS (50 μL/well) and incubate overnight at 4 °C (*see* **Note 10**).
2. Wash plates three times by filling them with water and pat them dry.

Table 3
Two-round nested PCR programs

	Step	Temperature (°C)	Time	
PCR1				
1	Denaturation	94	3 min	
2	Denaturation	94	45 s	Repeat steps 2–4 39 times
3	Annealing	50	60 s	
4	Extension	72	60 s	
5	Extension	72	10 min	
PCR2				
1	Denaturation	94	3 min	
2	Denaturation	94	45 s	Repeat steps 2–4 29 times
3	Annealing	50	60 s	
4	Extension	72	60 s	
5	Extension	72	10 min	

3. Saturate the plates with 150 µL/well of ELISpot blocking buffer for at least 2 h at 37 °C to avoid nonspecific binding.
4. Discard the ELISpot blocking buffer and pat the plates dry.
5. Add 100 µL of cell suspension at a concentration of 10^6 cells/mL (i.e., 10^5 cells/well) and 10^5 cells/mL (i.e., 10^4 cells/well) at least in triplicate (*see* **Note 11**).
6. Pool the remaining cells and dispense them in the control wells (negative controls: wells without coating, without secondary antibody or without cell; positive control: wells with a pool of cells).
7. Incubate plates for 5–20 h in a cell culture incubator at 37 °C with 5 % of CO_2.
8. Discard the cells and wash the plates three times with ELISpot washing buffer then two times with PBS only. Pat the plates dry with paper towel.
9. Add 50 µL/well of secondary antibody coupled to peroxidase diluted in PBS.
10. Incubate for 2 h at room temperature.
11. Wash the plates three times with ELISpot washing buffer then two times with PBS only. Pat the plates dry.
12. To develop the spots, prepare freshly a solution by dissolving a 20 mg AEC tablet in 2.5 mL of DMF.

13. Add to the dissolved AEC 47.5 mL of acetate buffer and 25 µL of fresh 30 % (w/w) hydrogen peroxide immediately prior to use (*see* **Note 12**).
14. Add 100 µL/well of the detection solution.
15. Wash abundantly under running water (*see* **Note 13**).
16. Let the plates to dry overnight at room temperature (*see* **Note 14**).
17. Read the plates using an ELISpot Reader according to the manufacturer's instructions (*see* **Note 15**) (Fig. 2b, c).

3.7 Sera Isolation and NP-Specific ELISA

3.7.1 Isolation of Sera from the Blood

1. Centrifuge blood briefly to collect blood at the bottom of the tube and incubate at 37 °C for 1 h or overnight at 4 °C to let the blood coagulate.
2. Centrifuge in a microcentrifuge at 2,000 rpm (500×*g*) at room temperature for 10 min.
3. Delicately remove the serum and transfer it in clean conical tubes. Store at −20 °C until required.

3.7.2 NP-Specific ELISA

1. The day before the experiment coat the ELISA plates with 5 µg/mL of NP(4)-BSA or NP(15)-BSA diluted in PBS (50 µL/well) and incubate overnight at 4 °C (*see* **Notes 10** and **16**).
2. Wash plates four times with ELISA washing buffer and pat them dry.
3. Saturate the plates with 150 µL/well of ELISA blocking buffer for 1 h at 37 °C to avoid nonspecific binding.
4. Discard the ELISA blocking buffer.
5. Wash plates four times with ELISA washing buffer and pat them dry.
6. Add 50 µL of ELISA blocking buffer in all the wells except for the first row.
7. Dilute the sera in PBS in order to obtain an initial concentration of 1/200.
8. Add 75 µL of the diluted sera in the first row of the plate. Add 75 µL of a control serum in each plate to be used to generate a standard curve.
9. Dilute serially the sera across the plate (i.e., transfer 25 µL from the first line to the following line, mix well, and repeat the process for the following lines. Discard the last 25 µL).
10. Incubate at room temperature for 2 h.
11. Wash plates four times with ELISA washing buffer and pat them dry.

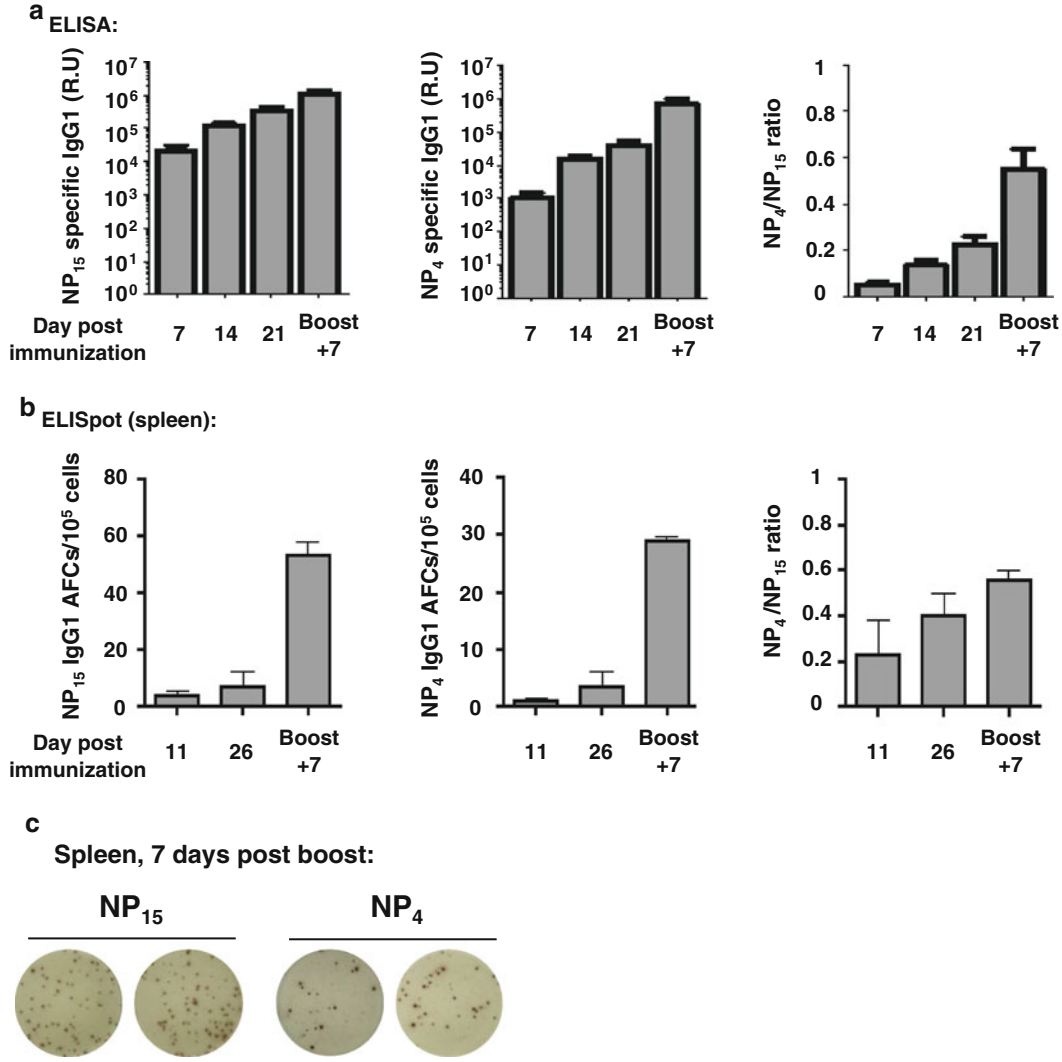

Fig. 2 Determination of affinity maturation by ELISA and ELISpot using the ratio NP_4/NP_{15}. (**a**) Representative results obtained when studying the kinetic of an immune response following immunization with NP-KLH in alum. The NP_{15}- (bound by both high and low affinity antibodies, *left panel*) and NP_4- (bound by high affinity antibodies only, *middle panel*) IgG1 serum titer were determined by ELISA 7, 14, and 21 days after primary immunization and 7 days after a secondary immunization. The antibody concentration is expressed in relative units (R.U.). Affinity maturation was then determined by calculating the ratio of the NP_4 titer by the NP_{15} titer (*right panel*). As the immune response progresses, affinity maturation increases as shown by the increase of the ratio NP_4/NP_{15}. (**b**) Representative results obtained when measuring the number of antibody forming cells (AFCs) in the spleen at different time points following primary and secondary NP-KLH immunization by ELISpot. The numbers of AFCs specific for NP_{15} (*left panel*) and NP_4 (*middle panel*) were determined 11 and 26 days after primary immunization with NP-KLH in alum and 7 days after a secondary immunization with NP-KLH only. Affinity maturation was then determined by calculating the ratio of the NP_4 spots by the NP_{15} spots (*right panel*). As the immune response progresses, affinity maturation increases as shown by the increase of the ratio NP_4/NP_{15}. (**c**) Representative ELISpot wells for splenic AFCs specific for NP_{15} (*left* wells) and NP_4 (*right* wells) 7 days after a secondary immunization (10^5 cells were loaded in each wells)

12. Add 50 µL of secondary antibody coupled with peroxidase diluted in PBS.
13. Wash plates four times with ELISA washing buffer pat them dry.
14. Reveal the plates by adding 100 µL/well of TMB and incubate for about 10 min.
15. Stop the reaction by adding 50 µL/well of 0.5 M sulphuric acid.
16. Read the optical density at 450 nm using a spectrophotometer (*see* **Note 17**) (Fig. 2a).

4 Notes

1. We have been using Imject Alum (Thermo Scientific) as adjuvant for all our experiments because we found it easy to use and efficient. However, aluminum hydroxide and aluminum phosphate can also be used to precipitate antigen.
2. It is good practice to have non-immunized control animals injected with alum or vehicle only in each experimental groups. This allows the precise gating of NP^+ B cells.
3. For studying the memory response mice can be challenged a second time with NP-KLH alone in absence of adjuvant.
4. The number of cells to prepare for the sort depends on the time point after immunization. The number and frequency of NP-specific GC B cells will progressively increase from day 9 until day 28.
5. Always wear gloves and use filtered tips to prepare the RT-Lysis buffer. Ideally, prepare this buffer under a hood or in a room dedicated to RNA extraction to avoid RNase contamination.
6. During the sorting you have to insure that the droplets containing the single cells are falling directly in the RT-lysis buffer.
7. For the reverse transcription you can also use heating blocks.
8. We generally use a classic Taq polymerase for our nested PCR with a final volume of 30 µL per tube. We did not observe polymerase-induced bias in our sequencing when we compared with a high-fidelity Taq.
9. Analysis of the V_H186.2 sequencing: the resulting sequence will have to be reverse complemented and blasted on the V_H186.2 germline sequence. We generally use the IgBLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/igblast/). Affinity maturation is measured by analyzing the frequency of the replacement of the tryptophan at position 33 by a leucine.

This mutation in the complementary determining region 1 (CDR1) is associated with 10 times higher affinity. The ratio of silent (S) and replacement (R) mutations in the CDR and framework regions (FWR) also gives an indication of the affinity with the ratio R/S increasing in the CDR as the affinity augments while it remains stable in the FWR (Fig. 1).

10. Using two differently haptenated-NP allows the determination of affinity maturation by ELISA and ELISpot. A weakly haptenated-NP (less than 5 NP molecules bound to each BSA molecule) will only be recognized by high affinity NP-specific antibodies while a highly haptenated NP (over 12 NP per BSA molecule) will be recognized both by high and low affinity NP-specific antibodies. Following immunization, affinity maturation progressively increases and so does the ratio NP(low)/NP(high) (Fig. 2).
11. We have observed that ELISpot plates with mixed cellulose membranes give less background than ELISpot plates with PVDF membrane.
12. The DMF dissolved AEC tablets can be kept for up to 1 week at 4 °C in a glass bottle and in the dark. The AEC powder or tablets are extremely toxic and should only be used with protective clothing and under a fume hood.
13. It is important to remove the plastic skirt at the bottom of the ELISpot plate during the last wash under running water to avoid dark marks on the membrane.
14. Dried ELISpot plates can be kept and analyzed several weeks after the experiments as long as they are protected from light and dust.
15. ELISpot analysis: the number of spots per well is determined using an ELISpot reader calibrated on the negative control wells (no cells added, no coating and no secondary antibody). In addition to the spot numbers, it is possible to study the spot surface that informs about the secreting capacity of the cells. Each spot represents a secreting cell. Affinity maturation is determined by doing the ratio of the number of antibody secreting cells specific for NP(low) and for NP(high). As the immune response progresses, the affinity increases and the ratio NP(low)/NP(high) goes from 0 to 1 (Fig. 2).
16. All the incubations in the ELISA protocol can be done either at 4 °C, room temperature or 37 °C. Adjust the incubation time accordingly (4 °C = overnight; room temperature = 2 h; 37 °C = 1 h).
17. ELISA analysis: use the O.D. (optical density) of the standard serial dilutions and corresponding arbitrary concentrations (e.g., starting at 1.000 and decreasing progressively following

your dilution factor) to make a standard curve. Fit a linear regression on the linear part of your standard curve (if your spectrophotometer does not do it automatically, that can be easily done on software like Excel) and use the associated equation to calculate the concentration in arbitrary units of the NP-specific antibodies in each serum. As for the ELISpots, affinity maturation is determined by the ratio of the NP(low) and NP(high) IgG1 concentration.

Acknowledgements

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Chapter 9

The SW_{HEL} System for High-Resolution Analysis of In Vivo Antigen-Specific T-Dependent B Cell Responses

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Abstract

T cell-dependent B cell responses generate optimal antibodies to combat foreign antigens. Naïve B cells responding to antigen undergo a complex series of differentiation events and cell fate decisions to provide long-lived memory B cells and plasma cells. Historically, B cell biologists have been challenged by the task of investigating rare antigen-specific B cells in an in vivo setting such that their interactions with antigen, regulation and migration may be accurately tracked. We have developed the SW_{HEL} experimental system capable of accurately monitoring B cells that interact with a protein antigen and then subsequently undergo isotype switching, somatic hypermutation, and affinity maturation within germinal centers (GC) to generate high-affinity antibodies. Here we provide a comprehensive description of the procedures involved in establishing and using the SW_{HEL} system to assess B cell responses to a foreign antigen. This system can provide a valuable measure of the functional capabilities of T follicular helper cells, whose role is ultimately to support and shape long-term humoral immunity.

Key words B cells, Antibodies, T-dependent, In vivo, Affinity maturation, Affinity-selection, Flow cytometry, Single cell sorting

1 Introduction

The ability to study antigen-specific B cell responses in vivo has been paramount in the quest to better understand humoral immunity. Many of the obstacles initially associated with investigating rare antigen-specific B cell clones in the primary repertoire have been overcome by the advent of transgenic and knock-in mice bearing high frequencies of lymphocytes with defined antigen specificities. We have developed the SW_{HEL} experimental system for high-resolution analysis of antigen-specific B cells that respond to hen egg lysozyme (HEL) within their normal cellular and molecular environments. The gene-targeting approach used to generate SW_{HEL} mice results in ~10–20 % of B cells carrying the anti-HEL specificity of the HyHEL10 monoclonal antibody.

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Importantly, SW_{HEL} B cells have the ability to undergo normal class switch recombination (CSR) and somatic hypermutation (SHM) of their immunoglobulin variable region genes.

The SW_{HEL} system is a means of examining numerous aspects of the B cell response following antigen encounter and activation: clonal expansion, cell fate decisions, cell migration and commitment into either extrafollicular antibody secreting cell (ASC) or early memory or GC B cell compartments. Since HEL-specific B cells in SW_{HEL} mice are capable of undergoing normal CSR and SHM, a major feature of the model is the ability to monitor affinity maturation and clonal selection as it occurs within a dynamic GC environment.

To mirror the low precursor cell frequencies typically expected in a primary response, small numbers of SW_{HEL} B cells are adoptively transferred, challenged with HEL, and identified in recipient mice using CD45 congenic approaches (Fig. 1). To assess B cell responses with physiologically relevant affinities for antigen, we have engineered recombinant HEL proteins carrying amino acid substitutions such that the B cell antigen receptor (BCR) interacts with antigen within a $K_a = 1.1 \times 10^7 - 2 \times 10^{10}$ (M^{-1}) affinity range. By challenging SW_{HEL} B cells with these lower-affinity HEL mutant proteins, the influence of initial BCR antigen affinity on key differentiation decisions may be examined. At various time points

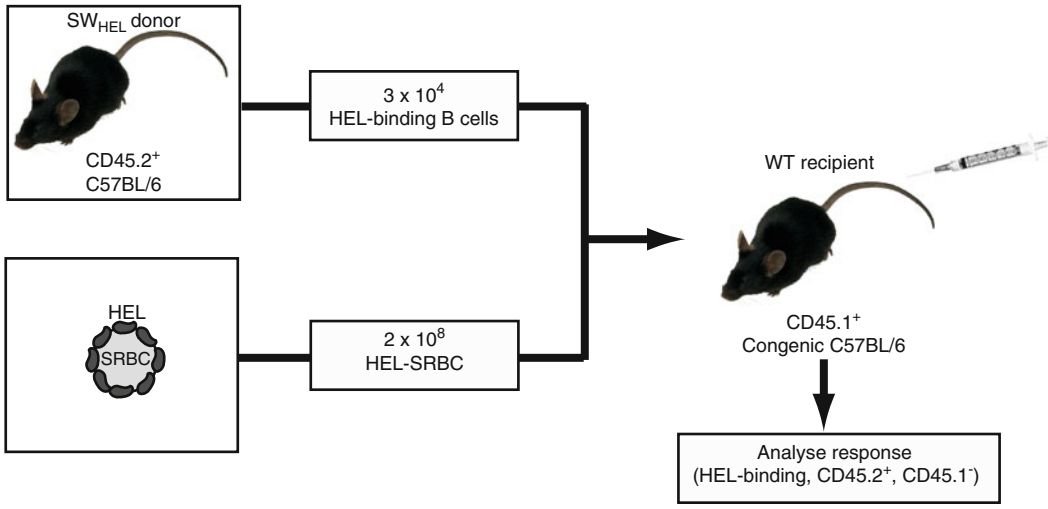


Fig. 1 Adoptive transfer strategy. SW_{HEL} donor ($CD45.2^+$) splenocytes were harvested and 3×10^4 HEL-binding B cells adoptively transferred into congenic SJL.*Ptprc*^{a} ($CD45.1^+$) recipients. These were simultaneously challenged with 2×10^8 HEL-conjugated SRBCs. Recipient spleens were harvested at various timepoints and analysed by flow-cytometry or immunofluorescent histology

post-immunization, spleens and serum from recipient mice may be assessed by (1) flow cytometry to quantitate and phenotype B cell subsets, (2) immunofluorescence histology to track their anatomical localization, and (3) ELISA to determine the circulating levels of antigen-specific antibody. Affinity maturation can be accurately monitored since the initial BCR specificity is known, and thus, any changes to the HyHEL10 variable region that occur via SHM are readily identified. Clonal selection can be tracked by flow cytometric sorting of single GC B cells to precisely identify BCR mutation patterns positively selected for survival within the GC. A powerful and versatile use of this system is that genetic deficiencies may be incorporated such that the role of virtually any gene of interest predicted to regulate the B cell response can be accurately investigated. In this chapter, we detail the reagents needed and procedures involved in genotyping SW_{HEL} mice, performing the adoptive transfer process and investigating the phenotype and affinity maturation of B cells.

2 Materials

2.1 Genotyping Materials

1. 10× Isolation Buffer: 670 mM Tris pH 8.8, 166 mM ammonium sulfate, 65 mM magnesium chloride, 10 % β-mercaptoethanol, 5 % Triton X-100. Combine 134 ml of 1 M Tris pH 8.8, 4.39 g ammonium sulfate, 13 ml of 1 M magnesium chloride, 20 ml β-mercaptoethanol, 10 ml Triton X-100, and dissolve in 23 ml Baxter Irrigation H_2O.
2. Proteinase K: Make up at 50 mg/ml in Proteinase K diluent.
3. Proteinase K diluent: 50 mM Tris–HCl (pH 8.0) and 10 mM calcium chloride in water.
4. GoTaq™ Green (or other equivalent Taq polymerase) and corresponding buffer.
5. dNTPs.
6. Genotyping Primers: Resuspend oligonucleotides at 200 μM. SW_{HEL} Heavy Chain (SHH) primer sequences are as follows: Upper primer (SHH-U) = gtctctgcaggtgagtcctaacttct, Lower primer 1 (SHH-L1) = caactatccctccagccataggat and Lower primer 2 (SHH-L2) = gttgattcttgtgtgacaccag. SW_{HEL} Light Chain (SHL) primer sequences are as follows: Upper primer 1 (SHL-U) = cagggccagccaaagtattg, Lower primer 1 (SHL-L) = tccaacctcttgtgggacagtt, Upper primer 2 (Sonic-U) = ctggctgtggaagcaggttt, and Lower primer 2 (Sonic-L) = cctgccagggactttctgaat.
7. DNA molecular weight marker.
8. Thermocycler/PCR machine.

2.2 Preparing Recombinant HEL

1. PD-10 columns.
2. Conjugation Buffer: 0.35 M D-mannitol, 0.01 M sodium chloride.
3. Retort stand.
4. Cuvette 50–2,000 μl.
5. Spectrophotometer.

2.3 HEL-SRBC Conjugation Materials

1. Hen Egg Lysozyme (HEL): Lysozyme from chicken egg white or recombinant HEL^{2X} and HEL^{3X} mutant proteins produced by the Brink laboratory.
2. Sheep red blood cells (in Alsever's Solution).
3. Dulbecco's PBS (DPBS).
4. Conjugation Buffer: 0.35 M D-Mannitol, 0.01 M sodium chloride.
5. EDCI: *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride.
6. HyHEL9 monoclonal antibody: HyHEL9 antibody was derived from a hybridoma and purification performed in the Brink laboratory.
7. Alexa Fluor® 647 Monoclonal Antibody Labeling Kit (Invitrogen Molecular Probes) or equivalent.
8. Hemocytometer.
9. Flow cytometer.

2.4 Reagents/ Buffers for Preparing SW_{HEL} Splenocytes and Adoptive Transfer

1. 2FR: 2 % (v/v) fetal bovine serum, 55 μM β-mercaptoethanol, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, made up in RPMI.
2. RBC Lysis Buffer: 0.15 M ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate (KHCO3), 0.1 mM sodium EDTA (Na_2EDTA), pH 7.2–7.4. Weigh 8.29 g NH_4Cl, 1.0 g KHCO3, 37.2 mg Na_2EDTA and dissolve in 800 ml water. Check pH and then top up with water to 1 l.
3. PBA (FACS) Buffer: 0.1 % (w/v) bovine serum albumin, 0.1 % (w/v) sodium azide, made up in PBS.
4. Fluorochrome-conjugated anti-B220 (RA3-6B2) antibody.
5. $OVA_{323–339}$ peptide (CGGISQAVHAAHAEINEAGR). Required only if using HEL-OVA to investigate T cell-B cell collaboration.
6. Succinimidyl-6-([ß-maleimidopropionamido] hexanoate) (SMPH). Required only to conjugate HEL-OVA.
7. Alum. Required only if investigating HEL-OVA responses.

2.5 Antibodies and Reagents for Flow Cytometry

1. Anti-mouse monoclonal antibodies: PE-conjugated anti-CD45R/B220 (RA3-6B2), PerCP-Cy5.5-conjugated anti-CD45.1 (A20), PE-Cy7-conjugated anti-CD45.2 (104), FITC-conjugated anti-CD38 (90). Use additional antibody clones and conjugates according to project aims.
2. Anti-CD16/32 (2.4G2) for Fc-blocking.
3. Fetal calf serum/fetal bovine serum (FCS/FBS).
4. 50 ml tubes.
5. 96-well round bottom plates.
6. 35 μm filter round-bottom FACS tubes.
7. FlowJo or equivalent software to analyze flow cytometry data.

2.6 Additional Reagents for Affinity Maturation Analysis

1. Antibodies as in Subheading 2.5 to detect B cell subsets of interest.
2. Recombinant HEL^{2X} and HEL^{3X} protein obtained from the Brink laboratory.
3. Biotinylated anti-mouse IgG1 (A85-1) monoclonal antibody or other isotype according to project aims.
4. Streptavidin Pacific Blue™ (SA-PB).
5. C57BL/6 mouse serum: Obtained either by cardiac puncture or purchased.

2.7 Single Cell FACS Sorting Materials

1. Digest Buffer (suitable for one 96-well plate): 100 μl 10× Taq PCR reaction buffer (no $MgCl_2$), 50 μl Proteinase K (10 mg/ml in water), 10 μl 10 mM EDTA, 10 μl Tween 20 (10 % solution), make up with 830 μl water to give a total of 1 ml (Table 2a). Allow an additional 10 % when calculating volumes for multiple plates.
2. 96-well Skirted PCR plates.
3. 96-well non-skirted PCR plates.

2.8 SHM Analysis Materials

1. Thermocycler/PCR Machine.
2. Adhesive plate seals (either foiled or non-foiled).
3. SHM Primary PCR Primers: Upper primer (SSC-U1) = gtt gta gcc taa aag atg atg gtg and Lower primer (SSC-L1) = gat aat ctg tcc taa agg ctc tga g (lower).
4. SHM Secondary PCR Primers: Upper primer (SSC-U2) = tct tct gta cct gtt gac agc cc. Upper primer (SSC-U2) = ttg tag ccta aaa gat gat ggt gtt aag tc. Lower primer (SSC-L2) = caa ctt ctc tca gcc ggc tc.
5. Taq polymerase or equivalent.
6. dNTPs.

7. illustra™ ExoStar™ or equivalent PCR Clean-up Reaction Kit.
8. Gel-doc/equivalent to visualize DNA under UV light.
9. Sequencing Primers: Same as SHM Secondary PCR Primers.
10. DNA Strider™ and Sequencher™ or equivalent sequence alignment software.

2.9 ELISA Reagents

1. NPP Buffer: 34.9 mM sodium bicarbonate ($NaHCO_3$), 15 mM sodium carbonate (Na_2CO_3), 3.1 mM sodium azide (NaN_3), 1 mM magnesium chloride ($MgCl_2$). Weigh 2.93 g $NaHCO_3$, 1.59 g $NaCO_3$, 0.2 g NaN_3, 0.095 g $MgCl_2$. Make up to 1 l with water.
2. 96-well plates for ELISA.
3. Tween 20: 0.05 % (v/v) solution in PBS.
4. Humidity chamber/plastic container lined with wet paper towel on the bottom.
5. 1 % BSA buffer: 1 % (w/v) in PBS.
6. 0.1 % BSA buffer: 0.1 % (w/v) in PBS.
7. Skim milk powder: 1 % (w/v) in 0.1 % BSA buffer.
8. Recombinant HyHEL10 isotype-specific standards obtained from the Brink laboratory.
9. Biotinylated anti-mouse IgG1 (A85-1) monoclonal antibody (or other isotype of interest).
10. Streptavidin-Alkaline Phosphatase.
11. p-nitrophenyl phosphate.
12. ELISA microplate reader.
13. 37 °C dry air incubator.

2.10 Immunofluorescence Histology Materials

1. Cryostat.
2. Microscope slides.
3. Acetone: 100 % solution, stored at –20 °C until required.
4. Normal horse serum (NHS): 30 % solution made up in PBS.
5. HEL.
6. Polyclonal rabbit anti-HEL sera or other anti-HEL antibody.
7. Cy5-labelled anti-rabbit IgG antibody.
8. Conjugated monoclonal antibodies: FITC-labelled anti-IgD (11-26c.2a) antibody and biotinylated anti-CD3 antibody.
9. Streptavidin-Alexa Fluor® 350 antibody.
10. Additional reagents if investigating HEL-OVA responses: Biotinylated anti-Thy1.1 (HIS51) antibody, Cy3-tyramide staining kit, FITC-labelled anti-rabbit IgG antibody, Alexa Fluor® 647-labelled anti IgD antibody.

11. Anti-Fade Solution: Weigh 2.66 g of 1,4-diazabicyclo-[2.2.2] octane (DABCO), add 900 ml Glycerol and 100 ml PBS (or equivalent anti-fade product). Store at room temperature, protected from light.
12. Coverslips.
13. Fluorescence microscope and relevant software.

3 Methods

3.1 SW_{HEL} Mice and Genotyping

3.1.1 SW_{HEL} Ig Transgenic Mice

Gene-targeted V_H10 mice were produced by Prof. Robert Brink and have been previously described (1). These mice can be maintained either on a C57BL/6 or C57BL/6-SJL.*Ptprc*^{a} congenic background and are to be used as donor mice in the adoptive transfer. Congenic mice (either C57BL/6-SJL.*Ptprc*^{a} or C57BL/6 respectively) are used as recipient mice.

3.1.2 Genotyping of SW_{HEL} Mice by PCR Amplification

1. Extract genomic DNA by purifying from a 1–2 mm length of tail tip. Resuspend tail tips in 2 μl of 1× Isolation Buffer (diluted from 10× stock in water) containing 2 μl of Proteinase K (50 mg/ml in appropriate diluent) and incubate at 65 °C for 4–16 h. Quick spin and cool to room temperature. Use 1 μl of the resulting solution to determine the genotype by PCR.
2. PCR amplify the genomic DNA, extracted as described in Subheading 2.1. Set up PCR reactions using Go Taq, GoTaq™ buffer, dNTPS at 0.2 mM, and primers at 0.4 μM (Fig. 2a).
3. Run SW_{HEL} Heavy Chain and SW_{HEL} Light Chain PCRs on a thermocycler according to the following PCR conditions: 94 °C for 4 min, (94 °C for 30 s, 63 °C for 30 s, 72 °C for 1 min) × 35 cycles, followed by 72 °C for 10 min.
4. Separate the resulting PCR products on a 1–2 % agarose gel containing 1 % ethidium bromide (or equivalent) and visualize under UV light. Determine which mice carry the correct genotype (Fig. 2b, **Notes 1–2**).

3.2 Preparation and Desalting of Recombinant HEL Proteins

A comprehensive protocol for the transformation of plasmid DNA into *Pichia pastoris*, protein expression and purification has been excluded due to space limitations. Purified recombinant HEL^{WT}, HEL^{1X}, HEL^{2X}, HEL^{3X}, and HEL^{4X} proteins (2, 3) are stored in PBS aliquots at −80 °C for long-term storage, and once thawed, stored at 4 °C for a maximum of 8 months (*see* **Notes 3–5**). HEL mutant proteins are desalted into Conjugation Buffer prior to SRBC conjugation.

1. Equilibrate a PD-10 column with ~30 ml Conjugation Buffer.
2. Load 100 μg of protein onto the center of the column and allow to settle in prior to adding the remaining volume up to 2.5 ml with Conjugation Buffer. This flow-through can be discarded.

a SW_{HEL} Heavy Chain Genotyping PCR reaction

Reagent	Initial Conc.	Volume (µl)
DNA prep	Undiluted	1.00
tdH_2O	-	17.37
GoTaq Buffer	5X	5.00
dNTPs	10mM	0.50
Primer mix*	10µM	1.00
GoTaq	5U/µl	0.13
		25.00

*Primer mix is comprised of SHH-U, SHH-L1, and SHH-L2 primers diluted 1/20 in tdH_2O

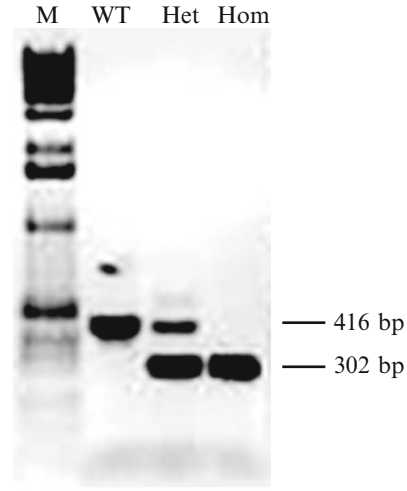
b SW_{HEL} Light Chain Genotyping PCR reaction

Reagent	Initial Conc.	Volume (µl)
DNA prep	Undiluted	1.00
tdH_2O	-	16.37
GoTaq Buffer	5X	5.00
dNTPs	10mM	0.50
Primer mix 1**	10µM	1.00
Primer mix 2***	10µM	1.00
GoTaq	5U/µl	0.13
		25.00

** Primer mix 1 is comprised of SHL-U, SHL-L diluted 1/20 in tdH_2O.

*** Primer mix 2 is comprised of Sonic-U, Sonic-L diluted 1/20 in tdH_2O.

c SW_{HEL} Heavy Chain Result



d SW_{HEL} Light Chain Result

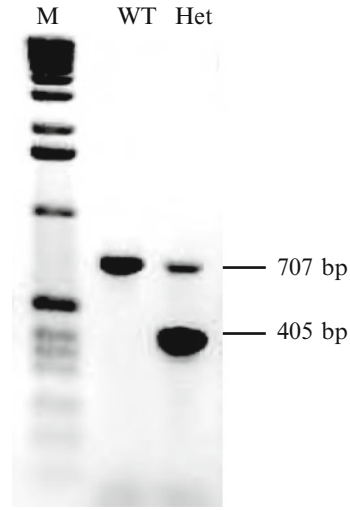


Fig. 2 Determination of SW_{HEL} mouse genotype. PCR reactions for determining the SW_{HEL} Heavy Chain (**a**) and Light Chain (**b**) genotype of SW_{HEL} mice, with expected PCR product sizes to determine zygosity for Heavy Chain (**c**) and Light Chain (**d**). Note that SW_{HEL} Light Chain is homozygous lethal and thus homozygous mice are not viable. *M* molecular weight marker X (Roche), *WT* wild-type, *Het* heterozygote, *Hom* homozygote

3. Elute the protein by adding 3.5 ml Conjugation buffer and keep the eluate as this will contain the HEL protein. Collect the eluate as fractions in the following volumes; Fraction 1 as 250 µl, Fraction 2 as 1,000 µl (this will contain the majority of protein), Fractions 3–7 as 250 µl.
4. Determine the absorbance/optical density of each fraction by spectrophotometry at 280 nm. Calculate the protein concentration.

3.3 Conjugation of HEL to Sheep Red Blood Cells

This protocol describes the conjugation of HEL to SRBC at 10 μg HEL/10×10^9 SRBC in a 1 ml reaction. All centrifugation steps for SRBC are performed at $1{,}111 \times g$ for 5 min at 4 °C.

1. Sterile aliquot approximately 8–10×10^9 SRBC into a 50 ml tube. Count SRBCs and adjust accordingly if SRBC number does not fall into this range (*see* **Notes 6** and **7**).
2. Wash SRBC in 30 ml DPBS and centrifuge. Repeat this twice. After the third wash, resuspend SRBC in 10 ml Conjugation Buffer and centrifuge.
3. During centrifugation, perform the following calculations:

   x = the volume representing 10 μg of mutant HEL protein (desalted)

   y = volume of Conjugation Buffer to resuspend in = 1,000 μl – 100 μl – x.
4. Resuspend SRBCs in volume y of Conjugation Buffer (*see* **Note 8**). This volume will ensure that a concentration of 10 μg HEL/ml is obtained in the reaction (allowing for volume of EDCI to be added and neglecting pellet volume).
5. Add 10 μg of HEL mutant protein (=x) and mix on ice on a platform rocker for 10 min.
6. Add 100 μl of 100 mg/ml EDCI (made up in Conjugation Buffer) and continue to mix on rocker for a further 30 min on ice.
7. Wash SRBCs in DPBS four times or until cell lysis has ceased (a colorless supernatant should be obtained).
8. Count SRBCs and resuspend accordingly such that a cell concentration of 1×10^9 cells/ml in DPBS is obtained. This allows 2×10^8 SRBC to be injected per mouse in a 200 μl volume.
9. Confirm that HEL-SRBC conjugation was successful by flow-cytometric analysis using Alexa Fluor® 647-conjugated HyHEL9 antibody (Fig. 3a).

3.4 Preparation of SW_{HEL} Splenocytes and Adoptive Transfer

All centrifugation steps for splenocytes in 50 ml tubes are performed at $440 \times g$ for 5 min at 4 °C.

3.4.1 Transfer of SW_{HEL} Splenocytes

1. Harvest the spleen from a SW_{HEL} donor mouse according to local ethical guidelines and collect in 2FR medium (*see* **Note 9**).
2. Prepare a cell suspension by using a 70 μm cell strainer compatible for a 50 ml tube.
3. Wash splenocytes in 8 ml of 2FR.
4. Lyse RBC by resuspending splenocyte pellet in 5 ml RBC Lysis Buffer and underlaying without delay with 1 ml FBS.
5. Centrifuge and resuspend SW_{HEL} cells in 5 ml of 2FR medium prior to counting.

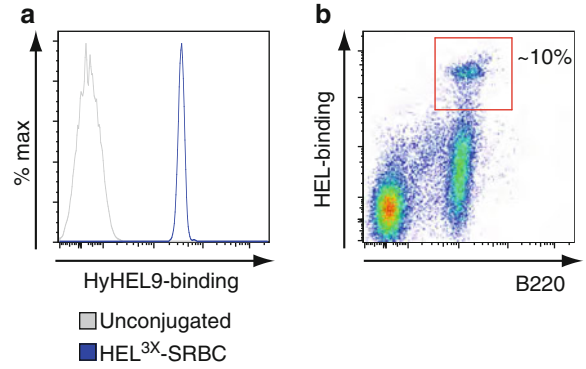


Fig. 3 Preparation of HEL^{3X}-SRBC and SW_{HEL} donor splenocytes. (**a**) Flow cytometry histogram showing HyHEL9-Alexa Fluor 647 staining of HEL^{3X}-conjugated Sheep Red Blood Cells. (**b**) Flow cytometry plot of splenocytes obtained from a SW_{HEL} mouse donor depicting ~10 % HEL-binding $B220^+$ cells

6. Quantitate HEL-binding B cells as follows: Stain 0.5×10^6 cells with 200 ng/ml HEL on ice for 20 min. Wash twice with PBA buffer followed by dual-staining with HyHEL9-Alexa Fluor® 647 and anti-B220-PE. Determine the percentage of HEL-binding B cells by flow cytometry (Fig. 3b, **Note 10**).
7. Adjust the concentration of splenocytes in suspension such that 3×10^4 HEL-binding B cells can be injected per recipient mouse in a 50 µl volume (6×10^5 HEL-binding B cells/ml).
8. Mix conjugated HEL-SRBC with SW_{HEL} cells in vitro immediately prior to tail vein injection. Each recipient mouse receives a total of 250 µl, which is comprised of 2×10^8 HEL-SRBCs in 200 µl and 3×10^4 HEL-binding B cells in 50 µl.
9. SW_{HEL} mice on the SJL/Ptprc^a ($CD45.1^+$) background are used as donors into 8-week-old C57BL/6 ($CD45.2^+$) recipient mice (or vice versa, *see* **Note 11**).

3.4.2 Co-transfer of SW_{HEL} Splenocytes and OT-II T Cells

1. To investigate collaborative responses between SW_{HEL} B cells and OT-II T cells, chemically conjugate HEL to $OVA_{323–339}$ peptide (CGGISQAVHAAHAEINEAGR) using the cross-linking agent SMPH (Fig. 4) (4).
2. Prepare a mixture of spleen cells from SW_{HEL} and OT-II mice containing 3×10^4 HEL-binding B cells and 3×10^4 $V\alpha2^+$ $CD4^+$ OT-II T cells.
3. Inject SW_{HEL} B cells and OT-II T cells intravenously into recipient mice, together with 30 µg HEL-$OVA_{323–339}$ conjugate.
4. Administer 100 µg OVA (Sigma) in Alum intraperitoneally on the same day of cell transfer.

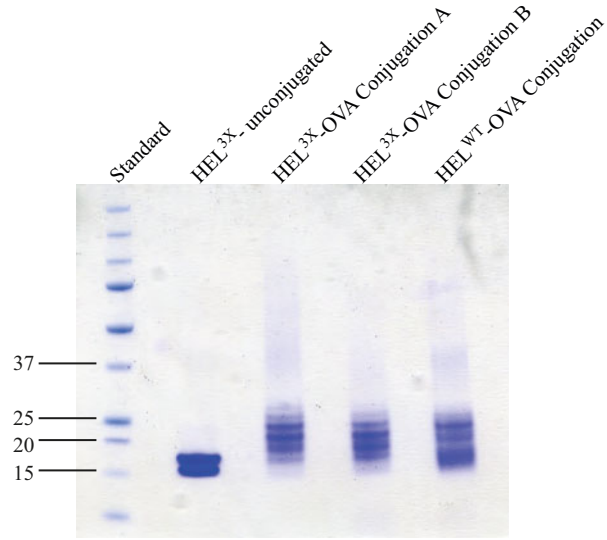


Fig. 4 Coomassie staining of HEL conjugated to OVA323–339 peptide, used as the immunizing antigen when studying HEL-specific B cell and Ovalbumin-specific T cell responses. Note the "ladder" of modified HEL proteins containing increasing numbers of conjugated peptides. Standard-BioRad Precision Plus™ Protein All Blue Standard

3.5 Flow-Cytometric Analysis of Antigen-Specific B Cell Subsets

This section describes the basic procedure involved to identify and quantitate B cell subsets by flow cytometry and is suitable for all time points post-immunization. For projects requiring affinity maturation analyses, please refer to Subheading 3.6. All centrifugation steps for tubes are performed at 440 × *g* for 5 min at 4 °C. All centrifugation steps for 96-well plates are performed at 863 × *g* for 10 s at 4 °C. All staining steps for flow cytometry are performed in 50 µl/well, in PBA buffer, on ice for 20 min. All wash steps for 96-well plates are performed by adding 200 µl PBA buffer to each well, centrifuging at 863 × *g* for 10 s at 4 °C to pellet the cells, then discarding the wash buffer by flicking and quickly inverting the plate on a paper towel.

3.5.1 Staining

1. Harvest recipient mouse spleens in 2FR at the desired time point according to project aims.
2. Prepare splenocyte suspensions using 70 µm cell strainers suitable for 50 ml tubes. Wash cell strainers with 2FR medium. Centrifuge tubes and remove supernatant.
3. RBC lyse by resuspending cell pellet in 5 ml RBC Lysis buffer and underlaying quickly with 1 ml FCS/FBS prior to centrifugation. Remove supernatant and resuspend in 5 ml of PBA buffer. Count cells.

Table 1
Staining protocol for flow cytometric analysis of responding SW_{HEL} B cells

Step	Antibody/reagent
(a)	
1	Fc-block (anti-CD16/32) HEL
2	HyHEL9-Alexa Fluor 647 Anti-B220 PE
3	Anti-CD45.1 PerCP/Cy5.5 Anti-CD45.2 PE/Cy7 Anti-CD38 FITC

Step	Antibody/reagent
(b)	
1	Fc-block (anti CD16/32) HEL^{3X}
2	Anti-IgG1 biotin
3	5 % mouse serum
4	HyHEL9-Alexa Fluor 647 Anti-B220 PE
5	Anti-CD45.1 PerCP/Cy5.5 Anti-CD45.2 PE/Cy7 Anti-CD38 FITC Streptavidin Pacific Blue

Dotted lines indicate wash steps are to be performed

4. Transfer 2.5×10^6 cells per well into 96-well round-bottom plates (*see* **Note 12**). Include the appropriate number of replicate wells by considering the desired number of events at acquisition on the cytometer and also the time point being analyzed. Centrifuge and wash twice in PBA.
5. Block Fc receptors with unlabelled anti-CD16/32. To detect antigen-specific cells, stain cells with saturating levels of HEL (200 ng/ml) (Table 1a). Wash twice.
6. Stain with HyHEL9-Alexa Fluor® 647 and PE-conjugated anti-B220 (*see* **Notes 13** and **14**). Wash twice.
7. Stain for congenic markers using PerCP Cy5.5-conjugated anti-CD45.1 and PE-Cy7-conjugated anti-CD45.2. Include FITC-conjugated anti-CD38 and any other stains according to project aims. Wash twice.

8. Following all staining and wash steps, cells are resuspended at a final concentration of approximately 10^7/ml prior to data acquisition on a flow cytometer.
9. Filter cells using a 35 μm filter round-bottom FACS tubes immediately prior to data acquisition on a flow cytometry analyzer. Collect ~2.5–4 × 10^6 events per sample.

3.5.2 Analysis

Analyze cytometer files with FlowJo software or equivalent.

1. Include light scatter gates on all samples to include lymphocytes but exclude dead cells and debris.
2. Apply a further doublet exclusion gate and exclude autofluorescent cells using a "dump" channel.
3. Separate donor-derived cells from endogenous recipient cells using CD45.1/ CD45.2 gates.
4. Apply subsequent HEL-binding gates prior to quantifying extrafollicular plasma cells, early memory B cells, Germinal center B cell, plasma cells, or memory B cells (Fig. 5a) (4).

3.6 Tracking Affinity Maturation Using the SW_{HEL} System

This section describes the procedures involved in tracking affinity maturation and is suitable for time points from day 7 onward, from which time point a mature GC has formed, and thus, affinity maturation may be readily identified.

1. Prepare splenocyte suspensions as in **steps 1–4** of Subheading 3.5.
2. For affinity maturation studies, stain cells with sub-saturating levels of HEL^{3X} (50 ng/ml) and unlabelled anti-CD16/32 (Table 1b) (*see* **Note 15**). Wash twice.

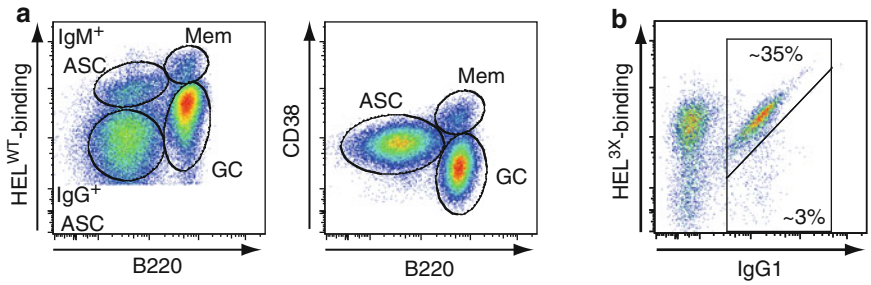


Fig. 5 Representative SW_{HEL} responses. (**a**) Day 5 response elicited by HEL^{2X}-SRBC. Forward and side light scatter gates were applied, doublets and autofluorescent cells excluded, and donor-derived cells revealed by gating on donor allotype-positive and recipient allotype-low cells. HEL-binding gates were applied prior to identifying SW_{HEL} responding B cell subsets at Day 5 as being Germinal Center B Cells (GC), Antibody-Secreting Cells (ASC), Early Memory B cells (Mem). (**b**) Day 14 response elicited by HEL^{3X}-SRBC. Light scatter gates, exclusion gates and donor-derived gates were applied as described for (**a**) prior to identifying SW_{HEL} responding B cells with high level HEL^{3X}-binding, indicative of an affinity-matured response

3. To identify isotype-switched cells, stain with biotinylated anti-mouse IgG1 or other desired antibody isotype (*see* **Note 16**). Wash twice.
4. Block with 5 % mouse serum (*see* **Note 17**). Wash twice.
5. Stain with HyHEL9-Alexa Fluor® 647 and PE-conjugated anti-B220. Wash twice.
6. Stain for congenic markers using PerCP Cy5.5-conjugated anti-CD45.1 and PE-Cy7-conjugated anti-CD45.2. Include FITC-conjugated anti-CD38 and Streptavidin Pacific Blue™. Wash twice.
7. Following all staining and wash steps, cells were resuspended at a final concentration of approximately 10^7/ml prior to data acquisition on a flow cytometer.
8. Filter cells and acquire data on a flow cytometry analyzer as described in Subheading 3.5. Collect ~3.5–7 × 10^6 events per sample.
9. Analyze cytometer files. For affinity maturation studies, apply GC B cell gates, and using the HEL^{3X}-binding stain counter-stained with $IgG1^+$, assess affinity-matured B cells according to their high level HEL^{3X}-binding (Fig. 5b) (*see* **Note 18**) (2, 5).

3.7 Single Cell FACS Sorting for SHM Analysis

This section describes the single cell sorting of donor-derived GC B Cells and should be modified to assess other B cell subsets according to project aims.

1. Prepare cell suspensions as previously described in Subheadings 3.5 and 3.6.
2. Block Fc receptors with unlabelled anti-CD16/32. Wash.
3. Stain cells with a FITC conjugated antibody directed against the relevant allotype marker, to detect donor-derived responders. Also stain with PE-conjugated anti-CD38 and APC-conjugated anti-B220 to identify B cell subsets.
4. Prepare 96-well skirted PCR plates for single cell sorting by adding 10 µl of Digest Buffer per well of a 96-well plate (*see* **Note 19**).
5. Single cell sort GC B cells (or other populations of interest) by applying appropriate sorting gates (Fig. 6).

3.8 SHM Analysis of HyHEL10 Heavy Chain Variable Region: PCR Amplification, DNA Visualization and Data Analysis

All centrifugation steps for single cells in 96-well plates are done at 1,500 rpm (440 × *g*) for 5 min.

1. After single cell sorting, cover cells with adhesive sealing foil and centrifuge without delay (*see* **Note 20**).
2. Heat plates to 56 °C for 40 min and 95 °C for 8 min, and recentrifuge prior to storage at –80 °C overnight (*see* **Note 21**).

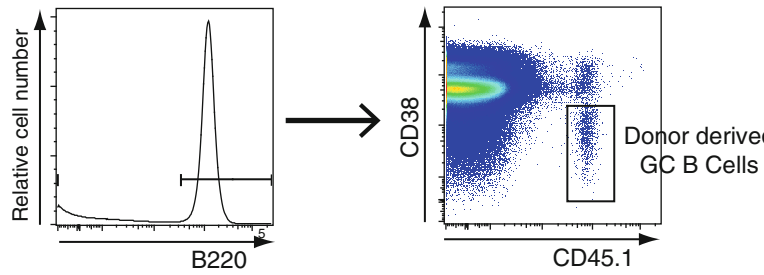


Fig. 6 Example Gating strategy employed for single-cell sorting and SHM analysis. Splenocytes from adoptive transfer recipients were stained as described in Subheading 3.7 and subjected to single-cell sorting into 96-well plates on a BD FACSAria. Forward and side light scatter gates were applied, doublets and autofluorescent cells excluded, prior to identifying B cells as $B220^{hi}$-expressing cells. Donor-derived Germinal Center B cells were then single cell sorted by applying gates on $CD45.1^{+}$ $CD38^{low}$ expressing cells

3. Thaw 96-well plates at room temperature for 5–10 min before subjecting to Primary Polymerase Chain Reaction (PCR) amplification of the V_H10 gene to analyze SHM.
4. Perform the Primary PCR reaction according to the volumes shown in Table 2b and the following PCR conditions: 94 °C for 3 min, (95 °C for 15 s, 55 °C for 1 min, 72 °C for 1 min) × 35 cycles, 72 °C for 10 min.
5. Perform the Secondary PCR reaction according to the volumes shown in Table 2c and PCR conditions: 94 °C for 3 min, (95 °C for 15 s, 62 °C for 40 s, 72 °C for 1 min) × 35 cycles, 72 °C for 10 min.
6. Determine which PCR wells contain the appropriately sized PCR product by subjecting to standard electrophoresis procedures, using 1–2 % agarose gels and detection via UV light (Fig. 7).
7. For wells containing amplified DNA, eliminate any unincorporated primers and inactivate nucleotides using illustra™ ExoStar™.
8. Dilute PCR products 1 in 10 prior to sequencing. Detect mutations by aligning sequence files with the HyHEL-10 sequence using DNA Strider™ or similar. Confirm sequence read by examination of the chromatogram trace files using Sequencher™ 3.11 or similar (*see* **Note 22**).

3.9 Determination of Antigen-Specific Serum Antibodies by ELISA

The serum levels of anti-HEL antibodies of the various Ig subclasses may be measured by ELISA. All wash steps are comprised of 3 × 300 µl 0.05 % Tween 20/PBS washes. Each step below is incubated in a humidity chamber for 1 h at 37 °C unless otherwise stated. This protocol is suitable for a 96-well plate format; however, volumes can be easily adapted to suit a 384-well plate format.

Table 2
Volumes required for SHM analysis of HyHEL10 heavy chain variable regions

Reagent	Initial Conc.	Volume/plate (μl)	Volume/4.4 plates (μl)
(a)			
Taq buffer (no $MgCl_2$)	10×	100	440
Proteinase K	10 mg/ml	50	220
EDTA	10 mM	10	44
Tween 20	10 %	10	44
tdH_2O	–	830	3,652
		1,000	4,400

Reagent	Initial Conc.	Volume (μl)
(b)		
DNA in 1 × Taq buffer (no $MgCl_2$)	Undiluted	10.00
Taq buffer (no $MgCl_2$)	10×	1.50
$MgCl_2$	50 mM	1.00
dNTPs	10 mM	0.50
Upper primer	10 μM	0.10
Lower primer	10 μM	0.10
Taq DNA polymerase	5 U/μl	0.15
tdH_2O	–	11.65
		25.00

Reagent	Initial Conc.	Volume (μl)
(c)		
Primary PCR in 1 × Taq buffer	Undiluted	2.50
Taq buffer (no $MgCl_2$)	10×	2.25
$MgCl_2$	50 mM	1.00
dNTPs	10 mM	0.50
Upper primer	10 μM	1.00
Lower primer	10 μM	1.00
Taq DNA polymerase	5 U/μl	0.10
tdH_2O	–	16.65
		25.00

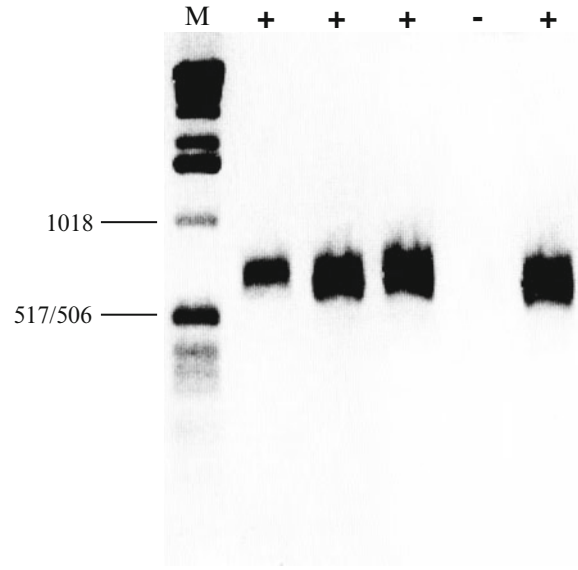


Fig. 7 Agarose gel bands showing wells containing (+) or not containing (–) secondary PCR product from single sorted SW_{HEL} B cells. *M* molecular weight marker X (Roche)

1. Coat 96-well ELISA plates with 10 μg/ml HEL in 60 μl NPP buffer and incubate overnight at 4 °C (*see* **Note 23**). Wash.
2. Block plate with 100 μl of 1 % BSA buffer. Incubate.
3. Perform serial twofold dilutions of sera in 50 μl 0.1 % BSA buffer in duplicate or triplicate. Use recombinant HyHEL10 standards (6) to quantitate antibody levels for each class of Immunoglobulin. Incubate.
4. Detect bound serum antibody using Ig heavy chain isotype-specific biotinylated antibody diluted in 50 μl 1 % skim milk powder diluted in 0.1 % BSA buffer. Incubate.
5. Add Streptavidin-Alkaline Phosphatase (SA-AP) diluted in 50 μl 0.1 % BSA buffer. Incubate.
6. Visualize with 1 mg/ml of substrate p-nitrophenyl phosphate in 100 μl NPP buffer by reading the absorbance at 405 nm on a microplate reader.
7. Construct a standard curve and determine the concentration of serum anti-HEL from the absorbance of dilutions that fall within the dynamic range of the curve (*see* **Note 24**).

3.10 Tracking Cell Localization by Immunofluorescence Histology

The following procedure is used to identify HEL-binding B cells within the context of surrounding B cell follicles and periarteriolar lymphoid sheath areas such that their migration can be accurately tracked (Fig. 8a). This procedure can be easily adapted to

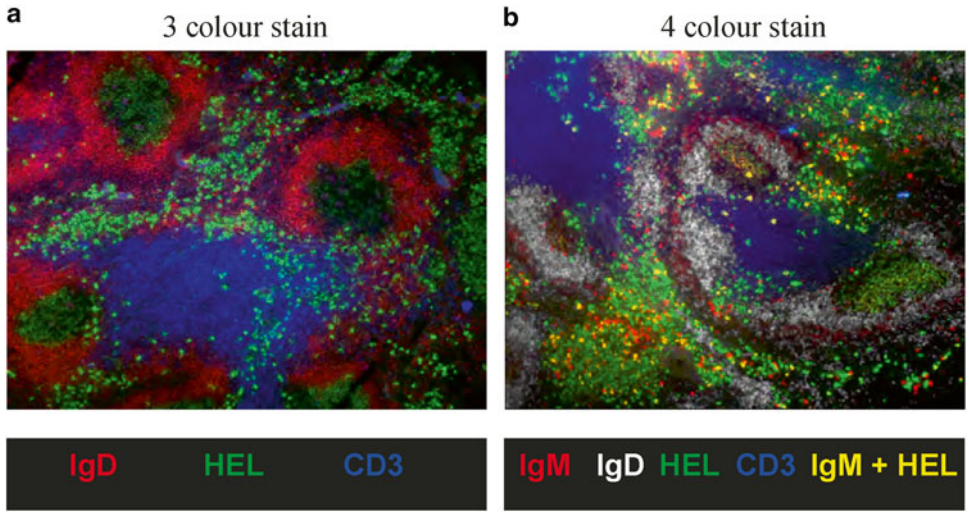


Fig. 8 Immunofluorescence Histology of SW_{HEL} responses. SW_{HEL} responses were elicited with HEL^{2X}-SRBC and recipients analyzed at Day 5 by spleen tissue sectioning and immunofluorescence histology. (**a**) Example of a 3-color stain. IgD^+ areas (*red*)—B cell follicles, IgD^- areas within B cell follicles—Germinal Centers, HEL-binding (*green*)—SW_{HEL} B cells, CD3 (*blue*)—T cells. (**b**) Example of a 4-color stain. IgM (*red*)—unswitched IgM+ cells, IgD (*white*) B cell follicles, HEL-binding (*green*)—SW_{HEL} B cells, CD3 (*blue*)—T cells, IgM + HEL (*yellow*)—colocalization of IgM and HEL-binding stains

accommodate different stains and fluorophores (Fig. 8b). Unless indicated otherwise, all stains are performed at room temperature for 20 min and all washes are of 3 × 5 min with PBS.

1. Cut mouse tissue into 6–7 μm sections using a cryostat.
2. Fix sections by submerging in chilled 100 % acetone for 20 min. At this point, store at −20 °C until the day of staining.
3. On the day of staining, thaw sections at room temperature for ~10 min (*see* **Note 25**). Block with 30 % NHS for 1 h at 37 °C. Wash.
4. To stain for HEL-binding cells, incubate sections firstly with 200 ng/ml HEL^{WT} (Sigma) (Table 3). Wash.
5. Stain sections with polyclonal rabbit anti-HEL sera. Wash.
6. Stain sections with Cy5-labelled anti-rabbit-IgG. Include a FITC-labelled anti-IgD (11-26c.2a) stain to identify follicular B cells and biotinylated anti-CD3-stain (eBiosciences; 500A2) to detect T cells in this step. Wash.
7. Stain with Streptavidin-Alexa Fluor® 350 (Molecular Probes) for 10 min.
8. Use an anti-fade solution to reduce photobleaching prior to mounting coverslips.
9. Visualize slides using a fluorescence microscope and appropriate software.

Table 3
Staining protocol for tracking antigen-specific responses by immunofluorescence histology

Step	Antibody/reagent	Concentration/dilution factor	Incubation duration (min)
1	PBS (rehydration)	Undiluted	20
2	Normal horse serum	30 %	30
3	HEL Anti-CD3 biotin	100 ng/ml 1:100	30
4	SA A350	1:1,000	20
5	Normal rat serum Rabbit anti-HEL	5 % 1:800	30
6	Anti-rabbit Cy5 Anti-IgD FITC	1:250 1:100	30

Dotted lines indicate wash steps are to be performed

For collaborative responses between SW_{HEL} B cells and OT-II T cells, detect OT-II transgenic T cells by staining with biotinylated anti-Thy1.1-biotin (HIS51) followed by a Cy3-tyramide staining kit. In this case, detect HEL-binding B cells using a FITC-labelled anti-rabbit-IgG and follicular B cells using an Alexa Fluor® 647-labelled anti-IgD-stain.

4 Notes

1. Expected PCR product sizes when genotyping are 302 bp for SW_{HEL} Heavy chain and 416 bp for WT Heavy Chain.
2. Expected PCR product sizes when genotyping are 405 bp for SW_{HEL} Light chain and 707 bp for WT Light Chain.
3. For long-term storage of mutant HEL proteins, keep at –80 °C and thaw when required. Aliquots in PBS may be stored for several months at 4 °C; however, HEL in Conjugation Buffer (mannitol/NaCl) should be stored for no greater than 2 months at 4 °C.
4. Confirm protein concentration of recombinant HEL by spectrophotometry at regular intervals, particularly post-thaw from –80 °C storage.
5. To calculate the concentrations of HEL^{1X}, HEL^{2X}, or HEL^{3X} use a molar absorption coefficient, Epsilon of 2.4.
6. As a rough guide, approximately 1.5–2 ml of SRBC (dependent on the supplier) will equate to $8–10 \times 10^9$ SRBC.

7. To count SRBC, perform several serial dilution steps to ensure greater accuracy of the count. Due to rapid settling of the SRBC, be sure to obtain an even suspension by inverting the tube several times before sampling.
8. Using this protocol, approximately 30 recipient mice can be injected after factoring in cell lysis and the loss associated with centrifugation steps. For recipient numbers >30 scale up the reaction as necessary but adhere to the same proportions of 10 μg HEL/10×10^9 SRBC per 1 ml conjugation reaction. By way of example if 60 recipients are to be injected, conjugate 20 μg of mutant HEL to 20×10^9 SRBC in a 2 ml reaction.
9. A spleen from a 6–10-week-old SW_{HEL} mouse will typically have ~10–20 % HEL-binding B cells. Younger mice will have a greater frequency of HEL-binding B cells.
10. When quantitating % HEL-binding B cells by flow cytometry, be sure to account for doublets and triplets by analyzing Forward Light Scatter.
11. Adoptive transfers can also be performed using SW_{HEL} mice on a C57BL/6 background and adoptively transferring these cells into C57BL/6.SJL.Ptprc^a recipients.
12. Round-bottom plates facilitate resuspension of cell pellets in buffer; however, V-bottom plates retain the original cell numbers with less associated cell loss. If cell numbers are limiting, V-bottom plates are recommended.
13. High-speed centrifugation of diluted antibody mixes can remove fluorophore aggregates, which when analyzed can appear as ultrabright false positives. Dilute antibodies at the required dilution factor as determined by antibody titration, then centrifuge in a benchtop microfuge at maximum speed (up to 25,000 × *g*) for 15 min at 4 °C.
14. Cells may be fixed and permeabilized as an alternative way of assessing plasma cells according to their high intracellular expression of immunoglobulins.
15. It is highly recommended to separate HEL^{WT} from lower affinity HEL mutant stains on a 96-well plate to avoid cross-well contamination during staining/centrifugation.
16. The HyHEL9 mAb is a mouse IgG1 antibody. Therefore, it is critical to block with 5 % normal mouse serum after staining with anti-mouse IgG1 to prevent false positive detection of high affinity IgG1 switched cells.
17. Mouse serum (used as 5 % v/v in PBA for blocking) can be purchased or obtained from the blood collection of a C57BL/6 mouse.
18. The canonical Y53D high affinity mutation can be read out by staining with 50 ng/ml of HEL^{3X}.

19. Prepare a master mix of Digest Buffer without Proteinase K, then add the Proteinase K just prior to single cell sorting if sorting large numbers of plates to ensure maximal enzyme digest efficiency.
20. Perform PCR Digest steps immediately following the sort.
21. Ideally perform the Primary PCR the day of or day following the sort. At this stage, the Primary PCR plates may be stored for several days at −80 °C.
22. For analyzing sequencing results, we recommend setting up an Excel spreadsheet to consolidate the results for easy visualization and graphing.
23. 384-well plates can be used instead of 96-well plates to accommodate a greater number of serum samples or when sample volume is limiting. Volumes for steps can be adjusted by using 30 µl instead of 60 µl, 40 µl instead of 100 µl, and 20 µl instead of 50 µl.
24. For affinity-matured antibody, serum samples should be titrated out to obtain a titration curve, with the relevant serum controls included such that endpoint titers may be calculated (7).
25. After thawing slides (if previously frozen), use a hydrophobic pen to outline the tissue section, such that smaller volumes of diluted antibody can be contained on top of the tissue section. As a rough guide, when making up diluted antibody mixes allow approximately 100 µl/tissue section.

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Chapter 10

Triggering Positive Selection of Germinal Center B Cells by Antigen Targeting to DEC-205

Giulia Pasqual, Alessandro Angelini, and Gabriel D. Victora

Abstract

Germinal centers (GCs) are the site of maturation of antibody affinity and are thus of key importance to humoral immunity. The study of B-cell dynamics and selection within the GC has been hampered by the limited number of techniques available to manipulate GC output in vivo. Here, we describe an approach to trigger positive selection of B cells in vivo by targeting antigen specifically to a subpopulation of GC B cells via the surface lectin DEC-205 and forcing their interaction with T follicular helper cells. Targeted GC B cells can then be followed over time as they progress through the stages of positive selection.

Key words Germinal center, T follicular helper cells, Antigen presentation, B-cell selection, DEC-205

1 Introduction

Germinal centers (GCs) are foci of rapidly dividing B cells that form within the B-cell follicles of secondary lymphoid organs upon antigenic exposure. Within these structures, B cells mutate their immunoglobulin genes and are subsequently selected for increased affinity for antigen, leading to affinity maturation [1]. Our knowledge of how this process is regulated is limited because GCs cannot currently be mimicked in tissue culture and thus can only be studied in vivo.

Loss-of-function manipulations of GCs in vivo using conditional genetic deletion or blocking antibodies have shed some light into the mechanism of B-cell selection in the GC. However, precise gain-of-function experiments in which selection can be triggered at will and studied over time have traditionally been lacking. Here, we present a protocol to trigger positive selection of GC B cells in vivo, and follow their fate over time, by targeting T-cell antigen directly to these cells using antibodies to the surface lectin DEC-205 (*Ly75*).

Marion Espéli and Michelle Linterman (eds.), *T Follicular Helper Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1291, DOI 10.1007/978-1-4939-2498-1_10,

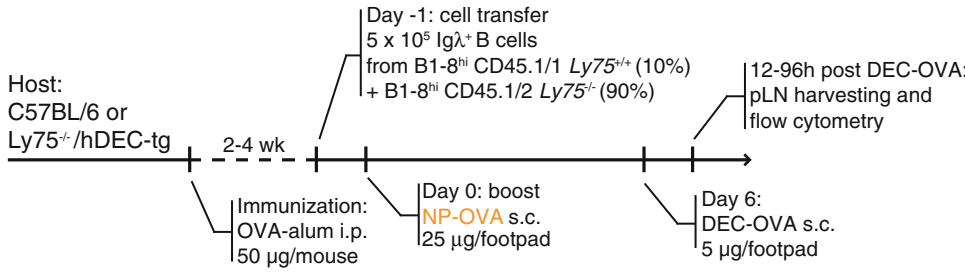

Fig. 1 Schematic representation of the experimental protocol

This protocol was initially developed to generate experimental evidence for the hypothesis that, in order to be positively selected in the GC, a B cell must obtain access to signals delivered by a limited number of GC-resident T follicular helper (Tfh) cells [2]. To accomplish this, we generate GCs in which all B cells have homogeneously high BCR affinity (by transferring B cells that express the 4-hydroxy,3-nitrophenylacetyl (NP)-specific B1-8^{hi} heavy chain), and are therefore minimally competitive, but which contain a minority of cells that express the surface lectin DEC-205 (*Ly75*$^{+/+}$) within a majority knockout (*Ly75*$^{-/-}$) population. Absence of strong competition is essential to ensure that the proportion of cells of each genotype within each GC remains similar to the proportion in the input (transferred cells). We then provide a minority of B cells with a high density of peptide-MHC-II by delivering antigen to this population through DEC-205 (thus bypassing the B-cell receptor), using a recombinant antibody to DEC-205 fused to the ovalbumin (or a control) protein (αDEC-OVA). This will skew Tfh cells toward preferentially interacting with *Ly75*$^{+/+}$ B cells, triggering their selection for exit as plasma cells or for cyclic reentry into the GC dark zone in a synchronized manner. Importantly, because the B cells are NP-specific, delivery of αDEC-OVA will not trigger BCR signaling in the B1-8^{hi} cells, thus isolating signals from T-cell help from any potential signals delivered via the BCR. This selective process can then be followed over time, allowing for a detailed dissection of the changes that B cells undergo upon positive selection. A schematic overview of the protocol is provided in Fig. 1.

2 Materials

2.1 Production of αDEC-205 Fusion Proteins

1. FreeStyle 293F cells (Invitrogen) and FreeStyle 293 Expression Medium (Gibco; *see* **Note 1**).
2. 125- and 500-ml sterile baffled vented cap Erlenmeyer flask.
3. Cell culture incubator (37 °C, 5 % CO_2) equipped with an orbital shaker (130 rpm).

4. High-quality, endotoxin-free DNA preparations (*see* **Note 2**) of mammalian expression plasmids encoding for:
   - αDEC-205 light chain (αDECLC).
   - αDEC-205 heavy chain (bearing mutations that reduce binding to Fc receptors [3]) fused to full-length ovalbumin (αDECHC-OVA).
   - αDEC-205 heavy chain fused to sequence comprising amino acids 57–346 from the circumsporozoite protein (CSP) of *P. yoelii* (αDECHC-CS) or other control protein.
   - Plasmids are available from our lab upon request.
5. Polyethylenimine (PEI) 1 mg/ml solution.
6. OptiPRO SFM medium.
7. Sterile disposable 500-ml centrifuge bottles and tubes (*see* **Note 3**).
8. Sterile disposable bottle top 0.45-μm filters with PES membrane.
9. Cell culture-grade 10× phosphate-buffered saline (PBS), pH 7.4.
10. 1.5 × 15 cm chromatography columns (*see* **Note 4**).
11. Protein G Sepharose 4 Fast Flow.
12. Sterile, Water for Injection Quality, cell culture-grade H_2O.
13. Washing buffer: cell culture-grade 1× PBS, pH 7.4.
14. Elution buffer: 100 mM glycine HCl, pH 2.7.
15. Neutralization buffer: cell culture-grade 1 M Tris–HCl, pH 9.0.
16. 50-kDa centrifugal filter units.
17. PD-10 desalting columns.
18. Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) Assay for endotoxin detection.

2.2 Mouse Strains

All strains should be on the same genetic background; we have not tested this protocol in any background other than C57BL6.

1. Donor mice: B1-8hi$^{+/-}$/*Ly75*$^{-/-}$/CD45.1/2 (BHD) and B1-8hi$^{+/-}$/CD45.1/1 (BHS) [4, 5].
2. Recipient mice: wild-type or *Ly75*$^{-/-}$/human DEC-205 transgenic [6] (optional; *see* **Note 5**).

2.3 Antigens and Injections

1. Ovalbumin (OVA).
2. Alum.
3. 3-nitro,4-hydroxyphenylacetyl (NP)-OVA (Biosearch Technologies; loading between 10 and 20 NP haptens per OVA molecule).
4. 1-cc insulin syringe and ½-cc insulin syringe 28-G ½ in. needle and 28-G ½ in. needle.

2.4 Cell Transfer

1. FACS buffer (FB): PBS, 2 % v/v fetal calf serum, 1 mM EDTA.
2. 70-μm nylon mesh cell strainer.
3. 60-mm tissue culture dish.
4. 3-ml syringes.
5. 15-ml conical tubes.
6. Red cell lysis buffer of choice.
7. B-cell negative magnetic selection reagents: In this protocol, we use reagents from Miltenyi, but other kits may be substituted and used according to the manufacturer's instructions. CD43 MACS beads, MACS LS magnetic columns, MidiMACS magnetic separators and stand.
8. Hemocytometer.
9. Antibodies: anti-mouse $Ig\lambda_{1-3}$ FITC, clone R26-46; anti-mouse IgMa PE, clone DS-1.
10. 1-cc insulin syringe 28-G ½ in. needle.

2.5 Flow Cytometry

1. 1.5-ml microfuge tubes.
2. Pestle for 1.5-ml microfuge tubes.
3. Anti-mouse CD16/32, clone 2.4G2, purified (Fc block).
4. Antibodies for flow cytometry as required.

3 Methods

3.1 Expression of Anti-DEC-205 Antibody Fusion Proteins

The procedure below describes the expression and purification of anti-DEC-205 fusion proteins in 1 l scale, usually allowing for the purification of ~5–7 mg of protein. The protocol can be scaled up or down according to experimental requirements.

3.1.1 FreeStyle 293F Cell Culture

1. Thaw 1 vial (1×10^7 cells) of FreeStyle 293F cells and immediately transfer to a 125-ml flask previously filled with 30 ml of FreeStyle 293 Expression Medium, without antibiotics. Incubate cells at 37 °C, 5 % CO_2, and 130 rpm orbital shaking.
2. Count and estimate concentration of cell suspension daily. Once the culture has reached $1–3 \times 10^6$ cells/ml (typically 2–4 days), subculture the cells by seeding shaker flasks at 3×10^5 cells/ml in pre-warmed FreeStyle 293 Expression Medium. Subculture cells a minimum of two additional times to allow them to recover from thawing before using them for transfections.
3. For transfection, grow cells to a density of 1×10^6 cells/ml (logarithmic growth phase) in four 1-l flasks (250 ml media volume each).

3.1.2 Cell Transfection and Protein Production

1. Dissolve 50 mg PEI in 50 ml cell culture-grade H_2O, heat at 55 °C for at least 30 min until dissolved and clear, and adjust pH to 7 with HCl. Filter sterilize, aliquot, and store at −80 °C.
2. Allow PEI, OptiPRO SFM medium, and DNA to reach room temperature.
3. Add 1 mg (500 μg LC + 500 μg HC) of DNA to 19 ml OptiPRO. Mix gently and incubate at room temperature for 15 min.
4. In a different tube, add 2 ml PEI to 18 ml of OptiPRO. Mix gently and incubate at room temperature for 15 min.
5. Add PEI solution to DNA solution and mix gently. Incubate 15 min at room temperature.
6. Slowly add 10 ml DNA:PEI solution to each flask drop by drop while mixing.
7. Return flask to the incubator and grow for 6–8 days before harvesting.
8. At the end of the 6–8-day production phase, harvest cells by centrifugation at 15,000 × *g* for 30 min at 4 °C. Collect the medium and remove any additional cell debris by filtration through 0.45 μm PES membranes.
9. Buffer the clarified medium by adding 1/10 vol. of 10× PBS, pH 7.4. This will ensure the optimal pH for IgG binding to the resin.
10. Proceed immediately with purification step. Alternatively, clarified medium can be stored at 4 °C overnight.

3.2 Purification of αDEC-205 Fusion Proteins by Affinity Chromatography

1. Transfer 5 ml of Protein G Sepharose 4 Fast Flow in the chromatography column.
2. Wash column with 10 CV (50 ml) of cell culture-grade H_2O.
3. Equilibrate column with 10 CV (50 ml) of cell culture-grade PBS, pH 7.4.
4. Load clarified medium. Collect flow-through and store at 4 °C until ready for **step 8**.
5. Wash column with 10 CV (50 ml) of cell culture-grade PBS, pH 7.4.
6. Elute protein by applying 4 CV (20 ml) of 100 mM glycine HCl, pH 2.7. During elution, progressively add to the eluate 2 ml of cell culture-grade 1 M Tris–HCl, pH 9.0, to promptly neutralize the acidic pH.
7. Store eluate at 4 °C until **step 9**.
8. Re-equilibrate column with 10 CV (50 ml) PBS, pH 7.4. To maximize the amount of protein recovered, we recommend to load the flow-through at least once. Repeat **steps 5** and **6** to wash the column and elute the protein.

9. Pool eluates together (total volume should be ~50 ml).
10. To dilute glycine and Tris buffers before concentration, add 50 ml of cell culture-grade PBS, pH 7.4. Load on four 50-kDa centrifugal filter units and concentrate protein by centrifugation (4,000 × *g*, 10 °C) to a total final volume of 10 ml (*see* **Note 6**).
11. Dilute sample again by adding 90 ml of cell culture-grade PBS, pH 7.4. Load on four 50-kDa centrifugal filter units and concentrate protein by centrifugation (4,000 × *g*, 10 °C) to a total final volume of 2.5 ml.
12. To remove all traces of glycine and Tris, clean sample using a PD-10 desalting column according to manufacturer's instructions. Briefly, equilibrate PD-10 desalting column with 25 ml of cell culture-grade PBS, pH 7.4. Add 2.5 ml of sample from **step 11** to the column. Discard the flow-through. To eluate the sample, apply 3.5 ml of cell culture-grade PBS and collect the eluate.
13. Using a spectrophotometer, measure A_{280} and estimate protein concentration. For anti-DEC-205-OVA, we estimated a mass extinction coefficient ε of 1.396 $l \times g\text{-}1 \times cm\text{-}1$.
14. Verify the purity and homogeneity of the purified anti-DEC-205 antibody fusion protein by SDS-PAGE using an 8 % polyacrylamide gel (nonreducing conditions).
15. Measure endotoxins using Endpoint Chromogenic LAL Assay according to manufacturer's manual. If endotoxin level is >15 EU/ml for a 1 mg/ml of anti-DEC-205-fusion protein solution (endotoxin dose limit is 5 EU/kg), proceed with endotoxin removal (*see* **Note 7**).
16. Aliquot and store anti-DEC-205 antibody fusion proteins at −80 °C for long-term storage. Aliquot in use can be kept at 4 °C for approximately 1 month.

3.3 Immunization (2–4 Weeks Prior to Boost)

1. Reconstitute 100 mg lyophilized OVA in 10 ml PBS for 10 mg/ml solution; filter using 0.22-μm syringe filter. Store at −20 °C in 1 ml aliquots for up to 2 years.
2. Resuspend Imject Alum thoroughly by shaking or vortexing.
3. In a 2-ml microcentrifuge tube, combine 60 μl OVA stock solution (equivalent to 600 μg) with 740 μl sterile PBS per ten mice to be immunized.
4. Place tube open on a slowly rotating vortexer and add 400 μl of alum suspension dropwise into the whirling protein solution.
5. Close tube, vortex on fast setting for an additional 10 s, and then incubate for 30 min at 4 °C on a tube rotator. The OVA-alum preparation is then ready to inject.
6. Inject each recipient mouse intraperitoneally with 50 μl OVA-alum into each side of the abdomen (total 100 μl = 50 μg/mouse).

3.4 Cell Transfer (1 Day Prior to Boost)

A typical transfer will require about 5×10^6 BHD B cells and 1×10^6 BHS B cells. A typical yield for $CD43^{low}$ B cells is ~3×10^7 cells per mouse. Therefore, transferring cells into ten host mice will require two BHD donors and one BHS donor (*see* **Note 8**). Carry out all procedures on ice unless otherwise specified.

1. Place one 60-mm tissue culture dish per spleen on an ice bath. In the dish, place a 70-μm cell strainer and 5 ml FB.
2. Euthanize donor mice using the method approved by your institution's animal ethics committee.
3. Harvest spleens and macerate into the dish with FB by forcing through the cell strainer using the back end of the plunger of a 3-ml syringe. Using the syringe, transfer splenocyte suspension into a 15-ml conical tube through the cell strainer, wash strainer and dish with another 5 ml FB, add wash to the same 15-ml conical tube, and pellet cells at $300 \times g$.
4. Lyse erythrocytes by resuspending pellet in 1 ml red cell lysis buffer solution and incubating for 3 min at RT; stop lysis by adding 10 ml FB and filtering through a cell strainer into a fresh 15-ml conical tube; pellet cells at $300 \times g$.
5. Isolate $CD43^{low}$ (resting) B cells by negative selection using Miltenyi CD43 microbeads and LS columns, according to the manufacturer's instructions (FB can be used instead of PBE in all steps). Combine cells from different mice of the same genotype at this point.
6. Count cells in the flow-through using a hemocytometer. Rest cells on ice.
7. Take a small aliquot (~1 % of the total) and stain for $Ig\lambda^+$ B cells by dispensing the aliquot into a FACS tube and add 100 μl of FB containing 0.5 μg/ml anti-$Ig\lambda_{1-3}$ FITC and 1 μg/ml anti-IgMa PE. Incubate at RT for 10 min, wash cells with 1 ml FB, and run on a flow cytometer. $Ig\lambda^+IgMa^+$ cells should constitute 10–20 % of all cells in the suspension.
8. Pellet cells left on ice at 300 g, and resuspend in sterile PBS (not FB) at a concentration of 5×10^5 $Ig\lambda^+$ B cells per 100 μl.
9. Combine cells in PBS at the correct ratio of BHD to BHS $Ig\lambda^+$ B cells (e.g., for a 90 %:10 % ratio, combine 90 μl BHD cells with 10 μl BHS cells per mouse; *see* **Note 9**).
10. Inject 100 μl of the final cell suspension intravenously into each recipient mouse by tail-vein or retro-orbital injection using a 1-cc insulin syringe, as approved by your institution's animal ethics committee.

3.5 NP-OVA Boost (Day 0)

1. Reconstitute lyophilized NP-OVA in sterile PBS at a concentration of 1 mg/ml; filter using 0.22-μm syringe filter. Store at −20 °C in 1 ml aliquots for up to 2 years.
2. Using a ½-cc 28-G insulin syringe, boost mice in one or both hind footpads (as approved by your institution's animal ethics committee) with 25 μl (=25 μg) NP-OVA solution per footpad.

3.6 αDEC-OVA Treatment (Day 6)

1. Immediately prior to treatment, dilute DEC-OVA and DEC-CS solutions (prepared as in Subheading 3.2) to a final concentration of 200 ng/μl in sterile PBS.
2. Using a ½-cc 28-G insulin syringe, inject mice into one or both hind footpads (as approved by your institution's animal ethics committee) with 25 μl (=5 μg) of αDEC-OVA (or αDEC-CS as a control).

3.7 Popliteal Lymph Node Harvesting and Flow Cytometry (Day 7–9)

1. Euthanize donor mice using the method approved by your institution's animal ethics committee.
2. Isolate the popliteal lymph node(s) draining the immunized footpad(s). Using a plastic pestle, gently macerate the LN against the wall of a 1.5-ml microfuge tube containing 100 μl of FB + 1 μg/ml of Fc block (*see* **Note 10**). Incubate on ice for 5 min.
3. Without washing, add 100 μl of 2× antibody solution for flow cytometry in FB, for a final volume of 200 μl. Cells can be stained for any marker of choice, but staining must at a minimum include antibodies to a B-cell marker (CD19 or B220), GC markers (e.g., CD38 and FAS), and markers for allelic discrimination of donor cells (CD45.1 and CD45.2). Our standard staining solution is detailed below for your reference:

Purpose	Marker	Conjugation	Clone	μg/ml	Manufacturer
Light vs. dark zone	CD83	Biotin	Michel-19	5.0 (1×)	BioLegend
Incubate 30 min at 4°C, add 1 ml FACS buffer, filter through a 70-μm strainer into a new tube, pellet cells, and resuspend in 200 μl of stain 2 (below)					
B cells	CD19	BV421	6D5	1.0	BioLegend
Germinal centers	CD38	APC	90	0.5	BioLegend
	FAS	PE-Cy7	Jo2	0.25	BD
Donors vs. host	CD45.1	FITC	A20	0.5	BD
	CD45.2	Alexa700	104	0.5	BioLegend
Light vs. dark zone	Streptavidin	APC	–	0.5	BD
	CXCR4	PE	2B11	0.5	BD
Incubate 30 min at 4°C, then pellet cells, and resuspend in 200 μl FB; cells are ready for flow cytometry analysis					

4 Notes

1. Although expression of anti-DEC-205 antibody fusion proteins can be performed in various cell lines, we recommend the use of FreeStyle 293F cells for two main reasons: (a) They are adapted to high-density suspension growth, making the handling of large-scale cultures less time- and space-consuming than adherent cells, and (b) this line can be maintained in serum-free conditions, allowing easier purification.
2. Plasmid DNA for transfection must be clean, sterile, and free from phenol, sodium chloride, and endotoxins. Contaminants may kill the cells, and salt will interfere with PEI complexing thus decreasing the transfection efficiency. To limit the amount of endotoxin in plasmid preparations, we recommend using EndoFree Plasmid Maxi Kit (Qiagen #12362) or NucleoBond® Xtra Maxi EF (Macherey-Nagel #740424.10). To ensure sterility, filter your DNA preparation through a 0.22-μm filter before use or EtOH precipitate it.
3. Wherever possible, use sterile, specifically endotoxin-free disposable plastics. If you need to use glassware or other materials that are not guaranteed endotoxin-free, you should soak them in 0.5 M NaOH and then rinse thoroughly with endotoxin-free water.
4. Use cell culture-grade, endotoxin-tested buffers for protein purification. When this is not possible, prepare buffers with cell culture-grade, endotoxin-tested H_2O and filter through a 0.22-μm filter before use.
5. Choice of recipient mice: In order for DEC-OVA to be targeted exclusively to a small subset of GC B cells, the ideal host would also be deficient for *Ly75*, so as to avoid additional targeting of OVA to host DCs and to the small proportion (~10 %) of residual host B cells that contribute to GCs when B1-8^{hi} cells are transferred. However, when establishing this protocol, we noticed that *Ly75*$^{+/+}$ GC B cells were lost from *Ly75*$^{-/-}$ mice beginning at 5–6 days postimmunization, which we attributed to immune-mediated rejection of cells bearing the DEC-205 molecule (which is nonself to a *Ly75*$^{-/-}$ recipient). Therefore, we chose to use WT C57BL6 mice as recipients, relying on the differences between *Ly75*$^{+/+}$ and *Ly75*$^{-/-}$ cells as evidence of the cell-intrinsic nature of our findings. We subsequently found that rejection of DEC-205 by *Ly75*$^{-/-}$ recipient mice could be circumvented by crossing these mice to a strain expressing the human DEC-205 protein under a CD11c promoter [6] (even though the anti-mouse DEC-205 antibody used for antigen targeting does not bind to the human homologue, presence of human DEC-205 is sufficient to tolerize

Ly75$^{-/-}$ mice to mouse DEC-205). Results obtained using these mice as hosts were comparable to those obtained using WT hosts, the only difference being that the residual endogenous GC cell population fails to expand in *Ly75*$^{-/-}$ hDEC-tg recipients. We therefore recommend the use of WT recipients for most experiments, unless there is reason to suspect indirect effects (which would be detected in both *Ly75*$^{+/+}$ and *Ly75*$^{-/-}$ B1-8^{hi} populations).

6. Dilution of glycine buffer before concentration is required to increase protein solubility and thus avoid protein precipitation during the concentration process.
7. Endotoxin can be removed from antibody preparations using Triton X-114 as described [7].
8. Donors and recipients must be sex matched as follows: Male recipients can receive cells from male or female donors or any mix thereof; female recipients can only receive cells from female donors (due to rejection of Y-chromosome determinants by female mice).
9. The ratio of BHS to BHD cells in the GC is inversely proportional to the strength of the signal delivered to these cells by Tfh after treatment with DEC-OVA because of competition for Tfh among the BHS themselves. Thus, a starting ratio of 5/95 (BHS/BHD) will lead to greater expansion of the BHS population than a ratio of 25/75.
10. Macerating pLNs with a pestle in a microfuge tube prevents the substantial loss of cells that occurs when using a cell strainer and a 15-ml conical tube.

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Chapter 11

Detection of Mouse Natural Killer T Follicular Helper (NKT_{FH}) Cells by Flow Cytometry

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Abstract

Natural killer T follicular helper (NKT_{FH}) cell, a recently identified B-cell helper innate cell population, can be easily missed due to its low frequency and the fact that it only forms upon immunization or infection with glycolipid-containing antigens or microbes. Here, we describe our in-house optimized protocol to detect these mouse NKT_{FH} cells by multiparameter flow cytometer.

Key words Natural killer T follicular helper (NKT_{FH}) cells, CD1d-α-GalCer tetramer, CXCR5, PD-1, Flow cytometry (FACS), Antibody staining

1 Introduction

Natural killer T follicular helper (NKT_{FH}) cells were identified 2 years ago. Because their antigen receptors, as with most NK T cells, are relatively invariant, they are considered an innate T-cell subset. NKT_{FH} cells elicit a unique type of antibody response, recently designated as type 2 thymus-dependent antibody responses (TD-2) [1]. Upon activation by glycolipid α-galactosylceramide-presenting dendritic cells, some NKT cells rapidly upregulate CXCR5 and PD-1, two surface markers that normally identify follicular T cells. The highest expression of CXCR5 and PD-1 is found on germinal center T_{FH} cells, which as we now know include NKT_{FH}. In order to become $CXCR5^{hi}$ $PD\text{-}1^{hi}$ cells in germinal centers, NK T cells effectively co-opt the T_{FH}-differentiation pathway by expressing Bcl6 and forming cognate interaction with CD1d-expressing B cells. This B-cell-NKT_{FH} cognate interaction leads to rapid production of extrafollicular IgM and IgG antibody responses [2, 3] as well as rapid formation of germinal centers. Nonetheless, unlike conventional TD responses to protein antigens, the TD-2 response that is elicited by NKT_{FH} does not lead to the generation of long-lived memory B cells or plasma cells [4–6].

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NKT_{FH} is a rare population and is thus challenging to detect. On top of that, commercially available anti-mouse CXCR5 monoclonal antibodies provide only weak fluorescent signals. Whereas we and others [4, 6] readily detected NKT_{FH} cells after glycolipid immunizations, Bai et al. [7] were unable to detect this rare population in their study in the context of encapsulated pathogen *Streptococcus pneumoniae*. Hence, the use of a standard and optimized protocol is expected to facilitate the accurate identification of mouse NKT_{FH} cells.

Here, we share our in-house optimized protocol for the detection of mouse NKT_{FH} cells. This protocol has been used successfully now by independent investigators.

2 Materials

2.1 Reagents

1. *Roswell Park Memorial Institute (RPMI) medium 1640:* commercially available.
2. Cell strainers.
3. *Red blood cell lysis buffer, pH 7.4:* 155 mM ammonium chloride (NH_4Cl), 0,1 M ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-Na_2), 10 mM potassium bicarbonate ($KHCO_3$).
4. *10× PBS, pH 6.8:* 0.038 M sodium phosphate monobasic (NaH_2PO_4), 0.162 M sodium phosphate dibasic (Na_2HPO_4), 1.5 M sodium chloride (NaCl).
5. *Sodium azide (*$NaN3$*): 10 % (w/v)* in dH_2O (*see* **Note 1**).
6. Heat-inactivated fetal calf serum (HI-FCS).
7. *FACS buffer:* 1× PBS, 0.002 % (vol/vol) NaN_3, 0.5 % (v/v) HI-FCS (*see* **Note 2**).
8. *Serum-supplemented antibody diluents:* FACS buffer, 2 % normal mouse serum (NMS), additional 2 % HI-FCS.
9. 96-Well round-bottomed plates.

2.2 Antibodies

1. Purified rat anti-mouse CXCR5 (clone 2G8).
2. Biotin-conjugated AffiniPure goat anti-rat (H + L).
3. Streptavidin-conjugated phycoerythrin-Cyanine 7 (SA-PECy7).
4. Peridinin chlorophyll-conjugated anti-mouse B220, clone RA3-6B2 (B220-PerCP).
5. Allophycocyanin Cyanine 7-conjugated anti-mouse B220, clone RA3-6B2 (B220-APC-Cy7).
6. Allophycocyanin Cyanine 7-conjugated anti-mouse CD4, clone GK1.5 (CD4-APC Cy7).

7. Alexa Fluor 700-conjugated anti-mouse CD4, clone RM4-5 (CD4-Alexa Fluor 700).
8. Phycoerythrin-conjugated anti-mouse PD-1, clone J43 (PD1-PE) (*see* **Note 4**).
9. Fluorescein isothiocyanate-conjugated anti-CD3, clone 145-2C11 (CD3-FITC) (*see* **Note 5**).
10. Alexa Fluor 700-conjugated anti-mouse CD45.1, clone A20 (CD45.1-Alexa Fluor 700).
11. Pacific Blue-conjugated anti-mouse CD45.1, clone A20 (CD45.1-Pacific Blue).
12. Pacific Blue-conjugated anti-mouse CD45.2, clone 104 (CD45.2-Pacific Blue).
13. Peridinin chlorophyll protein-cyanine 5.5 anti-mouse CD45.2, clone 104 (CD45.2-PerCPCy5.5).
14. Allophycocyanin-conjugated CD1d-α-GalCer tetramer.

2.3 Instrument

A flow cytometer with at least two lasers is required for detecting endogenous NKT_{FH}, and three lasers are required for tracking adoptively transferred NKT_{FH}.

3 Methods

3.1 Cell Suspension Preparation

1. Prepare spleen and lymph node single cell suspension by sieving mashed spleen or lymph node with RPMI over a cell strainer to remove clumps and debris and follow by gently pipetting the cells up and down (*see* **Note 3**).
2. Centrifuge cells at 350 × *g* for 4 min. Carefully discard supernatant by decanting or aspiration.
3. Treat spleen cells with 500 μl (per spleen) of red blood cell lysis buffer, and incubate at room temperature for 30 s.
4. Quench with 5 mL of cold RPMI and centrifuge at 350 × *g* for 4 min. Carefully discard supernatant by decanting or aspiration.
5. Resuspend pelleted white blood cells in 5 mL of RPMI media.
6. Count red blood cell-depleted spleen cells and lymph node cell suspensions.

3.2 CXCR5 Staining

All steps are incubated in the dark (i.e., covered by aluminum foil).

1. Plate two million cells per well in a 96-well round-bottomed plate, plating at least four replicate wells per stain (a total of eight million cells identically stained will be pooled prior to flow-cytometric acquisition to obtain enough events).

2. Centrifuge cells in the 96-well round-bottomed plate at 350 × *g* for 4 min. Discard supernatant.
3. Resuspend cells in 60 µL/well of 1:60 rat anti-mouse CXCR5 purified in freshly prepared serum-supplemented antibody diluents. Incubate in a refrigerator (4–8 °C) for 60 min (*see* **Note 4**).
4. Top up with 140 µL/well of FACS buffer and centrifuge at 350 × *g* for 4 min. Discard supernatant. Wash a second time by resuspending the cell pellet with 200 µL/well of FACS buffer, and centrifuge at 350 × *g* for 4 min. Discard supernatant.

3.3 Secondary Antibody Staining

1. Resuspend cell pellet in 60 µl of biotin-conjugated AffiniPure goat anti-rat (H + L) (1:600) in serum-supplemented antibody diluents for 30 min in the fridge.
2. Top up with 140 µL/well of FACS buffer, and centrifuge at 350 × *g* for 4 min. Discard supernatant. Wash a second time by resuspending the cell pellet with 200 µL/well of FACS buffer, and centrifuge at 350 × *g* for 4 min. Discard supernatant.

3.4 Tertiary Antibody Staining

1. Stain with tertiary antibody at 1:800 for SA-conjugated PE-Cyanine 7 in serum-supplemented antibody diluents for 30 min in the fridge. At this time, include all other surface stains. Here, B220 is included as a dump channel. Example of staining combination:

   The staining combinations below are useful in adoptive transfer or bone marrow chimera experiments involving congenically labeled CD45.1 and CD45.2 mice. For staining combinations used to detect only endogenous iNKT cells, CD45.1 and CD45.2 antibodies can be excluded.

   Example 1:

   SA-PE-Cy7

   B220-PerCP

   CD4-APC Cy7

   PD-1-PE (*see* **Note 5**)

   CD3-FITC (*see* **Note 6**)

   CD45.1-Alexa Fluor 700

   CD45.2-Pacific Blue

   Example 2:

   SA-PE-Cy7

   B220-APC Cy7

   CD4-Alexa Fluor 700

   PD-1-PE

   CD3-FITC

   CD45.1-Pacific Blue

   CD45.2-PerCPCy5.5

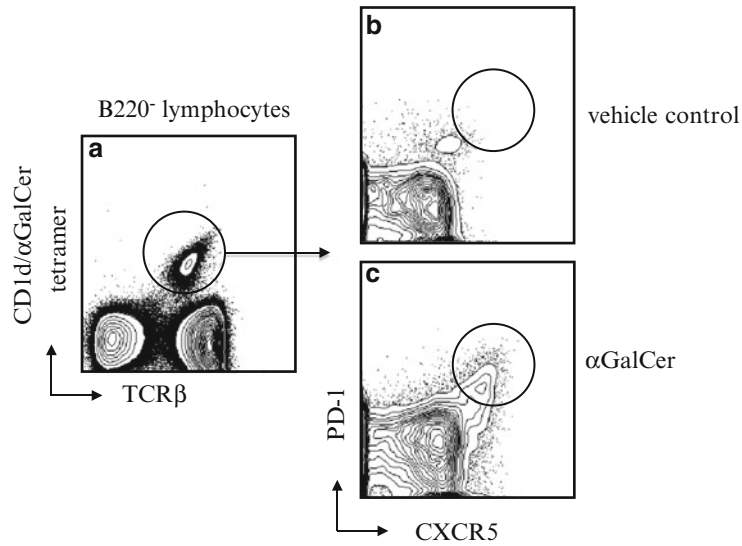

Fig. 1 Representative flow-cytometric contour plots showing gating of splenic CD1d/αGalCer tetramer$^+$ CD3$^+$/TCRβ^{int} NKT cells (**a**) from C57BL/6 mice 6 days postinjection with either vehicle control (tyloxapol buffer) (**b**) or αGalCer (**c**). The gate in (**c**) indicates the presence of NKT_{FH} (PD-1^{hi} CXCR5^{hi})

2. Wash two times with *FACS* buffer by resuspending the cell pellet with 200 μL/well of FACS buffer and centrifuge at 350 × *g* for 4 min. Discard supernatant.

3.5 CD1d-Tetramer Staining

1. Stain with CD1d-α-GalCer tetramer-APC in FACS buffer for 30 min in the fridge in the dark (*see* **Note 7**).
2. Wash two times with FACS buffer by resuspending the cell pellet with 200 μL/well of FACS buffer and centrifuge at 350 × *g* for 4 min. Discard supernatant.

3.6 Flow Cytometry Acquisition and Analysis

1. Pool the four wells containing cells stained with the same antibody combinations (4 wells × 2 million cells), and resuspend in 300 μl of *FACS buffer* (*see* **Note 8**).
2. Optimal gating strategy for NKT_{FH} population is shown in Fig. 1 [4].

4 Notes

1. Sodium azide is toxic and reacts with copper plumbing. Do not introduce into plumbing system. Store in the dark.
2. As multiple individual samples, each containing eight million cells, are run on the flow cytometer, only 0.5 % HI-FCS is added to the FACS buffer so as to minimize the chances of blocking the probe of the flow cytometer machine.

3. To reliably detect the modest shift of CXCR5, a control group of mice immunized only with vehicle must be included in every experiment.
4. Plates with cells incubating with anti-CXCR5 antibodies can be left for hours in the refrigerator if needed for convenience.
5. In our hands, mouse anti-PD-1-PE (clone J43) from eBioscience provides the brightest signal.
6. Anti-mouse CD3 and anti-mouse TCR-β are equally useful to identify invariant NKT cells which are $CD3^+$ and TCR-β intermediate; only one of the two is needed when used in combination with α-Gal-tetramer staining.
7. Spin down CD1d-α-GalCer tetramer in microcentrifuge at 14,000 × *g* for 3 min, and prepare the working dilution immediately prior to staining; this helps precipitate unbound particles and thus reduces nonspecific staining. Avoid staining CD1d-α-GalCer tetramer together with streptavidin, biotin, or purified antibody as this will increase the background.
8. Perform a quick clean with 10 % v/v freshly prepared bleach (or 10 % v/v detergent), and then water approximately once every eight runs to minimize probe blockages of the flow cytometer.

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Chapter 12

Identification of Foxp3$^+$ T Follicular Regulatory (Tfr) Cells by Flow Cytometry

Ana Raquel Maceiras and Luis Graca

Abstract

Flow cytometry is a technology that allows multiparametric analysis of individual cells. As a consequence, it is among the most commonly used tools for the study of immune cells. It is useful both for the study of ex vivo cell populations isolated from experimental animals or human tissue and for characterizing the phenotype of cultured cells. The phenotypic analysis is based on antibodies associated to different fluorophores that specifically bind to key molecules. Genetically modified mouse strains that express a reporter gene under the control of a promoter of interest offer an important alternative for the staining of intracellular molecules without the need to permeabilize the cell membrane. In this chapter, we describe how Foxp3$^+$ follicular regulatory T (Tfr) cells, a population of regulatory T (Treg) cells related to T follicular helper (Tfh) cells and involved in the regulation of germinal centers (GC), can be identified by flow cytometry.

Key words Flow cytometry, Antibodies, Foxp3, Bcl6, CXCR5, PD-1, CD4 T cells, T follicular helper cells (Tfh), Germinal center

1 Introduction

T follicular regulatory (Tfr) cells constitute a population of follicular CD4 T cells that have been recently described [1–3]. Tfr cells are located within the lymphoid tissue, and a circulating counterpart has also been described [4]. Tfr cells are involved in the regulation of the germinal center (GC) response, for example, it has been shown that Tfr cells can limit the production of antibodies specific for an immunizing self-antigen, such as chromatin antigens [5].

Tfr cells share phenotypic characteristics of regulatory T cells (Treg) and of T follicular helper (Tfh) cells: Tfr cells express characteristic Tfh markers such as CXCR5, PD-1, and Bcl-6, but also Treg-associated molecules including Foxp3, CD25, GITR, and CD103. As a consequence, Tfr cells are commonly described as CD4$^+$CXCR5$^+$PD-1$^+$CD25$^+$Foxp3$^+$ cells, although CD25$^-$ Tfr cells have also been identified [1–3].

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Flow cytometry is one of the most commonly used techniques in immunology. Its multiparametric analysis allows an in-depth study of the physical characteristics of cells and also the phenotypic analysis of different proteins expressed by single cells. Flow cytometry utilizes monoclonal antibodies that are specific to different proteins expressed by cells and are often directly conjugated to fluorophores, allowing the direct identification of molecules of interest in cells being analyzed. In addition, the level of expression of a given molecule can be inferred from the intensity of the staining. As a consequence, the staining pattern obtained from a combination of markers allows the identification of cell populations on sample, as well as the quantification of the relative abundance of these populations.

It is also possible to use flow cytometry to isolate cell populations, or single cells, based on their phenotype for subsequent studies. It should be noted, however, that functional studies that require the isolation of live cells are incompatible with the use of monoclonal antibodies to stain intracellular molecules. This limitation is a consequence of the need to permeabilize cells in order to gain access to their intracellular components, with permeabilization leading to cell death. This is a critical issue for the study of Tfr cells as one key marker for the identification of this cell population is Foxp3, a nuclear transcription factor. Mouse strains expressing reporter genes under the control of the *Foxp3* promoter offer an alternative strategy to identify and sort viable Foxp3$^+$ cells (namely, Tfr) based on flow cytometry.

In summary, flow cytometry is a useful tool for the identification of Tfr cells, based on their phenotypic characteristics, allowing subsequent functional studies of Tfr role in the regulation of antibody-mediated immune responses.

2 Materials

All solutions should be prepared using distilled water and analytical grade reagents. Solutions may be prepared and stored prior to utilization.

When flow sorting Tfr cells for functional assays, it is necessary to use strategies that overcome the need for intracellular staining (as it requires fixation and permeabilization of cells). As an alternative Foxp3 reporter mice should be used (*see* Table 1).

In some experiments, in particular following in vitro assays, it may be important to use a cell viability test, using a fluorescent reactive dye that allows discrimination of live and dead cells (live/dead staining). The reagents and extra steps for the live/dead staining are marked as optional.

Table 1
List of Foxp3 reporter mice available

Mouse strain	MGI designation	Reporter molecule	Reference
Foxp3-GFP	$Foxp3^{tm2Ayr}$	GFP	Fontenot et al. [6]
Foxp3-EGFP	$Foxp3^{tm2Tch}$	EGFP	Haribhai et al. [7]
Foxp3-IRES-GFP	$Foxp3^{tm1Kuch}$	GFP	Bettelli et al. [8]
Foxp3-IRES-mRFP	$Foxp3^{tm1Flv}$	mRFP	Wan et al. [9]
Foxp3-hCD2	$Foxp3^{tm1(CD2/CD52)Shori}$	Human CD2	Komatsu et al. [10]

2.1 Surface Staining Reagents

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 M KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2. Store at room temperature.
2. Fetal calf serum. Store at –20 °C.
3. Staining buffer: PBS with 2 % FCS. Store at 4 °C.
4. ACK lysing buffer: 0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2. Store at room temperature.
5. Surface antibodies: Anti-mouse CXCR5 biotin (clone 2G8), anti-mouse CD4 APC-eFluor® 780 (clone RM4-5), anti-mouse CD279 (PD-1) PE (clone J43), and anti-mouse CD25 PE Cyanine7 (clone PC61.5) (*see* **Note 1**). Store at 4 °C.
6. Streptavidin: Streptavidin PerCP-Cyanine5.5 (*see* **Note 1**). Store at 4 °C.

2.2 Foxp3 Reporter Mice

1. Several Foxp3 reporter mouse strains have been generated and may be available for use (Table 1), such as Foxp3-GFP [6], Foxp3-EGFP [7], Foxp3-IRES-GFP [8], Foxp3-IRES-mRFP [9], and Foxp3-hCD2 [10]. It was claimed that reporter mice expressing a Foxp3-GFP fusion molecule have impaired Treg induction and function when compared with alternative Foxp3 reporter mouse strains, such as Foxp3-IRES-GFP [11, 12].

2.3 Viability Staining (Optional)

1. Fixable viability staining (*see* **Note 1**).

2.4 Intracellular Staining

1. Intracellular fixation/permeabilization buffer (IFP buffer): Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience). Store at 4 °C.
2. Permeabilization buffer: 10× permeabilization buffer (eBioscience). Store at 4 °C.
3. Fc region blocking (clone 24G2 or 93).
4. Intracellular antibody: Anti-mouse/rat Foxp3 APC (clone FKJ-16 s) (*see* **Note 1**). Store at 4 °C.

3 Methods

3.1 Surface Staining

1. Single cell suspensions should be obtained prior to cell staining procedures (except for cell samples from in vitro cultures). This may be accomplished by mechanical dissociation such as mesh sieving or Dounce homogenization.
2. When using splenocytes, red blood cell lysis should be performed before starting the following steps.
3. Make sure the splenocytes are in a tube of at least 15 ml capacity. Centrifuge the cells at 300×*g* for the appropriate time (depending on the volume) for the cells to form a pellet. Discard the supernatant.
4. Resuspend in 1 ml per spleen of ACK lysing buffer and incubate for 5 min at 4 °C.
5. Add directly 9 ml of staining buffer and mix well. Centrifuge the cells for 5 min at 300×*g*. Discard the supernatant. When performing red blood cell lysis, wash on step number 7 may be skipped.
6. Count the cells. Do not forget that flow cytometry will only give relative proportions of the different populations, and it is likely that the staining procedure will lead to some cell loss. The starting number of cells is, therefore, essential to calculate the cell number of each subsequently identified population. Cells can be counted manually using a hemocytometer. Alternatively, counting beads can be added to the mixture allowing cell number quantification by flow cytometry.
7. Wash cells in staining buffer. Resuspend cells in an appropriate volume to obtain approximately 2×10^7 cells/ml.
8. Transfer 100 μl of each sample to a well of a U-bottom 96-well plate. Centrifuge the plate for 3 min at 300×*g*. Discard the supernatant (*see* **Note 2**).
9. Prepare the necessary amount of CXCR5 mix by diluting CXCR5 antibody 50× in staining buffer, in order to obtain at least 20 μl for each sample (*see* **Notes 3–5**).
10. Resuspend each cell sample in 20 μl of CXCR5 mix, and incubate at 37 °C for 10 min followed by 20 min at room temperature (*see* **Note 6**).
11. All the following steps should be performed protected from light and at 4 °C (or on ice). Keep the centrifuge at 4 °C.
12. (optional) Wash cells with PBS: Add 200 μl of PBS to each well and mix; centrifuge the plate for 3 min at 300×*g*. Discard the supernatant.
13. (optional) Reconstitute the fluorescent reactive dye by adding 50 μl of DMSO to each vial of the kit—dye stock solution (*see* **Note 7**).

14. (optional) Prepare the appropriate volume of the viability staining mix by diluting the previous dye stock solution 100× in PBS.
15. (optional) Resuspend each cell sample in 20 μl of the previous viability staining mix, and incubate for 30 min.
16. Wash cells with PBS (if the viability staining was performed) or staining buffer.
17. Prepare the appropriate volume of the second mix containing the remaining surface antibodies and the streptavidin. The mix must contain all antibodies and the streptavidin diluted 100× in staining buffer (*see* **Notes 3–5**).
18. Resuspend each cell sample in 20 μl of the previous mix and incubate for 30 min.
19. Wash cells with staining buffer.

3.2 Foxp3 Reporter Mice

1. If a Foxp3 fluorescent reporter mouse is used, the staining is complete. In this case, resuspend in 200 μl of staining buffer and transfer to an appropriate plate/tube for acquisition on a flow cytometer. To avoid blockages on flow cytometers, cell samples may be filtered at this step using a 70 μm pore filter.
2. If the reporter protein is not fluorescent, an antibody specific for the reporter protein conjugated to a fluorophore should be used together with the remaining antibodies during the surface staining **steps 17** and **18**. For example, in the case of Foxp3-hCD2, an antihuman CD2 conjugated to a fluorophore (e.g., antihuman CD2 APC, eBioscience) should be added to the mix prepared at step 17 of the surface staining part of the protocol.
3. Samples are ready to be acquired in a flow cytometer.

3.3 Intracellular Staining

1. Prepare the appropriate volume of IFP buffer by mixing 1 volume of the concentrate solution with 3 volumes of the diluent solution.
2. Resuspend each sample in 100 μl IFP buffer. Incubate for 30 min (*see* **Note 8**).
3. Centrifuge the plate for 3 min at 300 × *g*. Discard the supernatant.
4. Prepare the permeabilization buffer by diluting the 10× solution to 1× in distilled water.
5. Wash cells with permeabilization buffer.
6. Prepare the Fc blocking solution by diluting the Fc block 200× in permeabilization buffer.
7. Resuspend each sample in 20 μl Fc blocking solution. Incubate for 15 min.

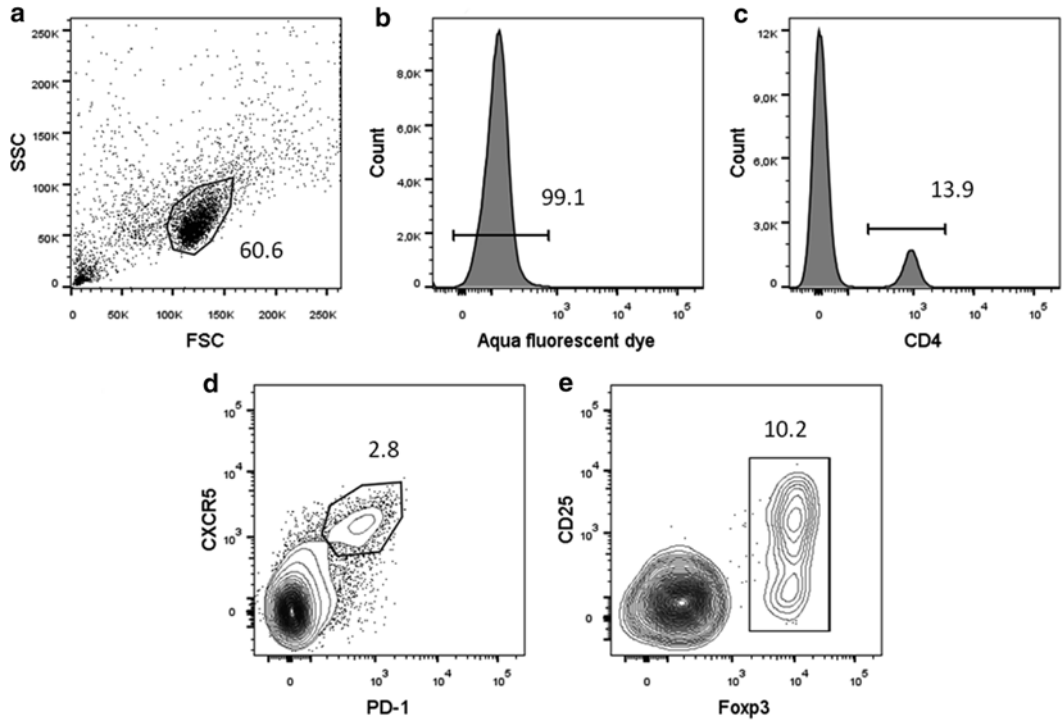

Fig. 1 *Tfr cells sequential gating strategy*. (**a**) Selection of cell based on the forward scatter (FSC) and side scatter (SSC) profiles. (**b**) Live cells are negative for the fluorescent reactive dye, so the negative population is selected. (**c**–**e**) Sequential selection of known Tfr markers: (**c**) $CD4^+$ cells, (**d**) $PD\text{-}1^+CXCR5^+$ cells, and (**e**) $CD25^+Foxp3^+$ cells

8. Prepare the Foxp3 mix by diluting the Foxp3 APC antibody 20× in permeabilization buffer (*see* **Notes 3**–**5** and **9**).
9. Add directly 5 μl of Foxp3 mix to each well containing the Fc blocking solution. Incubate for another 30 min.
10. Wash cells with permeabilization buffer.
11. Resuspend in 200 μl of staining buffer and transfer to an appropriate plate/tube for acquisition on a flow cytometer. To avoid clogs on flow cytometers, cell samples may be filtered at this step using a 70 μm pore filter.
12. Samples are ready to be acquired on a flow cytometer.

3.4 Gating Strategy

See Fig. 1.

4 Notes

1. The list of antibodies provided in this protocol is merely an example as other antibodies and conjugations to other fluorophores may be used as pleased. The same applies to the streptavidin used and the fixable cell viability dye, as streptavidin

conjugated to other fluorophores and different fluorescent reactive dyes may be used.

2. We found it easier to perform the following steps using 96-well plates, which also allows the use of smaller amounts of reagents. However, it may be performed in larger tubes, upscaling the volumes used accordingly.
3. An excess of all mixes should always be prepared, as pipetting errors may lead to insufficient volume for the last samples.
4. The antibody dilution stated may be used for the listed antibodies. However, the experimenter should test the optimal antibody working dilution.
5. To help define the negative populations, extra antibody mixes should be prepared so that each antibody is substituted by the corresponding isotype control.
6. In order to obtain a good staining for chemokine receptors, cells should be incubated for at least 10 min at 37 °C.
7. After reconstitution in DMSO, the dye stock solution may be stored at −20 °C and used later. If lower amounts are predicted to be used, store the solution in adequate aliquots in order to avoid repeated thawing and freezing cycles.
8. This incubation should preferentially be performed on ice.
9. Although the Foxp3 APC antibody is only diluted 20× in the Foxp3 mix, the final usage dilution will be 100× since the antibody is further diluted when added directly to the samples already in Fc blocking solution.

Acknowledgments

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Chapter 13

In Vitro Assay to Sensitively Measure TFR Suppressive Capacity and TFH Stimulation of B Cell Responses

Peter T. Sage and Arlene H. Sharpe

Abstract

T follicular helper (TFH) cells stimulate, whereas T follicular regulatory (TFR) cells inhibit, B cell responses. Despite the potent immunoregulatory roles for TFR cells in controlling the magnitude of antibody production, the precise mechanisms by which TFR cells exert their suppressive effects are not yet clear. The lack of specific assays to assess TFR cell function separately from differentiation has hindered progress in elucidating TFR cell function. This is due, in part, to difficulty in separating TFR cells from phenotypically similar, but functionally different, TFH cells. Here we describe an in vitro approach for sensitively and quantitatively assessing the capacity of TFR cells to suppress TFH-mediated B cell antibody production utilizing both ELISA and flow cytometry to measure B cell responses. Beyond assessing TFR function, this assay system can also be used to sensitively measure TFH stimulatory capacity as well as B cell function.

Key words T follicular regulatory cells, TFR, T follicular helper cell, TFH, Treg, B cell, Antibody, Suppression

1 Introduction

CD4+ T cells have long been appreciated to be essential in helping B cells to produce high-affinity antibodies. T follicular helper (TFH) cells are a subset of CD4+ T cells that have specialized functions in stimulating B cell responses [1–3]. Recently, an effector population of regulatory T cells (Tregs) called T follicular regulatory (TFR) cells has been identified. TFR cells have specialized functions in suppressing B cell responses [4–6]. TFR cells phenotypically resemble TFH cells in expression of CD4, CXCR5, ICOS, and PD1. The similarity in cell surface markers has resulted in TFR cells being hidden in traditional TFH gating strategies, which has hindered the development of assays to separately study and characterize TFH and TFR cell function. However, the differential expression of FoxP3 and GITR by TFR cells has enabled sorting strategies to highly purify TFH and TFR cells for functional studies [6, 7].

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Although TFH cells were identified over 20 years ago, specific assays for measuring TFH function are lacking. Many studies of TFH cells in murine models utilize knockout or otherwise manipulated mice and quantify TFH cells in lymphoid organs and correlate alterations with B cell phenotypes. This approach has yielded a number of exciting discoveries. However, methods for studying TFH effector function separately from differentiation have been challenging to develop. Assays to assess stimulatory capacity of TFH cells separately from differentiation are needed to advance our understanding of TFH cell biology. In vitro B cell stimulation assays have been used in human systems [8–10] and in a limited number of cases for murine studies [7, 11].

Similarly, the lack of specific assays for measuring TFR cell suppressive capacity in the effector phase represents a bottleneck for TFR cell research. Despite initial studies demonstrating that TFR cells potently suppress B cell responses, many questions still remain about how TFR cells regulate B cell responses. Additionally, relatively little is known about how TFR cells themselves are regulated. Only through the development of assays assessing TFR function can mechanisms of TFR cell maintenance and suppression be determined. We have published both in vitro and in vivo methods for assessing TFH and TFR cell function [7, 12]. Here we describe how to assay TFR suppression by assessing the capacity of TFR cells to suppress TFH-mediated B cell antibody production. We also explain how these assays can be employed to assess TFH cell stimulatory function and B cell activation.

2 Materials

2.1 NP-OVA Emulsion

1. 4-Hydroxy-3-nitrophenylacetyl-conjugated ovalbumin (NP-OVA) with an NP:OVA ratio of 15–30, diluted to 10 mg/ml in PBS (*see* **Note 1**).
2. Complete Freund's adjuvant *Mycobacterium tuberculosis* H37RA.
3. 3-way stopcock.
4. PBS.

2.2 Mice

1. 10–20 FoxP3-IRES-GFP reporter mice [13] (*see* **Note 2**). Non-reporter mice can also be used (*see* **Note 3**).
2. 1 cc syringe with a 27-gauge needle.

2.3 Materials for Cell Purification

1. Anti-CD4 magnetic beads and columns. Magnetic separation kits can be procured from numerous suppliers; here we will describe the protocol from Miltenyi. Kits from other suppliers will need to be used according to the manufacturer's instructions.

2. Anti-CD19 magnetic beads and columns. Magnetic separation kits can be procured from numerous suppliers; here we will describe the protocol from Miltenyi. Kits from other suppliers will need to be used according to the manufacturer's instructions.
3. Flow cytometry (FACs) buffer: PBS with 1 % FBS and 1 mM EDTA.
4. Conjugated antibodies (clone given in parentheses): anti-CD16/32 (Fc block), anti-CD4 PerCPCy5.5 (RM4-5), anti-CD19 APC/Cy7 (6D5), anti-ICOS PE (15 F9), anti-CXCR5 (2G8), streptavidin BV421. Optionally: anti-GITR FITC (DTA-1).
5. Aria (BD Biosciences) or Astrios (Beckman Coulter) cell sorter and Accuri cytometer (BD Biosciences) or other cell counter (*see* **Note 4**).

2.4 Assay Plating Reagents

1. 96-well round-bottom culture plates.
2. Anti-CD3 (2C11) and anti-IgM F(ab′)$_2$ (*see* **Note 5**).
3. R10 media: RPMI supplemented with L-glutamine, 10 % heat-inactivated fetal calf serum, 1× Pen-Strep, 1 mM HEPES, 50 μM beta-mercaptoethanol.

2.5 Antibody ELISA

1. Maxisorp plates.
2. Goat anti-mouse Ig coating antibody.
3. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (*see* **Note 6**).
4. Mouse IgG standard.
5. Phosphatase substrate, plus diethanolamine (DEA) buffer.
6. Plate reader.

2.6 Flow Cytometry

1. Conjugated antibodies (in addition to above): anti-IA-IE BV421 (M5/114.15.2), GL7 FITC, IgG1 PE (A85-1).
2. FoxP3 Fix/Perm and Perm/Wash buffer kit (eBioscience).

3 Methods

3.1 Preparation of Emulsion

1. Add 250 μl of 10 mg/ml NP-OVA to 2.25 ml PBS. Take up material into a 5 ml syringe and join syringe to one end of the 3-way stopcock, and expunge air.
2. Thoroughly mix H37RA CFA and take up 2.5 ml into a 5 ml syringe. Add to the other end of the 3-way stopcock.
3. Emulsify by sequentially pushing each syringe to mix contents into the neighboring syringe. Mix thoroughly, but cool to

4 °C if syringes become warm (excessive heat from friction will break down the antigen). Continue mixing until emulsion becomes somewhat difficult to move (~20 min of mixing). Check emulsion by putting a small amount in PBS and make sure it does not partially dissolve (it should have the consistency of toothpaste).

3.2 Immunization of Mice

1. Mix emulsion and load into a 1 cc syringe with a 27-gauge needle just before injecting into mice.
2. Inject 100 μl of emulsion subcutaneously on each flank of the mouse ~5 mm medial to the inguinal lymph node. Make sure the emulsion is *not* injected intraperitoneally. Injection should form a visible bolus.

3.3 T_{FH}, T_{FR}, and B Cell Sorting

1. Harvest inguinal lymph nodes from mice immunized 7 days previously (*see* **Note 7**).
2. Generate a single cell suspension by smashing through a 70 μm cell strainer that has been wet previously with R10 media, and then wash with R10 media.
3. Perform CD4-positive bead enrichment according to Miltenyi protocol and keep flowthrough from column. Purity should be ~90–95 %.
4. On flowthrough, perform CD19-positive bead enrichment according to Miltenyi protocol. Purity should be ~95 %.
5. For flow cytometry, incubate CD4-enriched cells with Fc Block and fluorochrome-conjugated anti-CD4, anti-CD19, anti-ICOS (each diluted 1:200 in FACs buffer), and anti-CXCR5-biotin (diluted 1:50 in FACs buffer) for 30 min on ice.
6. Wash twice by adding excess FACs buffer and centrifuging cells at 350×*g* for 5 min at 4 °C and removing the supernatant.
7. Incubate with streptavidin-BV421 1:500 for 15 min on ice. Wash 2× as described above in **step 6**.
8. Incubate CD19-enriched cells with Fc Block and fluorochrome-conjugated anti-CD4 and anti-CD19 (each diluted 1:200 in FACs buffer) for 30 min on ice. Wash 2×.
9. Sort T_{FH} (as $CD4^+ICOS^+CXCR5^+FoxP3^-CD19^-$) and T_{FR} (as $CD4^+ICOS^+CXCR5^+FoxP3^+CD19^-$) cells on cell sorter using maximal purity settings as per Fig. 1.
10. Sort B cells on cell sorter using maximal purity settings as $CD19^+$ and $CD4^-$.
11. Check purity and count cells with Accuri cytometer, counting only cells contained in the lymphocyte gate. Purity should be ~99 % (*see* **Note 8**).

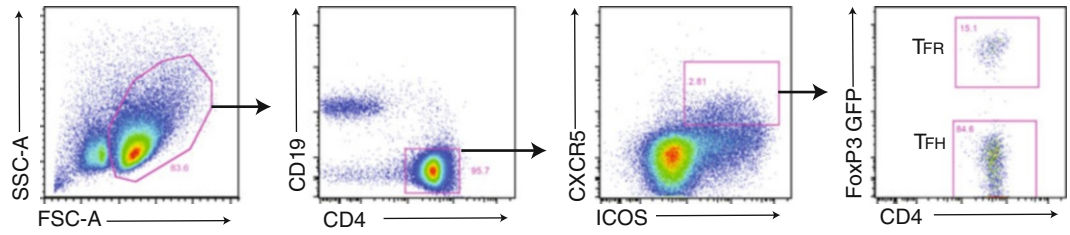

Fig. 1 Sorting strategy for isolation of TFH and TFR cells from draining lymph nodes. Mice were immunized with NP-OVA, and 7 days later draining lymph nodes were harvested and stained according to protocol. Sorting gates from an AriaII are shown. Plots are pregated on singlets

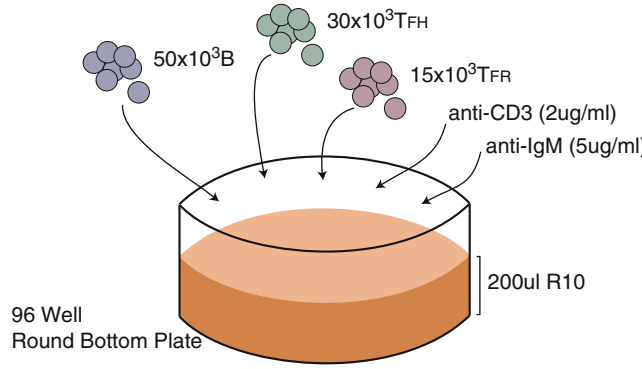

Fig. 2 Cell culture plating schematic. TFH, TFR, and B cells were sorted according to protocol and added together into cultures along with anti-CD3 and anti-IgM

3.4 Cell Plating

1. To a U-bottom 96-well culture dish, add 50×10^3 sorted B cells, 30×10^3 sorted TFH cells, and/or 15×10^3 sorted TFR cells in R10 media (*see* **Note 9**). Experimental groups should include B cells alone, B + TFH and B + TFH + TFR cells. By comparing B + TFH to B + TFH + TFR cell groups, TFR cell function can be measured. By comparing B + TFH to B cells alone, TFH cell function can be measured.
2. Add 2 μg/ml anti-CD3 and 5 μg/ml anti-IgM to wells and increase volume to 200 μl per well with R10 media (*see* **Note 10**) (Fig. 2).
3. Place in 37 °C 5 % CO_2 incubator for 6 days (*see* **Note 11**).

3.5 Flow Cytometry to Assess B Cell Function

1. After 6 days of culture, centrifuge at $370 \times g$ for 5 min and collect cells.
2. Resuspend cells in FACs buffer along with Fc Block (diluted 1:200 in FACs buffer).
3. Add anti-CD19 APC/Cy7, anti-IA-BV421, and anti-GL7-FITC (diluted 1:200 in FACs buffer) for 20 min on ice (*see* **Note 12**).

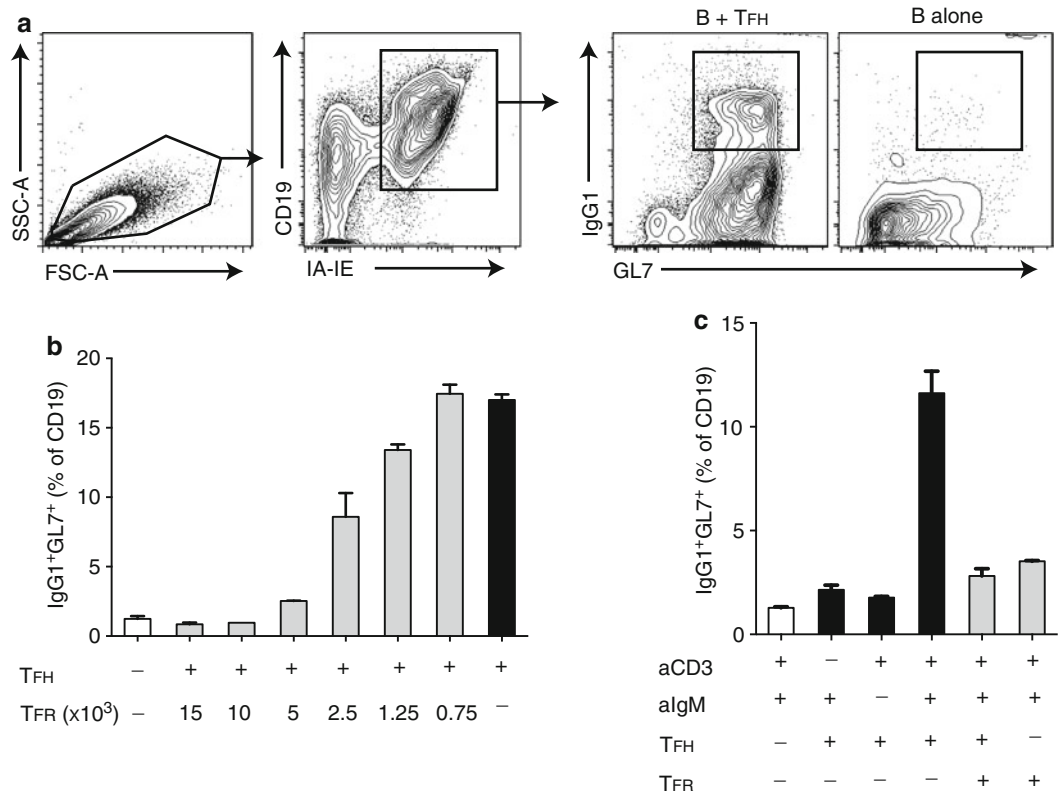

Fig. 3 Typical flow cytometric results from suppression assays. Suppression assay was performed as in protocol. (**a**) Flow cytometry gating strategy to identify IgG1 + GL7+ B cells (IgG1 and GL7 expressing germinal center-like B cells that have class switched) from cultures. (**b**) Suppression of IgG1 + GL7+ B cells by varying numbers of TFR cells. (**c**) Additional controls for suppression assay omitting anti-CD3, anti-IgM, or TFH cells

4. Wash 2× with FACs buffer.
5. Add Fix/Perm from FoxP3 intracellular kit; incubate 20 min on ice.
6. Wash 2× with Perm/Wash (diluted according to manufacturer's instructions) from FoxP3 intracellular kit.
7. Add IgG1 PE (diluted 1:200 in Perm/Wash); incubate for 20 min on ice.
8. Wash 2× with FACs buffer.
9. Run on flow cytometer and analyze according to gating strategy shown in Fig. 3.

3.6 IgG ELISA to Assess B Cell Function

1. Coat MaxiSorp plates with anti-mouse Ig overnight at 4 °C.
2. Wash 6× with 0.05 % Triton-X100 in PBS.
3. Block with 1 % BSA in PBS for 1 h at 37 °C.
4. Collect supernatants from cultures of B, TFH, and TFR cells, making sure not to collect cells.

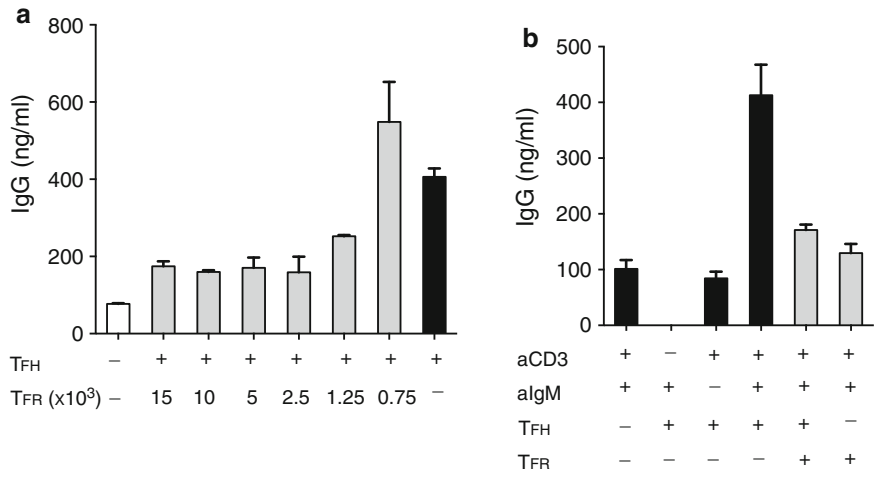

Fig. 4 Typical ELISA results from suppression assays. ELISA was performed on culture supernatants according to protocol. Supernatants were taken from the same cultures analyzed by flow cytometry in Fig. 3. (**a**) Suppression assay with varying numbers of TFR cells. (**b**) Suppression assay with additional indicated controls

5. Dilute cell culture supernatants 1:50 in 1 % BSA in PBS; add 100 μl per well.
6. Perform serial dilutions of IgG standard starting at 500 ng/ml on the same plate as samples. Incubate 45 min at RT.
7. Wash 6× times with 0.05 % Triton in PBS (*see* **Note 13**).
8. Add anti-mouse IgG AP in 1 % BSA to wells. Incubate 45 min at RT.
9. Wash 6× with 0.05 % Triton-X100 in PBS.
10. Add phosphatase substrate diluted in DEA buffer.
11. After color develops measure absorbance at 405 nm on plate reader (*see* **Note 14**) (Fig. 4).

4 Notes

1. NP-OVA in CFA is an efficient immunogen/adjuvant combination that will induce differentiation of both TFH and TFR cells in draining lymph nodes. This assay also can be used with TFH and TFR cells differentiated during infection or utilizing alternative adjuvant approaches. Some steps may need to be re-optimized in the context of other antigens/adjuvants.
2. The yield of TFH and TFR cells varies greatly depending on age of mice, variation in preparation of the emulsion, cell loss during preparation, and other factors. Typical yield may be around 30×10^3 TFH and 10×10^3 TFR per mouse. Adjusting the

number of mice may be necessary, depending on the number of experimental parameters to be tested.

3. Identification of TFR cells based on a FoxP3 reporter is optimal; however, in many settings a reporter strain is not available, particularly when assessing TFR and TFH cells from knockout strains. GITR can be used as a surrogate for a FoxP3 reporter, but care must be taken to ensure that sorting strategies result in high purity of TFR and TFH cells. This includes assessing FoxP3 expression after sorting using intracellular FoxP3 antibody staining. *See* [7, 12] for more details.
4. The Accuri cytometer is useful for accurately counting the cell concentration after sorting in order to precisely quantitate the number of cells for addition to culture. Ensuring proper ratios of cells is important in this assay. If a cell counter is not available, other ways of counting cells (e.g., hemocytometer) may be used.
5. Anti-CD3 and anti-IgM are used to activate T and B cells for determining the suppressive capacity of TFR cells or stimulatory capacity of TFH cells. Specific antigen (i.e., adding NP-OVA at 20ug/ml to the wells) can be used instead of anti-CD3 and anti-IgM in this assay, followed by the same downstream analysis.
6. Although this protocol assesses total IgG by ELISA, measuring individual IgG isotypes may also be informative.
7. Lymph nodes can be harvested at alternative time points depending on experimental question.
8. TFH and TFR cells sorted according to this protocol can also be used in adoptive transfer approaches to assess TFH and TFR function in vivo.
9. Cell numbers in the assay were optimized to allow long duration of B and TFH interaction, mimicking a germinal center-like reaction, but not deplete the cells of nutrients. In some cases, analyses of several cell ratios may be beneficial. For instance, adding 10×10^3 TFR cells and 30×10^3 TFH cells per well may result in a more sensitive measure of TFR function when comparing different groups of TFR cells (*see* also Figs. 3 and 4).
10. Both anti-CD3 and anti-IgM are necessary for optimal B cell class switching and antibody secretion (*see* also Figs. 3 and 4). Note that specific antigen may be used instead of anti-CD3 and anti-IgM.
11. Maximal B cell responses occur when cultures persist for 6 or more days without depleting the cells of nutrients in the well. It is important to take note of the color of the culture wells. If TFH + B cultures become very yellow before the end of the 6-day culture period, the cells may be harvested earlier for

flow cytometric analysis and supernatants analyzed for IgG by ELISA. Prolonging culture of TFH + B in nutrient-depleted cultures will result in inconclusive data. Lowering the anti-CD3 concentration or plating fewer TFH cells per well can help resolve this problem.

12. This protocol is for the most basic analysis of antibody production stimulated by TFH cells or suppressed by TFR cells. Other types of analyses of B cells such as B7-1, B7-2, and GL7 expression can be used as alternative measures of B cell activation. Additionally, TFH cells can be assessed for T cell activation by intracellular staining for Ki67 in conjunction with $CD4^+CD19^-FoxP3^-$ staining (TFH cells). In addition, culture supernatants can also be collected and cytokines assessed by ELISA or by cytometric bead array (BD Biosciences). Also, cultures may be stimulated with PMA/ionomycin and GolgiStop for intracellular cytokine staining of desired TFH cytokines such as IFNγ, IL-4, and IL-21.
13. The use of an automated plate washer is optimal; however, other methods of plate washing (e.g., using a multichannel pipette) are adequate.
14. In order to obtain reliable data, the culture supernatant absorbance should be within the linear range of the standards on the same plate. Typical developing time should be around 10–30 min.

Acknowledgments

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Part II

Human

Chapter 14

Flow Cytometric Detection and Isolation of Human Tonsil or Lymph Node T Follicular Helper Cells

Jan Misiak, Karin Tarte, and Patricia Amé-Thomas

Abstract

T-follicular helper (Tfh) cells have emerged as an independent $CD4^+$ helper T-cell lineage required for antigen-selected germinal center B-cell survival, class switch recombination, and differentiation into long-lived plasma cells. The quantification and function of Tfh subsets are currently extensively explored in humans with infectious diseases, cancer, or autoimmune disorders. Reliable methods to identify and isolate human Tfh cells in patients and healthy donors are necessary to perform these studies. Here, we propose a classical and robust flow cytometric method to detect and isolate Tfh cells from human secondary lymphoid organs based on the expression of CXCR5, PD-1, and CD25 in the $CD4^+$ T-cell population. An alternative protocol using anti-ICOS and anti-Bcl-6 antibodies and requiring fixation and permeabilization steps without a decrease of detection of membrane markers is also described.

Key words T-follicular helper cells, Flow cytometry, Cell sorting, CXCR5, PD-1, BCL-6

1 Introduction

T-follicular helper (Tfh) cells have emerged as an independent $CD4^+$ helper T-cell lineage, with distinct developmental and effector functions [1]. Like their murine counterpart, human Tfh cells are characterized by a strong expression of CXCR5 associated with a lack of CCR7 allowing their migration and retention into the CXCL13-rich light zone of germinal centers. In addition, they express high levels of programmed cell death-1 (PD-1) and inducible costimulator (ICOS) and could produce different cytokines such as IL-21, IL-4, IFN-γ, or IL-17, but also the chemokine CXCL13. These features are essentially associated with the expression of the transcription factor BCL-6, the master regulator of both murine and human Tfh cell differentiation [2, 3].

Currently, no single marker can provide an unequivocal identification of human Tfh cells in human secondary lymphoid organs. Two different phenotypic definitions have been used to characterize and isolate this helper T-cell subset, based on the strong expression

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of CXCR5 associated with the high expression of either ICOS or PD-1 [3, 4]. This last definition is now the most employed and is considered as the most relevant. More recently, a bona fide population of functional regulatory T cells presents in human germinal centers, and sharing classical Tfh features (like the expression of CXCR5, ICOS, PD-1, and BCL-6) has been revealed and was called follicular regulatory T cells (Tfr) [5, 6]. The phenotypic similarities of these two subsets motivate the addition of at least the membrane marker CD25 to flow cytometry panels, in order to properly define Tfh cells by excluding the $CD25^{high}$ population corresponding to Tfr [5, 7]. Finally, the recent development of efficient fixation and permeabilization solutions, allowing good entry of conjugated antibodies as well as new clones of antibodies targeting transcription factors that are usable for flow cytometry, enables convincing detection of BCL-6-expressing cells and definitively reinforces the definition of Tfh cells. Nevertheless, BCL-6 detection requires an intracellular stain that is incompatible with the recovery of viable cells and conduction of functional studies. Here, we describe classical and simple protocols developed for the detection and isolation of Tfh cells from human tonsil or lymph node samples.

2 Materials

2.1 Cell Preparation

1. Tissue medium (TM): RPMI 1640 Medium with GlutaMAX, 100 U/mL penicillin and 100 μg/mL streptomycin.
2. Lysis and viability solution (LV solution): mix 890 μL of diluted EasyLyse solution (Dako, 1/20 in distilled water) with 100 μL of trypan blue stain 0.4 %. Other lysis solutions exist and may be used according to the manufacturers' recommendations.
3. Petri dishes for tissue dissociation.
4. 5-mL syringe.
5. 23Gx1″ (0.6×25 mm) needle.
6. 100-μm nylon cell strainer.
7. 50-mL tube.
8. Density gradient medium such as Lymphoprep or Ficoll-Paque.
9. Fetal calf serum (FCS).
10. Freezing media: FCS, 20 % dimethyl sulfoxide (v/v).
11. Cryovials.
12. Thawing media: RPMI 1640, 20 % FCS (v/v).
13. 15-mL tubes.
14. Sterile scalpels.
15. DNase (1,000 U/mL).

2.2 Cell Staining

2.2.1 Basic Protocol

1. D-PBS 1× without calcium and magnesium.
2. AB serum: human serum, blood type AB obtained from the French Blood Institute.
3. 5-mL flow cytometry tubes.
4. DAPI nucleic acid stain.
5. Antibodies:

mAb/stain	Clone	Fluorochrome
Mouse IgG1 antihuman CD4	13B8.2	FITC
Mouse IgG1 antihuman CD25	M-A251	PE
Mouse IgG1 antihuman CD3	UCHT1	PE-CF594
Mouse IgG1 antihuman PD-1	J105	PE-Cy 7
Mouse IgG2b antihuman CXCR5	51505.111	APC

2.2.2 Variations of Basic Protocol

1. D-PBS 1× without calcium and magnesium.
2. AB serum: human serum blood type AB obtained from the French Blood Institute.
3. 5-mL flow cytometry tubes.
4. Antibodies:

mAb/stain	Clone	Fluorochrome
Mouse IgG1 antihuman BCL-6	K112-91	PE
Mouse IgG1 antihuman CD3	UCHT1	PE-CF594
Mouse IgG1 antihuman CD25	B1.49.9	PE-Cy 5.5
Mouse IgG1 antihuman PD-1	J105	PE-Cy 7
Mouse IgG2b antihuman CXCR5	51505.111	APC
Mouse IgG1 antihuman ICOS	ISA-3	Biotin
Mouse IgG1 antihuman CD4	RPA-T4	Brilliant Violet 421

5. LIVE/DEAD Fixable Yellow Dead Cell Stain.
6. Streptavidin APC-eFluor 780.
7. Transcription Factor Staining Buffer Set.

2.3 Data Collection and Analysis

1. Antibody capture beads: CompBeads anti-mouse Ig.
2. Flow cytometer: many flow cytometers are suitable for this procedure. We use a Gallios flow cytometer (Beckman Coulter) with three lasers (violet 405 nm, blue 488 nm, and red 633 nm).
3. Flow cytometry acquisition and analysis software, for example, Kaluza software version 1.3 (Beckman Coulter).

2.4 Cell Sorting

2.4.1 Enrichment of $CD4^+$ Cells

1. Enrichment buffer: D-PBS 1× without calcium and magnesium, 0.1 % human albumin (v/v), 0.6 % natrium citrate dihydrate (v/v), 2,500 U DNase, 6 mM $MgCl_2$.
2. Antibodies:

Mab	Clone
Mouse IgG1 antihuman CD19	J3.119
Mouse IgG1 antihuman CD16	3G8
Mouse IgG1 antihuman CD14	RMO52
Mouse IgG1 antihuman CD8	B9.11

3. Anti-mouse IgG MicroBeads (Miltenyi Biotec).
4. 40-μm nylon cell strainer.
5. Optional: MACSmix Tube Rotator (Miltenyi Biotec).

2.4.2 Cell Staining and Cell Sorting

1. D-PBS 1× without calcium and magnesium.
2. Fetal calf serum (FCS).
3. 70-μm nylon cell strainer.
4. Cell sorter, for example, BD FACSAria II cell sorter (BD Biosciences).

3 Methods

This is a basic protocol for the identification and isolation of Tfh cells by flow cytometry, using a simple cocktail of antibodies against CD3, CD4, CXCR5, PD-1, and CD25. Alternatively, ICOS could be used instead of PD-1.

3.1 Cell Preparation

All lymph node or tonsil samples are handled in a class 2 biosafety hood and under sterile conditions.

3.1.1 Dissociation of Lymph Node or Tonsil Samples

1. Collect specimens in cold TCM and store them on ice until processing.
2. Cut human lymph node or tonsil samples into pieces with a sterile scalpel in a Petri dish on ice.
3. Flush the cells outside the capsule with cold TM using a 5-mL syringe and a 23Gx1″ needle. Forty milliliters of TM is necessary for a pair of tonsils (*see* **Note 1**).
4. Filter the eluted cell suspension through a 100-μM cell strainer placed on a 50-mL tube.
5. Determine the cell concentration by diluting the cell suspension with LV solution and using a hemocytometer, like a Malassez counting chamber.

3.1.2 Optional: Isolation of Mononuclear Cells by Density Gradient

1. Dilute the cell suspension at a concentration of 25×10^6 cells/mL in TM.
2. Transfer 25 mL of Lymphoprep to a 50-mL tube. Ficoll-Paque could be alternatively used as a density gradient medium.
3. Gently layer 25 mL of the diluted cell suspension on the density gradient medium.
4. Centrifuge for 20 min at $800 \times g$ at room temperature with the brake disengaged.
5. Collect the mononuclear cells from the interface between the density gradient medium and the TM and transfer them into 50-mL tubes.
6. Wash cells twice with D-PBS by centrifugation at $800 \times g$ for 10 min at room temperature.
7. Resuspend cells in D-PBS and determine the cell concentration.

3.1.3 Optional: Freezing and Thawing of Total or Mononuclear Cells from Tonsil or Lymph Nodes

1. Centrifuge cells at $600 \times g$ for 5 min at 4 °C. Remove and discard the supernatant.
2. Resuspend the cell pellet in an appropriate volume of cold FCS at a concentration of $10–50 \times 10^6$ cells/mL.
3. Add an equivalent volume of the freezing medium dropwise to the cell suspension.
4. Transfer 1 mL of the cell suspension in freezing medium in each 2-mL cryovial.
5. Freeze the cryovials in −80 °C at least overnight.
6. Place the cryovials in liquid nitrogen for long-term storage (*see* **Note 2**).
7. Quickly thaw one cryovial in a 37 °C water bath, and transfer cells to a 15-mL tube containing 10 mL of prewarmed thawing media.
8. Centrifuge cells at $600 \times g$ for 5 min at room temperature. Remove and discard the supernatant.
9. Resuspend cells in 3 mL of the thawing medium.
10. Add 300 μL of DNase and incubate at 37 °C for 15 min.
11. Add 7 mL of thawing medium and centrifuge at $600 \times g$ 5 min at room temperature. Remove and discard the supernatant.
12. Resuspend cells in D-PBS and determine the cell concentration.

3.2 Cell Staining

3.2.1 Basic Protocol

The basic staining protocol is a six-color panel suitable for three-laser flow cytometers (violet 405 nm, blue 488 nm, and red 633 nm) enabling optimized discrimination of viable $DAPI^-$ tonsil or lymph node $CD4^+$ $CD3^+$ T-cell subsets (*see* **Note 3**) based on the expression of PD-1, CXCR5 (*see* **Note 4**), and CD25.

1. Centrifuge cells at $600 \times g$ for 5 min at 4 °C. Remove and discard the supernatant.

2. Resuspend cells at 1×10^7 cells/mL in 30 % human AB serum/PBS (v/v) (*see* **Note 5**).
3. Transfer 100 μL of cell suspension to 5-mL flow cytometry tubes.
4. Incubate 10 min at 4 °C.
5. Add the appropriate volume of the selected antibodies and thoroughly mix by vortexing (*see* **Note 6**).
6. Incubate at 4 °C in the dark for 25 min or at room temperature for 15 min.
7. Wash cells twice with 2 mL of D-PBS (centrifugation at $600 \times g$ for 5 min). Remove and discard the supernatant.
8. Resuspend the pellet in 300 μL of D-PBS.
9. Add 1 μL of DAPI at a concentration of 1 μg/mL to each tube and immediately acquire on the flow cytometer.

3.2.2 Variations of Basic Protocol

This extended protocol is an eight-color panel optimized for the analysis of intracellular molecules (*see* **Note 7**). The selected fluorochromes allow the detection of an additional marker using an FITC- or Alexa Fluor 488-conjugated antibody.

1. Wash cells twice with 2 mL of D-PBS by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
2. Resuspend cells at a concentration of 1×10^6 cells/mL.
3. Transfer 1 mL of the cell suspension to 5-mL flow cytometry tubes.
4. Add 1 μL of the reconstituted LIVE/DEAD Fixable Yellow Dead Cell Stain per tube and mix by vortexing.
5. Incubate at room temperature in the dark for 30 min.
6. Wash cells with 2 mL of D-PBS by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
7. Resuspend cells in 100 μL of 30 % human AB serum in PBS (v/v) (*see* **Note 5**).
8. Incubate 10 min at 4 °C.
9. Add the appropriate volume of cell-surface stain antibodies and thoroughly mix by vortexing (*see* **Note 6**).
10. Incubate at 4 °C in the dark for 25 min or at room temperature for 15 min.
11. Wash cells with 2 mL of PBS by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
12. (Optional) Add the appropriate volume of streptavidin if a biotinylated antibody was used, and thoroughly mix by vortexing.
13. (Optional) Incubate at room temperature for 15 min.

14. (Optional) Wash cells with 2 mL of D- PBS by centrifugation at 600 × *g* for 5 min. Remove and discard the supernatant, and resuspend the cell pellet by vortexing.
15. Add 1 mL of 1× BD Fix/Perm buffer and mix by vortexing.
16. Incubate at 4 °C in the dark for 45 min.
17. Wash cells twice with 2 mL of 1× BD Perm/Wash buffer by centrifugation at 800 × *g* for 5 min. Remove and discard the supernatant.
18. Add the appropriate volume of intracellular stain antibodies (e.g., antihuman BCL-6) and thoroughly mix by vortexing (*see* **Note 6**).
19. Incubate at 4 °C in the dark for 45 min.
20. Wash cells twice with 2 mL of 1× BD Perm/Wash buffer by centrifugation at 800 × *g* for 5 min. Remove and discard the supernatant.
21. Resuspend the cell pellet in 300 μL of D-PBS.
22. Acquire the samples on a flow cytometer.

3.3 Data Collection and Analysis

1. Acquire non-stained lymph node or tonsil cells to allow the photomultiplier voltage settings for all the different parameters: forward scatter (FSC), side scatter (SSC), and the selected fluorescence channels.
2. Prepare compensation controls using antibody capture beads incubated with each of the selected antibodies and cells with DAPI. Collect at least 10^4 events of each.
3. Acquire at least 10^5 events (excluding debris, dead cells, and doublets) of the sample of interest on flow cytometer. For tissue samples with very low $CD4^+$ T-cell frequency, and/or if you want to study subsets of Tfh cells, it may be necessary to collect more than 10^5 viable singlet events.
4. Data analysis is a crucial step in Tfh cell detection. Figure 1 highlights our gating strategy for a classical CD3/CD4/CD25/CXCR5/PD-1 staining of a tonsil sample. The first three gates allow exclusion of debris, doublets, and dead cells from the acquired events. After gating of $CD3^+$ $CD4^+$ T cells and the exclusion of $CD25^+$ cells, the classical CXCR5/PD-1 dot plot is drawn.
5. Figure 2 shows the classical CXCR5/PD-1 profile of human tonsil $CD4^+$ helper T cells after fixation and permeabilization steps. Similarly, the CXCR5/ICOS profile is not altered by the permeabilization procedure. Almost all Tfh cells defined as $CXCR5^{high}$ $PD\text{-}1^{high}$ cells express high level of ICOS. Finally, the intracellular expression of BCL-6 could be explored and is highly expressed in the vast majority of $CXCR5^{high}$ $PD\text{-}1^{high}$ cells.

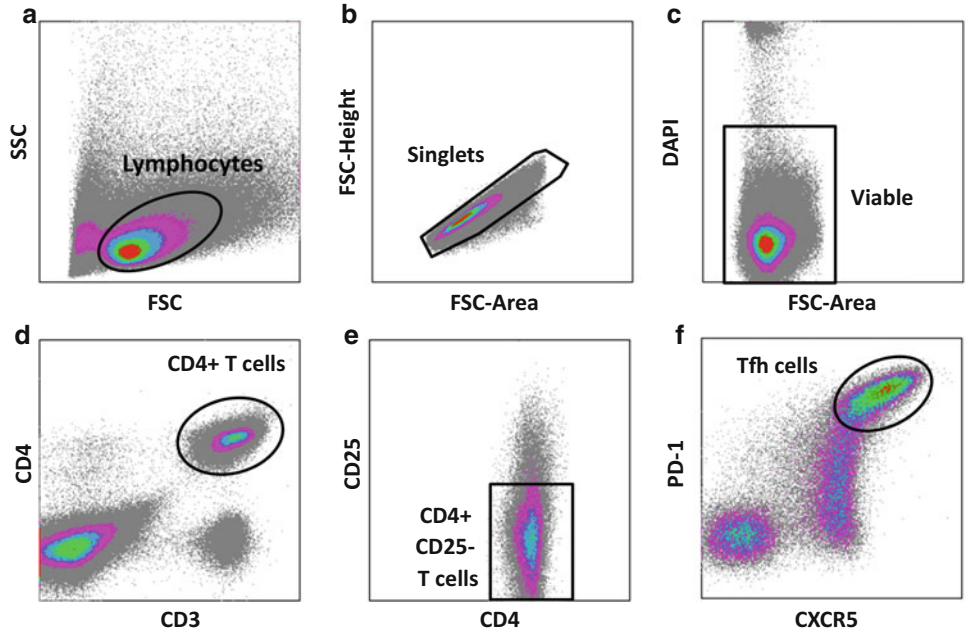

Fig. 1 Standard gating for Tfh cells in a human tonsil after staining for CD3, CD4, CD25, CXCR5, PD-1, and DAPI. (**a**) Lymphocytes are gated on the basis of low forward scatter (FSC) and side scatter (SSC), before (**b**) exclusion of doublets using pulse area versus height for FSC. (**c**) $DAPI^{+}$ dead cells are excluded, and (**d**) $CD3^{+}$ $CD4^{+}$helper T cells are selected from viable cells. (**e**) and (**f**) Tfh cells are gated based on their $CD25^{-}$ $CXCR5^{high}$ $PD\text{-}1^{high}$ phenotype

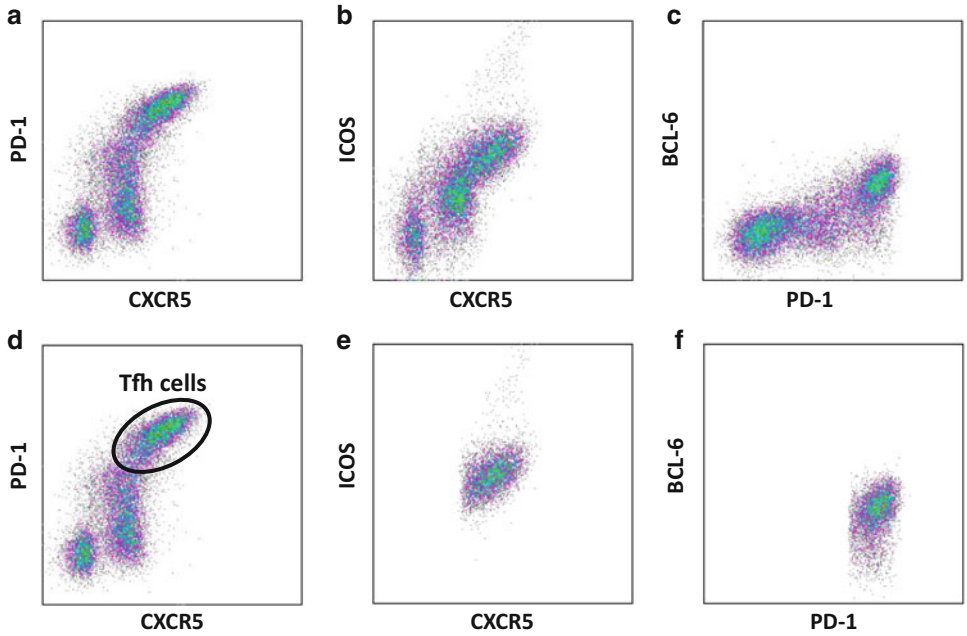

Fig. 2 Density plot representation of CXCR5, PD-1, ICOS, and Bcl-6 after intracytoplasmic staining of human tonsil single cell suspension based on the alternative protocol described in 3.2.2. A similar gating strategy as in Fig. 1 was used. Lymphocytes were gated on the basis of low forward scatter (FSC) and side scatter (SSC), before exclusion of doublets using pulse area versus height for FSC. Dead cells were excluded, and $CD3^{+}$ $CD4^{+}$helper T cells were select gated on viable cells. (**a**), (**b**), and (**c**) represent CXCR5/PD-1, CXCR5/ICOS, and PD-1/Bcl-6 expression profiles of total $CD4^{+}$ T cells. (**e**) and (**f**) represent the specific CXC5/ICOS and PD-1/Bl-6 profiles of gated Tfh cells based on their $CXCR5^{high}$ $PD\text{-}1^{high}$ expression (**d**)

3.4 Cell Sorting

The relatively low frequency of germinal center Tfh cells necessitates an enrichment step preceding the FACS sorting in order to keep a manageable time frame of the procedure and to maintain high viability of the sorted cells. Therefore, $CD4^+$ T cells are first enriched by MACS depletion of CD19-, CD16-, CD14-, and CD8-expressing cells, before staining and sort procedures.

3.4.1 Enrichment of $CD4^+$ T Cells

1. Suspend the thawed cells in the enrichment buffer at a concentration of 5×10^7 cells/mL.
2. For 10^7 total cells, add 2.5 μg of antihuman CD19 Ab and 0.25 μg of antihuman CD8, CD14, and CD16 antibodies. All these antibodies must be reconstituted with sterile distilled water at a final concentration of 200 μg/mL.
3. Incubate at 4 °C for 25 min under agitation. Optional: MACSmix Tube Rotator could be used to allow optimal agitation.
4. Wash cells with the enrichment buffer by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
5. Resuspend the pellet at a concentration of 12.5×10^7 cells/mL in enrichment buffer.
6. Add 20 μL of anti-mouse IgG MicroBeads/10^7 total cells.
7. Incubate at 4 °C for 20 min with agitation. Optional: MACSmix Tube Rotator could be used to allow an optimal agitation.
8. Wash cells with the enrichment buffer by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
9. Resuspend the pellet at 10^7 to 2×10^7 cells/100 μL in enrichment buffer and filter through a 40-μm nylon cell strainer.
10. Run a DepleteS program on autoMACS Pro, or perform the depletion using LD MACS columns according to the manufacturers' instructions.
11. Wash cells with the enrichment buffer by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
12. Resuspend cells in D-PBS and determine the cell concentration.
13. Adjust concentration to 10×10^7/mL of D-PBS.

3.4.2 Cell Staining and Cell Sorting

1. Add the appropriate volume of antibodies from the basic panel to the pre-enriched cells (*see* **Note 6**).
2. Incubate at 4 °C in the dark for 25 min.
3. Wash the cells twice with 2 mL of D-PBS by centrifugation at $600 \times g$ for 5 min. Remove and discard the supernatant.
4. Resuspend cells at a concentration of $2–4 \times 10^7$ cells/mL in D-PBS supplemented with 2 % FCS and filter through a 70-μm nylon cell strainer.
5. Place 500 μL of FBS in empty tubes prepared for the collection of the sorted populations.

6. Sort the population(s) of interest in a temperature-controlled chamber at 4 °C with agitation using an 85-μm nozzle (*see* **Note 8**).
7. Acquire at least 1×10^4 cells for a post-sort assessment of sorting purity.
8. Perform a cell count and assess the viability of the cells.

4 Notes

1. We recommend against the use of some enzymes like type IV collagenase that are classically used for enzymatic dissociation of tissue and modify the structure of CXCR5. Using two different clones (51505.111 and RF8B2), we were unable to reveal the expression of this molecule after this kind of treatment. Of note, DNase does not affect the structure of any of the markers described here.
2. Our comparison of results obtained with fresh and frozen total or mononuclear cells has indicated that virtually identical fluorescence intensity and population percentages are obtained for all the markers described here.
3. Classically, activation of T cells with an anti-CD3 monoclonal antibody no longer permits the use of another clone of antihuman CD3 conjugated with a fluorochrome for the detection step.
4. After activation of total tonsil or lymph node cells or specific T-cell subsets isolated from these tissues with PMA and ionomycin, the huge variability of CXCR5 expression found for $CD4^+$ T cells (*see* Fig. 1) is reduced which makes it difficult to distinguish the Tfh cell subset from the others, even if short-time activation (4 h) has been performed.
5. Human serum is used in the staining buffer to block nonspecific binding of the murine antibodies from the labeling panel. We selectively chose human serum blood type AB (AB serum) that does not contain antibodies against red blood cells.
6. Conjugated antibodies could give variable signal levels depending on the manufacturer and the lot. The titration of each antibody and the verification of the appropriate cocktail are two important steps to guarantee the validity of the obtained profiles. Instead of relying on the number of tests per vial listed by the manufacturer, you are advised to optimize the final concentration of each antibody in your cocktail using the concentration recommended on the specific datasheet as a starting point, and proceed with dilutions.

7. In our experience, membrane staining with the antihuman CD4 clone RPA-T4 gives the best signal after the permeabilization step. Similarly, the expression profile of CXCR5 is not altered by the permeabilization step when the 51505.111 clone is used, unlike the other clones we tested.
8. We perform the sort in a temperature-controlled chamber at 4 °C and at medium speed using an 85-μm nozzle to minimize the mortality of the sorted cells.

Acknowledgments

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Chapter 15

Human Tfh and Tfr Cells: Identification and Assessment of Their Migration Potential

Chang H. Kim, Seika Hashimoto-Hill, and Seung G. Kang

Abstract

The ability of follicular T cells to migrate into B-cell follicles is central for them to participate in germinal center responses. The chemokine receptor CXCR5 is expressed by both Tfh and Tfr cells and is the defining marker for follicular T cells. In addition, Tfh and Tfr cells express additional chemokine receptors to enable them to interact with B cells and other cell types. $CXCR5^+$ Tfh and Tfr cells are divided into $CCR7^+$ perifollicular cells and $CCR7^-$ follicular cells. Most of the $CXCR5^+$ $CCR7^-$ Tfh cells reside in germinal centers and are called GC T cells. The methods to identify human Tfh and Tfr cell subsets based on chemokine receptors and other antigens and assess their migration potential are provided in this article.

Key words Human, Tfh, Tfr, CXCR5, CCR7, Foxp3, Germinal center, Chemotaxis

1 Introduction

Migration and tissue localization characteristics of immune cells are important for their effector functions. This is particularly true for follicular T helper cells (Tfh), which play central roles in regulating T cell-dependent antibody responses [1–4]. Tfh cells are distinguished from non-Tfh $CD4^+$ T cells by their expression of the follicle homing receptor CXCR5. CXCR5 is the sole chemokine receptor for CXCL13 (also called BLC or BCA-1), a chemokine expressed by follicular dendritic cells, myeloid dendritic cells, and T cells in germinal centers [5–7]. CXCL13 is also expressed in non-lymphoid tissues in situations where tertiary lymphoid structures form [8]. Tfh cells are heterogeneous in effector function, surface phenotype, and microenvironmental localization. Depending on the expression level of the T-zone homing receptor CCR7, Tfh cells can localize in the T-B boundary, the mantle zone, and the light zone of germinal centers (GC) [9, 3]. Follicular T helper cells can be functionally divided into helper (Tfh) and regulatory (Tfr) T cells [3, 9]. Tfr cells are Foxp3-expressing T cells that share phenotypic characteristics of Tfh cells [9–11]: decreased CCR7 and

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CD127 expression and increased expression of programmed cell death (PD)-1, inducible T-cell costimulator (ICOS), and B- and T-lymphocyte attenuator (BTLA) [3, 12, 13, 2, 1]. CXCR5$^+$ Foxp3$^+$CD4$^+$ cells are found mostly in the mantle zone and perifollicular areas, but a small number of these cells have a CXCR5$^+$CCR7$^-$ phenotype and localize in GCs. In this chapter, the methods to identify and enrich human Tfh cell subsets and assess their migration potential are described.

2 Materials

2.1 Antibody Staining and Flow Cytometry

1. 4-mL round bottom or 1.5-mL tubes to perform the staining.
2. Phosphate buffered saline (PBS), pH 7.2: Milli-Q filtered H_2O containing 8 g/L NaCl, 0.2 g/L KCL, and 1.15 g/L Na_2HPO_4 stored at room temperature.
3. FACS buffer: 1× Dulbecco's phosphate buffered saline (PBS w/o $MgCl_2$ and $CaCl_2$), 0.5 % (w/v) bovine serum albumin, and 0.09 % (w/v) NaN_3 filter sterilized using a 0.2-μm filter.
4. Antibodies: CD4 (OKT4, fluorochrome conjugated), CD45RO (UCHL1, fluorochrome conjugated), CD45RA (HI100, fluorochrome conjugated), CD8 (HIT8a, fluorochrome conjugated), CD19 (HIB19, fluorochrome conjugated), CD14 (HCD14, fluorochrome conjugated), Foxp3 (259D, fluorochrome conjugated), ICOS (C398.4A, fluorochrome conjugated), CD56 (HCD56, fluorochrome conjugated), CD11B (ICRF44, fluorochrome conjugated), CD11C (3.9, fluorochrome conjugated), CCR7 (TGB, fluorochrome conjugated), CXCR5 (R&D Systems 51505, unconjugated/PE/APC), mouse IgG2b control antibody (MPC-11, unconjugated/PE/APC), biotinylated goat anti-mouse IgG (polyclonal), and PE/PerCP/APC-streptavidin. For more details on the potential antibody combinations for staining cells, *see* **Note 1**; for antibody concentration, *see* **Note 2**.
5. Intracellular staining kit. In this protocol we use the Fix/Perm set from eBioscience, although many kits are available and we have not tested them all in parallel to determine the best kit.
6. Blocking buffer: 5 % mouse serum, PBS (v/v).
7. FACS fix: 2 % paraformaldehyde, PBS (v/v).

2.2 T-Cell Enrichment by Sheep Red Blood Cell (SRBC) Rosetting

1. 50-mL and 14-mL sterile conical tubes.
2. Sterile metal mesh.
3. Sterile petri dishes (10 cm).
4. Phosphate buffered saline (PBS), pH 7.2: Milli-Q filtered H_2O containing 8 g/L NaCl, 0.2 g/L KCL, and 1.15 g/L Na_2HPO_4 stored at room temperature.

5. Sheep red blood cell blood suspension preserved in Alsever's solution.
6. 4 % 2-(2-aminoethyl) isothiourea dihydrobromide (AET) solution: 4 % (w/v) in Milli-Q filtered H_2O, filter sterilized through a 0.2-μm filter.
7. RPMI-1640 medium.
8. Heat-inactivated fetal calf serum: inactivated in a water bath at 56 °C for 30 min.
9. Histopaque-1077 or equivalent density gradient solution.
10. Red blood cell lysis solution: 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA. Adjust pH to 7.2 ~ 7.4. Sterilize the solution through a 0.2-μm filter and keep it at 4 °C.

2.3 T-Cell Enrichment by Magnetic Sorting

The magnetic sorting system used in this protocol is from Miltenyi. Other kits are marketed but we have not tested them all in parallel to determine the best kit.

1. MACS buffer: 1× Dulbecco's phosphate buffered saline (without $MgCl_2$ and $CaCl_2$), 0.5 % (w/v) bovine serum albumin, and 2 mM EDTA (500 mM stock, pH 8.0) filter sterilized using a 0.2-μm filter.
2. PE-conjugated Abs to CD19, CD11C, CD11B, and CD56.
3. Anti-PE magnetic isolation beads: In this protocol we use the Miltenyi Anti-PE MicroBeads.
4. Magnetic cell separator: Here we describe the use of either the autoMACS system (depletion-S program) or a depletion column (e.g., LS/LD column) and a Miltenyi manual separator (e.g., VarioMACS™ Separator).

2.4 Chemotaxis Assay

1. Chemotaxis buffer: RPMI-1640 medium, 0.5 % BSA (w/v), filter sterilized through a 0.2-μm filter. Store at 4 °C.
2. Chemokines: The human chemokines desired for the specific research question, for example, CXCL13 and/or CCL19 reconstituted and stored following manufacturer's instructions.
3. Transwell chemotaxis chambers: polycarbonate membrane cell culture inserts manufactured by Corning (5-μm pore size).
4. Sterile 24-well culture plates.
5. Incubator at 37 °C with 5 % CO_2.

3 Methods

3.1 Identification of Tonsil Tfh and Tfr Cells by Flow Cytometry

Tfh cells have the $CXCR5^+Foxp3^-$ phenotype (Fig. 1a). These cells can be further divided into T-B border area- and GC-residing cell subsets based on expression of CCR7 (Fig. 1b). PD-1 can replace CXCR5 because all $CXCR5^{bright}$ cells are $PD\text{-}1^{bright}$ (Fig. 1c).

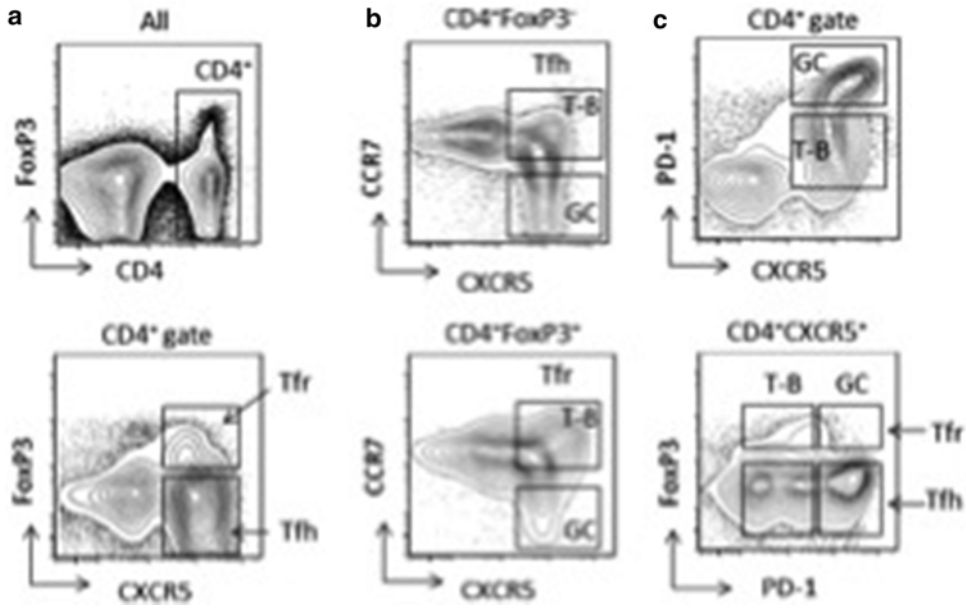

Fig. 1 Identification of Tfh and Tfr cells by flow cytometry based on expression of CXCR5 (or PD-1) and Foxp3. (**a**) Tfh cells are $CD4^+CXCR5^+$ $Foxp3^-$ cells. Tfr cells are $CD4^+CXCR5^+$ $Foxp3^+$ cells. (**b**) Tfh and Tfr cells are further divided into $CCR7^+$ T-B border area and $CCR7^-$ GC T cells. (**c**) PD-1 can be used as a surrogate marker for $CXCR5^+$ T cells. PD-1^{bright} cells are GC T cells, and PD-1^{medium} cells are T-B border area T cells. Anti-CD19 can be additionally used to exclude a small number of T- and B-cell aggregates in the Tfh cell pool

Tfr cells have the $CXCR5^+Foxp3^+$ phenotype (Fig. 1a). These cells can be further divided into T-B border area- and GC-residing subsets based on CCR7 expression. Two methods to assess CXCR5 expression are described below (*see* **Note 1**). Direct staining of T cells by fluorochrome-conjugated anti-CXCR5 yields somewhat weak CXCR5 staining on Tfh cells. Researchers can use an alternative three-step staining method for brighter CXCR5 staining.

3.1.1 Identification of $CXCR5^+$ T Helper Cells by One-Step Staining and Flow Cytometry

1. Place 2×10^6 tonsil cells (*see* Subheading 3.2) into a tube (4-mL round bottom or 1.5-mL tubes). Spin down for 5 min at $400 \times g$, 4 °C (≈2,000 rpm on a typical benchtop swing bucket centrifuge). Discard the supernatant.
2. Resuspend cell pellets in 30–40 μL of FACS buffer, add antibodies (typically 2 μL, *see* **Note 2**) for surface antigens (e.g., CD4-FITC, CXCR5-APC, PD-1-PerCP), and incubate for 30 min at 4 °C. From this point, protect the samples from light.
3. Wash the cells by adding 1–4 mL of FACS buffer depending on tube size, spin down for 5 min at $400 \times g$, 4 °C, and discard supernatant.
4. Resuspend cell pellets in 400 μL of Fix/Perm buffer and incubate overnight at 4 °C (*see* **Note 3**).

5. Fill tubes with Perm buffer and spin down for 5 min at 400 × *g*, 4 °C. Discard the supernatant. Repeat this wash step one more time.
6. Resuspend cells in Perm buffer and add anti-Foxp3-PE (2 μL). Incubate for 20–30 min at 4 °C.
7. To wash, fill with Perm buffer and spin down cells for 5 min at 400 × *g*, 4 °C. Discard the supernatant. Resuspend cell pellets in 400-μL Perm buffer.
8. Analyze the stained cells with a flow cytometer (e.g., BD Canto II) equipped with 488-nm and 633-nm lasers and detectors appropriate for fluorochromes such as FITC, PE, PerCP, and APC.
9. Identify Tfh and Tfr cells based on the criteria described in Fig. 1.

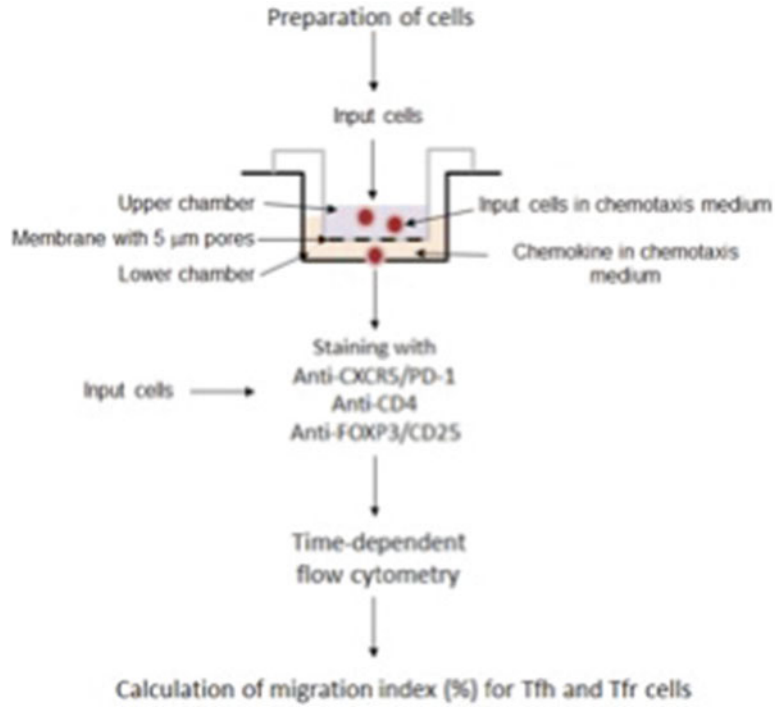

Fig. 2 Chemotaxis assays for Tfh and Tfr cells. Transwells with pore size of 5 μm are used. T cells are added to the upper chamber, and chemokines (CXCL13, CCL19, or others) are added to the lower chamber. The cells are allowed to migrate through the porous transwell membrane for 2–3 h. The cells that migrated to the lower chamber are stained with antibodies for Tfh and/or Tfr markers (e.g., CD4, PD-1/CXCR5, and Foxp3/CD25). Absolute numbers of the T helper subsets that migrated to the lower chamber are calculated based on a time-based flow cytometry. Input cells and cells migrating in the absence of chemokines are also examined at the same time to calculate net migration efficiency (% of input)

3.1.2 Identification of CXCR5⁺ T Cells by Three-Step Antibody Staining and Flow Cytometry

Often, the one-step staining method for CXCR5 (Subheading 3.1.1) does not allow for clear separation of positive from negative CXCR5-expressing cells. This problem can be overcome by using the three-step method described below (*see* **Note 4**).

1. Place 2×10^6 tonsil (*see* Subheading 3.2) or peripheral blood mononuclear cells (*see* **Note 5**) into a tube. Spin down the cells for 5 min at $400 \times g$, 4 °C, and aspirate out the supernatant.
2. Resuspend cell pellets in 40 μL of FACS buffer, add mouse anti-human CXCR5 or isotype control antibody, and incubate for 30 min at 4 °C.
3. Fill tubes with FACS buffer, spin down for 5 min at $400 \times g$, 4 °C, and discard supernatant.
4. Resuspend cell pellets with 40 μL of FACS buffer, add biotinylated goat anti-mouse IgG (H + L) antibody (1 μg), and incubate for 20 min at 4 °C.
5. Wash the cells as in **step 3**.
6. Resuspend cell pellets in 40 μL of FACS buffer, add APC-streptavidin (0.2 μL), and incubate for 20 min at 4 °C. From this point, protect the samples from light.
7. Add 2 μL of mouse serum (5 % final) to the cells and incubate for 10 min at 4 °C. This is to block the antigen-binding sites of the secondary antibody.
8. Add 2 μL of each fluorochrome-conjugated antibody to surface antigens (CD4/FITC, PD-1/PerCP, etc.) and incubate the cells for 20–30 min at 4 °C.
9. Wash the cells as in **step 3**, resuspend them in 400 μL of Fix/Perm buffer, and incubate for overnight at 4 °C.
10. Fill tubes with Perm buffer and wash the cells as in **step 3**. Repeat this wash one more time.
11. Resuspend the cells in 40 μL of Perm buffer and add anti-Foxp3-PE (2 μL).
12. Keep the cells at 4 °C for 30 min.
13. Fill tubes with Perm buffer and wash the cells as in **step 3**. Resuspend the cells in 400 μL of Perm buffer.
14. Analyze the stained cells by flow cytometry (*see* **Note 6**).
15. Use the criteria described in Fig. 1 to identify Tfh and Tfr cells.

3.2 Preparation of Enriched T Cells from Tonsils

The majority of tonsil cells are B cells, and, therefore, the use of enriched CD4⁺ T cells depleted of B cells and other cells makes it easier to perform subsequent assays such as chemotaxis assay and isolate Tfh and Tfr cells. Two methods to enrich T helper cells are described here. The first method has been used for decades and is probably the most cost-effective way to enrich T cells.

The second method is to utilize magnetic depletion of non-T cells. Cells isolated by either method are on average ~95 % pure based on CD3 expression.

3.2.1 Enrichment by Sheep Red Blood Cell (SRBC) Rosetting

1. First prepare the 4 % (AET) solution.
2. Place 25 mL of sheep blood Alsever's suspension in a 50-mL tube and add PBS to 50 mL. Centrifuge for 10 min at 400×*g* at room temperature. Repeat the centrifuge wash once.
3. Resuspend 1 volume of the packed sheep red blood cells (SRBC) in 4 volumes of 4 % AET solution and incubate for 20 min in a 37 °C water bath. The color will change to dark red.
4. Fill the tube with ice-cold PBS and centrifuge for 10 min at 400×*g*, room temperature. Repeat this centrifuge wash once.
5. Resuspend the packed AET-SRBC (8 mL) with 42 mL of RPMI-1640 medium.
6. Place an autoclaved sterile metal mesh (5-cm diameter) on a petri dish (10 cm) and add 10 mL of cold PBS.
7. Cut fresh tonsil tissues into small pieces (2–3 mm), transfer them onto a mesh, and grind tissue fragments using the flat end of a 5-mL plastic syringe plunger.
8. Transfer cells into a 50-mL conical tube, fill the tube with cold PBS, and count the cells using a hemocytometer. Spin down the cells for SRBC rosetting.
9. Resuspend the cell pellet with RPMI-1640 medium (40 million/mL).
10. Add 10 mL of heat-inactivated fetal calf serum and 20 mL of the AET-treated SRBC prepared above (step 5) to every 10 mL of tonsil mononuclear cells, and spin down the cell mixture for 5 min at 200×*g* at 4 °C. Incubate the cell-SRBC mixture for 1 h on ice.
11. Prepare a new 50-mL centrifuge tube containing 15 mL of Histopaque-1077.
12. Take the cell-SRBC mixture slurry and lay it on the Histopaque solution. Centrifuge for 30 min at 400×*g* at room temperature without brake.
13. Using a pipette, remove the clear RPMI medium over cells. Take the interface, which contains predominantly non-T cells (B cells and myeloid cells), and transfer it to a fresh 50-mL tube.
14. Aspirate out remaining supernatant and resuspend the rosetted SRBC-T-cell pellet at the bottom with 25 mL of RBC lysis solution.

15. When the solution becomes transparent, which takes a few minutes, immediately fill the tube with cold PBS. Count the rosetted T cells, spin down for 4 min at 400×*g*/room temperature, and resuspend the cells in a desired buffer (FACS or chemotaxis buffer). For chemotaxis assay, prepare cells at 5 million/mL in the chemotaxis buffer.

3.2.2 Preparation of Enriched T Cells from Tonsils by Magnetic Sorting (An Alternative Method)

1. Continue from **step 2** of Subheading 3.2.1.
2. Resuspend the tonsil cells in MACS buffer (250 μL for 50 million tonsil cells) in a 14-mL tube.
3. Add PE-conjugated Abs to CD19, CD11C, CD11B, and CD56 (50 μL each) per 50 million tonsil mononuclear cells. Incubate for 30 min on ice.
4. Top off the tube with MACS buffer and spin down at 400×*g* for 5 min at 4 °C. Discard the supernatant.
5. Add 60 μL of Miltenyi Anti-PE MicroBeads to 50 million tonsil mononuclear cells. Incubate 30 min on ice.
6. Top off the tube with MACS buffer and spin down at 400×*g* for 5 min at 4 °C. Discard the supernatant.
7. Isolate depleted cells using an autoMACS system (depletion-S program) or a depletion column (e.g., LS/LD column) and a Miltenyi manual separator (e.g., VarioMACS™ Separator). The yield will vary depending on samples. In general, 5–15 million cells enriched with $CD4^+$ T cells are obtained.
8. Add cold MACS buffer to the cells and spin down again. Resuspend the cells in chemotaxis buffer at desired cell concentrations (e.g., 5×10^6 cells/mL).

3.3 Chemotaxis of Tfh and Tfr Cells to Chemokines

The chemotactic response of Tfh and Tfr cells to chemokines such as CXCL13 (CXCR5 ligand) and CCL19/CCL21 (CCR7 ligands) can be determined by this method. It is necessary to duplicate or triplicate each chemotaxis run.

1. Add chemokines at desired concentrations to the chemotaxis buffer (*see* **Note 7**). Optimal concentrations are 3–5 μg/mL (CXCL13) or 2 μg/mL (CCL19) (*see* **Note 8**).
2. Place 600 μL of chemotaxis buffer containing a chemokine into the lower chamber. Some wells should have just the chemotaxis buffer without any chemokine for negative and input controls.
3. Place transwell inserts into each well (of a 24-well plate) and add 100 μL of the cells into each transwell (upper chamber) (*see* **Note 9**). Add the same number of cells to empty wells containing the chemotaxis buffer without transwell inserts for the input control (*see* **Note 10**).

4. Allow the cells to migrate for 3 h in a 5 % CO_2 incubator at 37 °C (*see* **Note 11**).
5. Remove transwell inserts and transfer cells from lower chambers into 1.5-mL tubes.
6. Count input and migrated cell numbers using a hemocytometer.
7. Spin down cells at 400 × *g*, 4 °C, and resuspend them in 40 μL of FACS buffer containing antibodies. Incubate cells on ice for 20 min. Antibodies to surface antigens such as CD4, CXCR5/PD-1, and CD25 can be used.
8. Wash with FACS buffer and spin down.
9. For assessment of $Foxp3^+$ Tfr cells, resuspend the cells in Fix/Perm buffer and stain for Foxp3 as described in Subheading 3.1. Staining with anti-CD25 can be used as a surrogate for Foxp3 to detect Tfr.
10. Spin down the cells and resuspend them in 300 μL of 1–2 % paraformaldehyde. It is important not to lose any cells during the procedure.
11. Acquire the stained cells with a flow cytometer for a fixed time period (e.g., 30 s) for all samples.
12. Analyze the flow cytometry data by FACS analysis software (e.g., FACSDiva or FlowJo). Calculate the absolute numbers of input and migrated CXCR5/PD-1^{++}Foxp3/$CD25^-$ Tfh cells or CXCR5/PD-1^+Foxp3/$CD25^+$ Tfr cells detected during the acquisition time period.
13. Based on the absolute numbers of Tfh or Tfr cells, calculate "net migration (% of input)" as below:

    Net migration (% of input) = [# of migrated Tfh cells to $CXCL13^-$ # of migrated Tfh cells in the absence of CXCL13]/[# of Tfh cells in the input sample]. The same can be applied for Tfr cells or other chemokines.

4 Notes

1. Heterogeneity in surface phenotype of Tfh and Tfr cells. The common surface antigen is CXCR5, but these T cells are highly heterogeneous in expression of additional antigens reflecting their activation and developmental stages. Tfh cells can be subdivided based on the expression of additional trafficking receptors (CCR7, CXCR3, and CCR4), effector molecules (ICOS and CD40L), costimulatory/suppressive/cytokine receptors (PD-1, BTLA, and CD127), and activation markers (CD69 and CD25). Thus, various combinations of

these and other antigens can be examined depending on your experimental objective.

2. For most antibodies, 1–2 μL work well to stain one million cells. For anti-CCR7, use 20 μL. Refer to manufacturer's guidelines for specifics.
3. While the manufacturer (eBioscience) recommends fixation from 30 min to overnight for staining of human Foxp3, we observed that overnight fixation yields brighter and more reproducible staining.
4. One-step versus three-step staining of CXCR5. Identification of $CXCR5^+$ T cells requires a sensitive staining method to visualize CXCR5 expression. The most sensitive way is to perform the three-step staining of CXCR5. This method requires a series of staining with anti-CXCR5 (mouse primary Ab), biotin-labeled anti-mouse IgG (secondary Ab), and streptavidin-conjugated tertiary fluorescent dyes such as PE/PerCP/APC to amplify the fluorescence signal. The benefit of the three-step staining method is clear identification of $CXCR5^+$ T cells well separated from $CXCR5^-$ T cells. A key point is to block the secondary anti-mouse IgG with mouse serum (or IgG) so that it won't bind other mouse antibodies to cell surface antigens such as CD4 and PD-1. The 1-step method is good for chemotaxis assay for fast handling and acquisition. The three-step is ideal for more accurate characterization and phenotyping of Tfh and Tfr cells.
5. Characterization of peripheral blood Tfh and Tfr cells. Most peripheral blood Tfh and Tfr cells express both CXCR5 and CCR7 [3, 9], which is somewhat similar to the Tfh cells found in the T-B border area of the tonsils in terms of CXCR5 and CCR7 expression. Blood mononuclear cells should be prepared by performing RBC lysis followed by Histopaque gradient centrifuge (**step 12** in Subheading 3.2.1). These cells can be used directly for chemotaxis or after enrichment for T cells by magnetic sorting or SRBC rosetting. Staining methods for flow cytometry for peripheral blood Tfh and Tfr cells are similar to those described in Subheading 3.1. However, do not use PD-1 to replace CXCR5 for peripheral blood Tfh and Tfr cells as they do not significantly express PD-1.
6. Use of an isotype-matching control antibody for the three-step staining of CXCR5. It is important to include a negative control stained with an isotype-matching control antibody. This allows accurate determination of the background level of CXCR5 staining. Simply, use an isotype-matching control antibody instead of anti-CXCR5 and proceed as described in Subheading 3.1.2, **step 2**. Expect 1–3 % of background staining

levels using the three-step staining method. The background fluorescent signal is dimmer than the true CXCR5 expression signal.

7. Chemotaxis medium is typically made of culture medium (e.g., RPMI-1640) supplemented with BSA (0.5 %). BSA can be replaced with FBS (1 %).
8. Optimal chemokine concentrations. Typically, chemotactic responses to titrated concentrations of chemokines are bell shaped because the signaling of G-protein-coupled chemokine receptors is desensitized at higher than optimal chemokine concentrations. Because of this issue and variable specific activity of chemokines depending on vendors and production batches, experimenters need to titrate to find optimal concentrations. Therefore, an initial titration experiment to find optimal chemokine concentrations is recommended. Researchers should be aware that optimal concentrations significantly vary among chemokines and chemokine receptors.
9. Consideration of transwell pore size and coating with extracellular matrix (ECM) proteins. 5-μm transwells work well for most chemotaxis experiments of human T cells. Experimenters can use transwells coated with extracellular matrix proteins such as fibronectin (10 μg/mL in PBS for a few hours). The coating allows cells to better adhere and migrate through the membrane. Expect higher % migration on ECM-coated transwells. Background migration also increases with ECM-coated transwells. Use of 3-μm transwells lowers the background migration.
10. Chemokinesis (random migration) of Tfh and Tfr cells can be studied using the same transwell system. For this, chemokines can be added also to the upper chamber at various concentrations, and cell migration from the upper to lower chamber can be assessed.
11. Time consideration. Time length of the chemotaxis assay depends on cell and membrane types and pore size. For T cells, 3 h should be fine but this can be changed to 2 or 4 h. The effective chemotactic gradient from the upper to lower chamber of the transwell system is lost in about 4–5 h [14]. Therefore, chemotaxis assays taking longer than 4 h are not recommended. The whole procedure, composed of cell preparation, chemotaxis, cell harvest, and flow cytometry, can take 1–2 days. Identification of Tfh cells and Tfr cells using the three-step staining method and flow cytometry typically takes 5–6 h. For additional Foxp3 staining, add 4–12 h for overnight fix and permeabilization of human T cells.

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Chapter 16

Analysis of Human Blood Memory T Follicular Helper Subsets

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Abstract

Human blood contains a memory counterpart of T follicular helper (T_{FH}) cells. Blood T_{FH} cells are composed of subsets that differ in phenotype and function. Recent studies show that analysis of blood circulating memory T_{FH} cells can provide clues to understand the mode of actions of vaccines and the pathogenesis of human autoimmune diseases. We will describe here a detailed protocol to analyze the memory T_{FH} subsets in human whole blood samples. We will also describe a protocol to assess the helper capacity of blood memory T_{FH} subsets.

Key words Human, Blood, T follicular helper cells, Subsets, CXCR5

1 Introduction

Our knowledge of the biology of T follicular helper (T_{FH}) cells has been significantly increased during the past decade (reviewed in refs. 1, 2). Studies of T_{FH} cells in humans remained relatively limited until recently, mainly due to difficulties in assessing the dynamics of T_{FH} cells in human lymphoid organs. However, progress in our understanding of the biology of blood circulating memory T_{FH} cells has provided clues to assess the alteration of T_{FH} responses in human diseases.

Blood memory T_{FH} cells, defined as $CXCR5^+$ $CD45RA^-$ $CD4^+$ T cells in human blood, contain long-lived memory cells [3–5]. Human blood memory T_{FH} cells are composed of heterogeneous populations with different phenotype and functions (refer to [6] for a detailed review). While approaches and markers to define subsets often differ among laboratories, blood memory T_{FH} cells can be universally assessed by these three parameters: (1) the chemokine receptors CXCR3 and CCR6, (2) the immunoregulatory molecule PD-1, and (3) the co-stimulatory molecule ICOS.

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The first parameter defines a set of three major subsets: CXCR3$^+$CCR6$^-$ cells that share properties with T_H1 cells (hereafter called blood memory $T_{FH}1$ cells), CXCR3$^-$CCR6$^-$ cells resembling T_H2 cells (hereafter called blood memory $T_{FH}2$ cells), and CXCR3$^-$CCR6$^+$ cells resembling T_H17 cells (hereafter called blood memory $T_{FH}17$ cells) [4]. Blood memory $T_{FH}2$ and $T_{FH}17$ cells are able to induce naïve B cells to produce immunoglobulins and to switch isotypes through cytokine secretion, while blood memory $T_{FH}1$ cells lack the capacity to help naïve B cells [3, 4, 7]. While blood memory $T_{FH}2$ cells promote IgG and IgE secretion, blood memory $T_{FH}17$ cells are efficient at promoting IgG and, in particular, IgA secretion [4]. Thus, $T_{FH}2$ and $T_{FH}17$ cells represent efficient helpers among blood memory T_{FH} cells with distinct capacity to regulate Ig isotype switching.

The second (PD-1) and third (ICOS) parameters define another set of three subsets: one activated subset (ICOS$^+$PD-1^{++}) and two quiescent subsets (ICOS$^-$PD-1$^+$ and ICOS$^-$PD-1$^-$). ICOS expression is limited to a small population (often <1 % in healthy subjects) and defines blood memory T_{FH} cells with activated phenotype that highly express PD-1 and the cell proliferation marker Ki-67 [3, 7–9]. ICOS$^-$ cells are further divided into PD-1$^+$ cells (~30 % of blood memory T_{FH} cells) and PD-1$^-$ cells, both of which lack the expression of Ki-67 and thus are in a quiescent state [3, 8].

The combination of the three parameters defines nine blood memory T_{FH} subsets with distinct functional properties [6]. By using this approach, we have recently identified a potential biomarker for the generation of successful antibody responses in seasonal influenza vaccination. Unexpectedly, we found that influenza vaccination exclusively increases the ICOS$^+$PD-1^{++} $T_{FH}1$ subset in blood (which peaks at 7 days postvaccination). Increase of blood ICOS$^+$PD-1^{++} $T_{FH}1$ subset correlated with the generation of protective antibody response. Mechanistically, ICOS$^+$ blood memory $T_{FH}1$ cells helped memory B cells, but not naïve B cells, to differentiate into plasma cells via secretion of IL-21 and IL-10 [9]. These observations suggest that ICOS$^+$PD-1^{++} $T_{FH}1$ cells are a major CD4$^+$ T cell subset associated with the generation of antibody response in influenza vaccination.

In this chapter, we will describe a detailed protocol to determine the blood memory T_{FH} subsets in human blood samples. We will also describe a protocol to assess the helper capacity of blood memory T_{FH} subsets.

2 Materials

2.1 Determination of Blood Memory T_{FH} Subsets by Flow Cytometry

1. Human blood samples in ACD (acid citrate dextrose) collection tubes.
2. 1× phosphate buffered saline (PBS).
3. 5-ml polystyrene round bottom tube.

Table 1
Antibodies for blood T_{FH} panel

Antibody	Fluorochrome	Clone
CD45	Pacific Orange	HI30
CD3	AF 700	UCHT1
CD4	Q655	S3.5
CD8	APCH7	SK1
CXCR5	AF 647	RF8B2
ICOS	PECy7	C398.4A
CD45RA	ECD	2H4LDH11LDB9
CXCR3	BV421	G025H7
CCR6	PE	11A9
PD-1	FITC	EH12.2H7

4. 50-ml centrifuge tube.
5. Ficoll-Paque PLUS solution (GE-Healthcare Life Sciences).
6. BD FACS lysing solution.
7. Fluorochrome-conjugated monoclonal antibodies (Table 1).
8. BD stabilizing fixative.
9. Flow cytometer, for example, BD Fortessa or LSR II (BD Biosciences).
10. Flow cytometry analysis software, for example, FlowJo (FlowJo, LLC).

2.2 Assessment of B Helper Capacity

1. Peripheral blood mononuclear cells (PBMCs).
2. PBS.
3. Complete RPMI medium: RPMI medium 1640 with 2 mM L-glutamine, 10 % heat-inactivated fetal bovine serum (FBS) (v/v), 100 units per ml penicillin, 100 μg per ml streptomycin, 1 mM sodium pyruvate, 25 mM HEPES, 0.05 mM 2-mercaptoethanol, and 1× MEM nonessential amino acids.
4. Vacuum-driven sterile filter polyethersulfone (PES) membrane 0.22 μm.
5. 70-μm nylon cell filter.
6. Trypan blue.
7. Hemocytometer.
8. MACS buffer: PBS, 2 % heat-inactivated FBS (v/v), and 2 mM EDTA.

9. MACs columns for cell separation (MS or LS columns, Miltenyi Biotec).
10. MACs magnetic separator (Miltenyi Biotec).
11. Human $CD4^+$ T cell magnetic isolation kit (Miltenyi Biotec).
12. Human CD19 MicroBeads (Miltenyi Biotec).
13. 5-ml polystyrene round bottom tube.
14. 5-ml polypropylene round bottom culture tube.
15. 96-well plate polystyrene round bottom culture plate.
16. 96-well V-bottom plates.
17. Staphylococcal enterotoxin, SEB (Toxin Technology).
18. Live/Dead fixable aqua dead cell stain kit (Life Technologies).
19. Flow sorter: BD FACSAria II (BD Biosciences) or equivalent.
20. Flow cytometer: BD Fortessa or LSR II (BD Biosciences) or equivalent.
21. Antibodies:
    (a) For sorting of blood memory T_{FH} cells: anti-CD4 APC-Cy7 (OKT4), anti-CXCR5 AF488 (RF8B2), anti-CCR6 PE (11A9), anti-CXCR3 BV421 (G025H7), anti-ICOS PE-Cy7 (C398.4A), anti-CD45RA AF700 (HI100), an APC dump cocktail composed of anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD56 (B159), anti-CD8 (SK1), and anti-CD11c (S-HCL-3).
    (b) For sorting of B cells: IgD PE (IA6-2), anti-CD20 FITC (2H7), anti-CD3 AF700 (UCHT1), and anti-CD27 APC-Cy7 (O323).
    (c) For assessment of plasma cells in the T-B culture: anti-CD4 Pacific Blue (RPA-T4), anti-CD3 AF700 (UCHT1), anti-CD38 PE-Cy7 (HB7), and anti-CD138 APC (MI15).

3 Methods

3.1 Determination of Blood Memory T_{FH} Subsets by Flow Cytometry

3.1.1 Staining of Blood Cells

1. Aliquot the antibody mixture (Table 1, *see* **Note 1**) to a 5-ml polystyrene tube (*see* **Note 2**).
2. Add 200 μl of whole blood to the antibody mixture and mix with a pipette (*see* **Note 3**).
3. Incubate for 15 min at room temperature in the dark.
4. Add 1.5 ml of 1× BD FACS lysing solution, mix with a pipette, and incubate for 10 min at room temperature in the dark.
5. Centrifuge for 7 min at 350 × *g* and discard the supernatant carefully.

6. Add 1.5 ml of PBS, and mix gently.
7. Centrifuge for 7 min at 350×*g* and discard supernatant carefully.
8. Resuspend in 250 μl of PBS for immediate FACS analysis or with 250 μl of 1× BD stabilizing fixative, and store at 4 °C until acquisition (up to 24–36 h).

3.1.2 Acquisition and Analysis of Flow Data

1. Acquire the flow data with BD Fortessa, LSR II, or an equivalent flow cytometer (*see* **Note 4**).
2. Analyze flow data by FlowJo (Fig. 1a).
   (a) Create a CD45 vs. SSC panel: Gate to the $CD45^+$ viable hematopoietic cells.
   (b) Create a FSC vs. SSC panel: Gate to the $FSC^{lo}SSC^{lo}$ lymphocyte population.
   (c) Create a CD3 vs. SSC panel: Gate to the $CD3^+SSC^{lo}$ T cell population.
   (d) Create a CD4 vs. CD8 panel: Gate to the $CD4^+CD8^-$ T cell population.
   (e) Create a CD45RA vs. CXCR5 panel: Gate to the $CD45RA^-CXCR5^+$ blood memory T_{FH} cells (*see* **Note 5**).

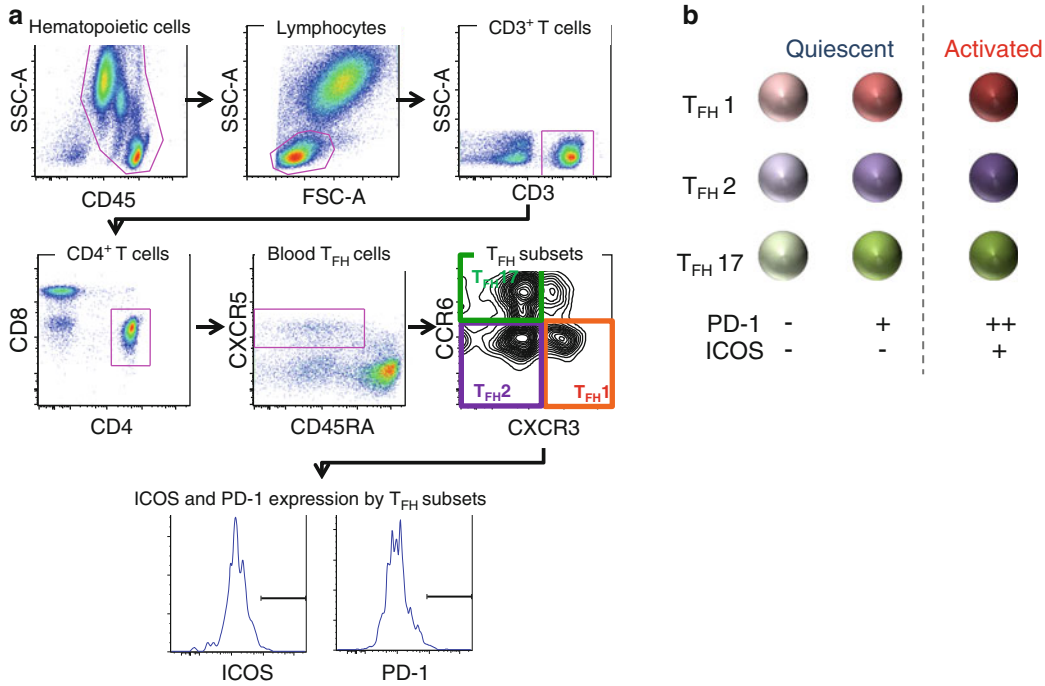

Fig. 1 The nine human blood memory T_{FH} subsets. (**a**) Gating strategy to define the blood memory T_{FH} subsets. (**b**) The matrix combination of the three parameters defines nine blood memory T_{FH} subsets with distinct functional properties

(f) Create a CXCR3 vs. CCR6 panel: Gate to the CXCR3$^+$ CCR6$^-$ $T_{FH}1$ cells, CXCR3$^-$CCR6$^-$ $T_{FH}2$ cells, and CXCR3$^-$ CCR6$^+$ $T_{FH}17$ cells (*see* **Notes 6** and **7**).

(g) Create a histogram for ICOS and PD-1 expression by blood memory $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ cells: Determine the percentage of cell population expressing ICOS and/or PD-1 among each blood memory T_{FH} subset (*see* **Note 8**).

(h) Determine the frequency of ICOS$^-$PD-1$^-$, ICOS$^-$PD-1$^+$, and ICOS$^+$PD-1$^+$ cells among blood memory $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ cells (the nine blood memory T_{FH} subsets (Fig. 1b)).

3.2 Assessment of B Helper Capacity

Here we will describe the methods to assess the capacities of blood memory T_{FH} subset to provide help to autologous B cell subsets (naïve and memory B cells). To induce the cognate interactions between T and B cells, a superantigen staphylococcal enterotoxin B (SEB) is added to the cultures. A similar strategy can be used to assess the helper function of tonsillar T_{FH} cells.

Assessment of the function of each blood memory T_{FH} subset requires large amounts of blood samples, such as from blood apheresis and buffy coat. To minimize the sort time, we first enrich CD4$^+$ T cell and B cells from blood samples before sorting. When small blood draw samples are used for the experiments, we recommend sorting total blood memory T_{FH} cells and B cells directly from PBMCs.

3.2.1 Sorting Blood Memory T_{FH} Cell Subsets

1. Obtain PBMCs from blood samples (*see* **Note 9**).
   (a) Dilute the blood 1:3 with PBS.
   (b) Add 15 ml of Ficoll-Paque PLUS solution in a 50-ml tube.
   (c) Layer up to 35 ml of the blood/PBS mixed solution onto the surface of Ficoll-Paque PLUS solution gently.
   (d) Centrifuge for 30 min at 400 × *g* (acceleration = 2, brake = 0) at 20 °C.
   (e) After centrifugation, the sample separates into two phases: a top phase of diluted plasma and a bottom phase of the Ficoll-Paque PLUS containing erythrocytes. Lymphocytes form a ring between the two phases. Transfer the interface containing lymphocytes into a 50-ml tube.
   (f) Wash with 40 ml PBS (8 min, 380 × *g*, 20 °C).
   (g) Wash with 40 ml PBS (8 min, 200 × *g*, 20 °C).
   (h) Resuspend cells in PBS and take an aliquot for counting.
2. Isolate CD4$^+$ T cells using human CD4$^+$ T cell isolation kit.
   (a) Resuspend PBMCs in 40 μl of MACS buffer per 10^7 PBMCs.
   (b) Add 10 μl of CD4$^+$ T Cell Biotin-Antibody Cocktail per 10^7 PBMCs.

(c) Mix well and incubate for 5 min at 4 °C.

(d) Add 30 μl of MACS buffer per 10^7 PBMCs.

(e) Add 20 μl of $CD4^+$ T Cell MicroBead Cocktail per 10^7 PBMCs.

(f) Mix well and incubate for an additional 10 min at 4 °C.

(g) Wash cells by adding 1–2 ml of MACS buffer per 10^7 cells and centrifuge at $350 \times g$ for 7 min.

(h) Aspirate supernatant completely.

(i) Resuspend cells in 500 μl of MACS buffer per 10^8 cells.

(j) Place a suitable MACS separator column (MS or LS depending on the number of PBMCs, *see* **Note 10**) in the magnetic field.

(k) Rinse the column with 500 μl (for MS column) or 3 ml (for LS column) of MACS buffer.

(l) Apply cell suspension onto the column.

(m) Collect unlabeled cells that passed through the column, and wash the column three times with 500 μl (for MS column) or 3 ml (for LS column) of MACS buffer.

(n) Centrifuge the collected cells ($CD4^+$ T cells) at $350 \times g$ for 7 min.

(o) Resuspend $CD4^+$ T cells in 200 μl PBS in a 5-ml polystyrene tube.

3. Stain blood $CD4^+$ T cells with fluorescence-labeled mAbs: anti-CD4 APC-Cy7, anti-CXCR5 AF 488, anti-CCR6 PE, anti-CXCR3 BV421, anti-ICOS PE-Cy7, anti-CD45RA AF 700, an APC dump cocktail composed of anti-CD16, anti-CD19, anti-CD56, anti-CD8, and anti-CD11c, and Live/Dead fixable aqua.
4. Incubate cells for 20 min at 4 °C in the dark.
5. Wash cells in 5 ml PBS.
6. Centrifuge the cells at $350 \times g$ for 7 min.
7. Resuspend cells in MACS buffer at $20–25 \times 10^6$ per ml in a 5-ml polystyrene tube.
8. Filter cells before sorting to prevent clogging (70- or 100-μm filter).
9. Sort blood memory $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ cells into 5-ml polypropylene collection tubes containing 1 ml PBS supplemented with 50 % FBS by using the gating strategy indicated in Subheading 3.1.2 (*see* **Note 11**).
10. Centrifuge the cells at $350 \times g$ for 7 min.
11. Resuspend cells in RPMI complete medium at a concentration of 2×10^5 cells per ml.

3.2.2 Sorting Blood B Cell Subsets

1. Isolate B cells from autologous PBMCs using human CD19 MicroBeads (*see* **Note 12**). Resuspend PBMCs in 80 µl of MACS buffer per 10^7 PBMCs.
2. Add 20 µl of CD19 MicroBeads per 10^7 PBMCs.
3. Mix well and incubate for 15 min at 4 °C.
4. Wash cells by adding 1–2 ml of MACS buffer per 10^7 cells and centrifuge at $350 \times g$ for 7 min.
5. Aspirate supernatant completely.
6. Resuspend cells in 500 µl of MACS buffer per 10^8 cells.
7. Place a suitable MACS separator column (MS or LS) in the magnetic field.
8. Rinse the column with 500 µl (for MS column) or 3 ml (for LS column) of MACS buffer.
9. Apply cell suspension onto the column.
10. Collect unlabeled cells that passed through the column, and wash the column three times with 500 µl (for MS column) or 3 ml (for LS column) of MACS buffer.
11. Collect $CD19^+$ B cells that bound to the column by pushing them out with 1–5 ml of MACS buffer with the column removed from the magnet.
12. Centrifuge the collected cells (B cells) at $350 \times g$ for 7 min.
13. Resuspend B cells in 200 µl PBS.
14. Stain cells with IgD PE, anti-CD20 FITC, anti-CD3 AF700, anti-CD27 APC-Cy7, and Live/Dead fixable aqua.
15. Incubate cells for 20 min at 4 °C in the dark.
16. Wash cells in 5 ml PBS.
17. Centrifuge the cells at $350 \times g$ for 7 min.
18. Resuspend cells in MACS buffer at $20–25 \times 10^6$ per ml in 5-ml polystyrene tube.
19. Filter cells before sorting to prevent clogging (70- or 100-µm filter).
20. Sort blood naïve and memory B cells into 5-ml polypropylene collection tubes containing 1 ml PBS supplemented with 50 % FBS.
    (a) Create a FSC vs. SSC panel: Gate to the $FSC^{lo}SSC^{lo}$ lymphocyte population.
    (b) Create a SSC vs. Live/Dead fixable aqua: Gate to Live/Dead aqua negative viable cells.
    (c) Create a CD3 vs. CD20 panel: Gate to the $CD3^-CD20^+$ B cell population.
    (d) Create a IgD vs. CD27 panel: Sort IgD^+CD27^- naïve B cells and IgD^-CD27^+ memory B cells.

21. Centrifuge the cells at $350 \times g$ for 7 min.
22. Resuspend cells in RPMI complete medium at a concentration of 2×10^5 cells per ml.

3.2.3 T and B Cell Coculture and Readouts

1. Plate 100 µl (2×10^4 cells) of blood T_{FH} cell suspension and 100 µl (2×10^4 cells) of blood B cell suspension per well in a 96-well round bottom plate. Perform in triplicate at a minimum (*see* **Notes 13** and **14**).
2. Add SEB to the culture at a final concentration of 1 µg/ml.
3. Incubate the plates at 37 °C in 5 % CO_2 incubator for 6 days for the culture with memory B cells and for 12 days for the culture with naïve B cells.
4. Harvest supernatant to measure IgM, IgG, and IgA concentrations by ELISA.
5. Count the recovered viable cells with trypan blue.
6. Transfer cells into a 96-well V-bottom plate.
7. Centrifuge the recovered cells at $350 \times g$ for 7 min.
8. Stain cells for 30 min with 50 µl PBS containing Live/Dead fixable aqua, anti-CD4 Pacific Blue, anti-CD3 AF700, anti-CD38 PE-Cy7, and anti-CD138 APC.
9. Incubate cells for 20 min at 4 °C in the dark.
10. Wash cells with 200 µl PBS.
11. Centrifuge cells at $350 \times g$ for 7 min.
12. Analyze the plasma cell population by flow cytometry.
    (a) Create a FSC vs. SSC panel: Gate to the $FSC^{lo}SSC^{lo}$ lymphocyte population.
    (b) Create a SSC vs. Live/Dead fixable dye: Gate to Live/Dead aqua negative viable cells.
    (c) Create a CD3 vs. CD4 panel: Gate to the $CD3^-CD4^-$ B cell population.
    (d) Create a CD38 vs. CD138 panel: Gate to the $CD38^{++}CD138^+$ plasma cells.
13. Determine the absolute number of plasma cells per well by calculation. Use the cell number of recovered viable cells and the percentage of plasma cells among viable cells determined by the flow cytometer.

4 Notes

1. Each antibody needs to be titrated on whole blood for optimal results.
2. Staining can be done with 2-ml round bottom deep-well plates.

3. For staining of PBMCs, 100 μl of PBMC suspension (isolated by Ficoll density gradient separation) in PBS (cell concentration: up to 1×10^7 per ml) can be used. We recommend (1) to substitute CD45 Pacific Orange to Live/Dead fixable aqua and (2) to titrate antibody amounts on PBMCs for optimal results. For PBMCs staining, **steps 4** and **5** (RBC lysis) should be omitted.
4. When a 10-color flow cytometer (such as BD Fortessa or LSR II) is not available, the panel needs to be simplified. The candidate markers to be excluded from the panel are CD45, CD8, and CCR6.
   - The panel of CD45 vs. SSC often helps to remove debris from the analysis of whole blood samples. However, CD45 is not essential.
   - The risk of CD8 exclusion from the panel is the contamination of a minor $CD4^+CD8^+$ T cell population in the analysis.
   - CCR6 exclusion from the panel still permits determination of $T_{FH}1$ and non-$T_{FH}1$ subsets [3].
5. $CD45RA^+CXCR5^{++}$ cells often contain contaminated B cells and therefore need to be removed from the analysis.
6. The expression of CXCR3 and CCR6 mAbs by blood $CD4^+$ T cells should show bimodal patterns, and therefore it is usually not difficult to put an appropriate quadrant gate for $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ subsets (*see* Fig. 1a). When the subsets are not clearly separated, these possibilities can be considered: (1) Quality of mAb is suboptimal, (2) FACS configuration for the detection of the fluorescence is suboptimal, and (3) The compensation of the flow panel is not adequate.
7. To examine whether CXCR3 and CCR6 staining is working, we recommend to compare the expression between naïve $CD4^+$ T cells ($CD45RA^+CXCR5^-$) and blood memory T_{FH} ($CD45RA^-CXCR5^+$) cells. Naïve $CD4^+$ T cells usually do not contain cells that highly express CXCR3 or CCR6.
8. Setting up the gate for ICOS and PD-1. The expression of ICOS and PD-1 by blood $CD4^+$ T cells does not usually show a bimodal pattern. Therefore, the gating strategy for ICOS and PD-1 is often problematic. Here we describe our method:
   - In the CD45RA vs. CXCR5 panel, gate to $CD45RA^+$ $CXCR5^-$ naïve $CD4^+$ T cells.
   - Create a histogram for ICOS and PD-1 by naïve $CD4^+$ T cells, and set the border by excluding all the naïve cells.
   - Apply the same gates to assess the frequency of ICOS- and PD-1-expressing cells among blood memory $T_{FH}1$, $T_{FH}2$, and $T_{FH}17$ cells.
9. Frozen PBMCs can be used for this assay.

10. In most cases, LS columns can be used for isolation of $CD4^+$ T and B cells. Maximum 1×10^8 labeled cells can be attached to the LS column (maximum input 2×10^9 PBMCs), while maximum 1×10^7 labeled cells can be attached to the MS column (maximum input 2×10^8 PBMCs).
11. Naïve $CD4^+$ T cells ($CD45RA^+CXCR5^-$) sorted at the same time can be used as a negative control.
12. When the number of PBMCs is relatively limited, B cells can be first isolated by positive selection with MACS. Blood memory T_{FH} subsets can be sorted from the B cell negative fraction. As the APC dump channel contains markers for B cells, monocytes, and dendritic cells, the protocol should be sufficient to remove the risk of contamination of other antigen-presenting cells.
13. To avoid evaporation of culture media during the long culture, the empty wells in the 96-well plate should be filled with 200 μl PBS.
14. Although early studies used mitomycin C (MMC) to inhibit the T cell growth in T-B culture, we do not recommend it. In our experience, MMC treatment significantly modifies the property of T cells (e.g., cytotoxicity (Bentebibel et al, unpublished observations)).

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Chapter 17

Flow Cytometric Analysis of Circulating Follicular Helper T (Tfh) and Follicular Regulatory T (Tfr) Populations in Human Blood

Yunbo Wei, Jinhong Feng, Zhaohua Hou, Xin Maggie Wang, and Di Yu

Abstract

Follicular helper T (Tfh) cells are the helper T-cell subset that localizes in germinal centers within secondary lymphoid organs. They support B cells to produce high-affinity antibodies and generate B-cell memory. By contrast, follicular regulatory T (Tfr) cells localize in germinal centers to suppress B-cell responses. Coordinately regulating antibody responses, Tfh and Tfr cells play a critical role for vaccination, infectious disease control, and the development of autoimmune diseases. Although *bona fide* Tfh and Tfr cells are hardly detected in human blood, circulating Tfh and Tfr memory cells in blood can be used to investigate their functions in health and disease. We have developed a comprehensive flow cytometric analysis to define different circulating Tfh and Tfr populations within $CXCR5^+$ $CD4^+$ T-cell population in human blood, based on numerous cell surface markers including CD25, CD127, CCR7, PD-1, CXCR3, and CCR6.

Key words Tfh, Tfr, Human blood, Flow cytometry, CXCR5

1 Introduction

Follicular helper T (Tfh) cells are a helper T-cell subset specialized in the regulation of antibody responses derived from the germinal center response. Tfh cells support germinal center response and provide selection signals to germinal center B cells to differentiate into memory B cells and long-lived plasma cells [1, 2]. Due to the essential role of Tfh cells in regulating high-affinity antibody production and B-cell memory formation, deficiency of Tfh cells leads to immunodeficiency, while excessive Tfh cells contribute to the development of autoimmunity and lymphoid malignancy [3, 4]. How to measure the activity of Tfh cells in patients is a question of crucial importance to dissect their function in human diseases and eventually develop immunological interventions to target Tfh cells.

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Tfh cells are generated, and carry out their function, in secondary lymphoid organs, such as spleens and lymph nodes. However, blood is the most common and sometimes the only available sample from patients; thus monitoring a population of circulating Tfh-like cells in the blood is a powerful tool to investigate Tfh biology in human health and disease. Circulating Tfh cells in blood are different from mature Tfh cells in germinal centers. Notably, circulating Tfh cells do not express the key Tfh transcription factor Bcl-6 [5]. Studies, including ours, suggest that circulating Tfh cells are memory Tfh cells, mainly generated before the terminal differentiation into mature Tfh cells [6].

Recently, follicular regulatory T (Tfr) cells were identified as a Treg cell subset that localizes in the germinal center to suppress B-cell response. Sharing certain key features of Tfh cells, Tfr cells also express the transcription factor Bcl-6 and the chemokine receptor CXCR5 [7–9]. In addition, circulating Tfr cells are detected in blood during an immune response [10].

CXCR5 on $CD4^+$ T cells is upregulated after activation and maintained at high levels during Tfh cell differentiation [1, 2]. As one of the key features of Tfh cells, CXCR5 is widely used to define circulating Tfh and Tfr cells in blood [5, 11–14]. These studies, however, also demonstrate that $CXCR5^+$ $CD4^+$ T-cell population in blood is heterogeneous [6]. In this chapter, we will describe how to stratify $CXCR5^+$ $CD4^+$ T cells in blood to measure Tfh cell active differentiation based on the additional markers CCR7 and PD-1 [5], to measure Tfh cell polarization based on the additional markers CXCR3 and CCR6 [12] and to measure circulating follicular regulatory T (Tfr) cells based on the additional markers CD25 and CD127 [15, 16]. A comprehensive flow cytometric analysis is required to appropriately and reliably monitor the function of Tfh cells in health and disease.

2 Materials

2.1 Cell Preparation

1. Phosphate buffered saline (PBS). Sterile and stored at room temperature.
2. Complete medium: RPMI medium 1640 with L-glutamine, 10 % deactivated fetal bovine serum (FBS) (v/v), 100 unit/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 0.055 mM 2-mercaptoethanol, and 1 % MEM nonessential amino acids (v/v).
3. 2 mL K2-EDTA tube for blood collection.
4. Ficoll-Paque™PLUS or other equivalent density gradient solutions.
5. 15-mL sterile tubes.
6. 70-μm nylon cell strainer.
7. Refrigerated centrifuge.

2.2 Cell Staining

1. 96-well U-bottom plate.
2. Medium for cell staining (FACS buffer): sterile PBS, 2 % FBS (v/v), 0.1 % sodium azide (NaN_3) (w/v), and 2 mM EDTA.
3. 70-μm nylon cell strainer.
4. 5-mL round-bottom tubes.
5. A full staining panel of antibodies: Here we use a panel of antibodies compatible with a flow cytometer with a 4-laser combination (e.g., BD FACS Aria III: blue/488 nm; yellow green/561 nm; red/633 nm; violet/405 nm) (Table 1).

2.3 Data Acquisition and Analysis

1. Flow cytometer. This protocol utilizes a BD FACS Aria III with four lasers for our full staining panel. Depending on the panel design for a particular experiment, as discussed below, a flow cytometer capable of 5–14 channels for fluorescence can be used. BD CompBeads can be used for compensation.
2. Analysis software. This protocol uses FlowJo (Version 10, Treestar) to analyze FACS data. Other analysis software with similar functions can also be used.

Table 1
The antibody staining panel for follicular helper T (Tfh) and follicular regulatory T (Tfr) cells

Laser	Filter	Fluorochrome	Antigen	Clone
Blue	530/30	FITC	TCR α/β	IP26
Yellow green	582/15	PE	CCR6	G034E3
	610/20	PE-CF594	CD25	M-A251
	670/14	7-AAD	/	/
	780/60	PE-Cy7	CCR7	G043H7
Red	660/40	AF647	CXCR5	RF8B2
	730/45	AF700	CD4	RPA-T4
	780/60	APC-Cy7	CD45RA	HI100
Violet	450/50	BV421	CXCR3	G025H7
	525/50	BV510	CD8	RPA-T8
	610/20	BV605	CD127	A019D5
	660/40	BV650	CD3	OKT3
	710/50	BV711	PD-1	EH12.2H7
	780/60	BV786	CD14	M5E2
		BV786	CD19	SJ25C1

3 Methods

3.1 Cell Preparation (Isolation of Mononuclear Cells from Human Peripheral Blood)

This protocol is described based on a single venous blood sample collection. All blood samples are handled in a Class 2 biosafety hood.

1. Collect 2 mL of blood in a 2-mL K2-EDTA blood collection tube and gently mix.
2. Dilute the blood 1:1 with PBS (2-mL blood and 2-mL PBS).
3. Add the same volume (4 mL) of Ficoll-Paque PLUS solution in a 15-mL tube.
4. Layer the 4-mL blood/PBS mixed solution onto the surface of Ficoll-Paque PLUS solution gently.
5. Centrifuge for 30 min at 400×*g* (acceleration=2, brake=0), 20 °C.
6. After the centrifugation, the sample separates into two phases: a top phase of diluted plasma and a bottom phase of the Ficoll-Paque PLUS containing erythrocytes. Transfer the interface containing lymphocytes into a 15-mL tube.
7. Wash twice with 10-mL cold PBS (10 min, 350×*g*, 6 °C).
8. Resuspend cells in PBS and take an aliquot for counting.

3.2 Cell Staining

1. Transfer 1×10^6 cells to one well of a 96-well U-bottom plate (*see* **Note 1**).
2. Prepare the staining antibody mixture in FACS buffer as in Table 1 or a modified staining panel (*see* **Notes 2–4**).
3. Centrifuge the plate (4 min, 300×*g*, 6 °C) to pellet the cells. Discard supernatant carefully.
4. Add 50uL of the staining antibody mixture to each well and mix with cells by gentle pipetting.
5. Incubate for 30 min at room temperature. Cover the plate with foil to minimize exposure to light and prevent evaporation.
6. Wash cells twice with FACS buffer (3 min, 350×*g*, 6 °C).
7. Filter cells with 70-μm nylon mesh into 5-mL FACS tubes before data acquisition.

3.3 Data Acquisition and Analysis

1. Prepare compensation controls for each fluorochrome. We use BD CompBeads but PBMCs can also be used for this purpose.
2. After data acquisition on the flow cytometer, the raw data will be saved and exported under the format FCS 3.0.
3. Raw data is imported into FlowJo or the analysis software of your choice. The compensation is checked and adjusted manually if necessary (*see* **Note 5**).

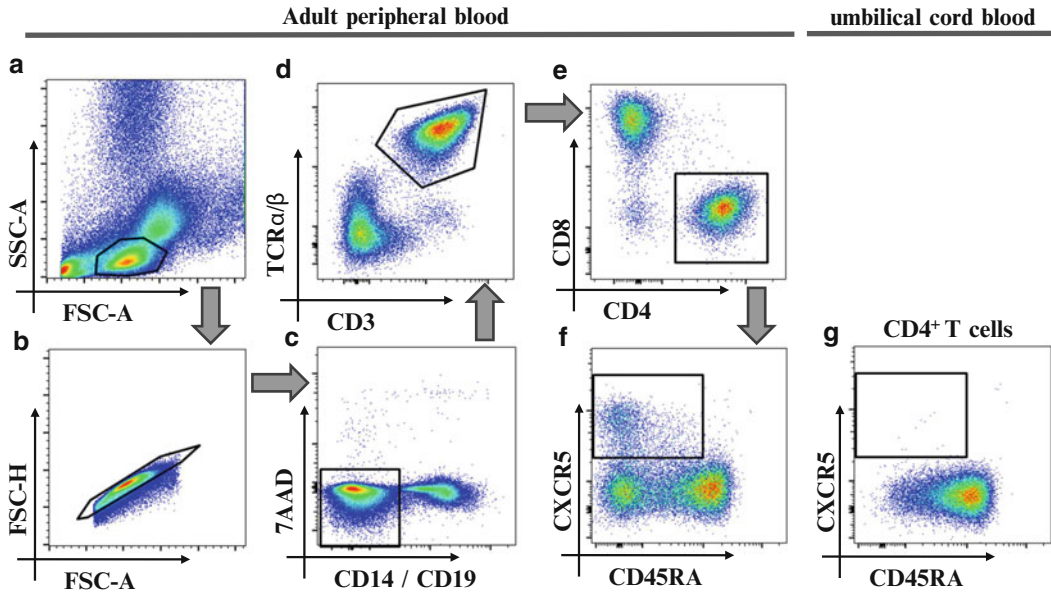

Fig. 1 The standard gating for CXCR5$^+$ CD4$^+$ T cells in human blood. (**a**) Forward scatter (FSC) and side scatter (SSC) are used for the lymphocyte gating. (**b**) FSC-A and FSC-H are used to discriminate cell aggregates from single cells. (**c**) 7AAD and CD14/CD19 are used to exclude nonviable cells and B cells and monocytes. (**d**) CD3 and TCRα/β are used to gate α/β T cells. (**e**) CD4$^+$ T cells. (**f**) CXCR5$^+$ CD4$^+$ T cells downregulate CD45RA. (**g**) CXCR5$^+$ CD4$^+$ T cells are absent in umbilical cord blood

4. Figure 1 shows the gating strategy for CXCR5$^+$ CD4$^+$ T cells, 7-AAD$^-$CD14$^-$CD19$^-$/CD3$^+$TCRα/β$^+$/CD8$^-$CD4$^+$/CXCR5$^+$CD45RA^{low}. Forward scatter (FSC) and side scatter (SSC) are used for the lymphocyte gating. FSC-A and FSC-H are used to discriminate cell aggregates from single cells. 7AAD and CD14/CD19 are used to exclude nonviable cells and B cells and monocytes. CD3 and TCRα/β are used to gate α/β T cells. CXCR5$^+$ CD4$^+$ T cells downregulate CD45RA expression. CXCR5$^+$ CD4$^+$ T cells are present in adult peripheral blood but absent in umbilical cord blood.
5. Figure 2 shows the gating strategy for circulating Tfh and Tfr populations based on CD25 and CD127. Circulating Tfr cells are CD25^{high}CD127^{low} consistent with the previously published phenotype for identification of Treg cells without the requirement for intracellular Foxp3 staining [15, 16]. Circulating Tfh cells express heterogeneous amount of CD25 (negative to low) and CD127 (low to high).
6. Figure 3 shows the gating strategy for CCR7^{low}PD-1^{high} precursor Tfh cells (or effector memory Tfh cells) and CCR7^{high}PD-1^{low} central memory Tfh cells. The naïve CD4$^+$ T cells (CD45RA^{high}CXCR5$^-$) are CCR7^{high}PD-1^{low}, which can help to set up the gating. High percentages of CCR7^{low}PD-

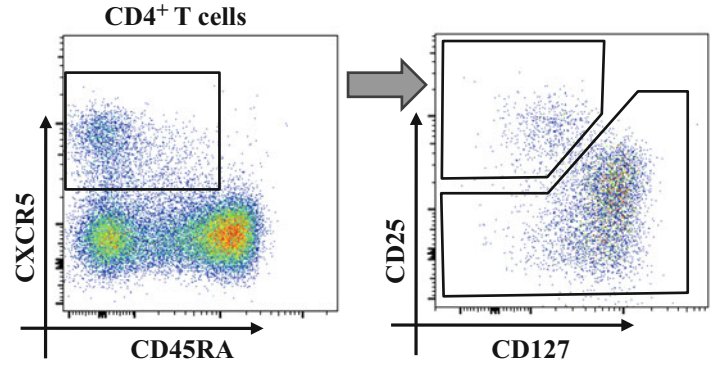

Fig. 2 The standard gating for Tfh and Tfr cells within CXCR5^{+} CD4^{+} T cells in human blood. Within CXCR5^{+} CD4^{+} T cells in blood, Tfr cells in blood are CD25^{high}CD127^{low}, and Tfh cells express heterogeneous amount of CD25 (negative to low) and CD127 (low to high)

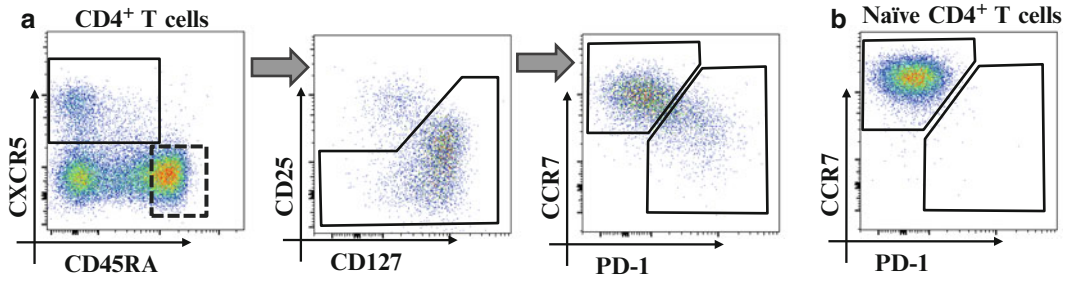

Fig. 3 The standard gating for precursor Tfh cells (or effector memory Tfh cells) and central memory Tfh cells. (**a**) Within circulating T cells in blood, precursor Tfh cells (or effector memory Tfh cells) are CCR7^{low}PD-1^{high}, and central memory Tfh cells are CCR7^{high}PD-1^{low}. (**b**) CD45RA^{high}CXCR5^{-} naïve CD4^{+} T cells, as shown in *dotted line square* in (**a**), are CCR7^{high}PD-1^{low}

1^{high} precursor/effector memory Tfh cells indicate active Tfh cell differentiation (*see* **Note 6**).

7. Figure 4 shows the gating strategy for Th1-type (CXCR3^{+}), Th2-type (CXCR3^{-}CCR6^{-}), Th17-type (CCR6^{+}), and Th1-/Th17-type (CXCR3^{+}CCR6^{+}) Tfh cells.

4 Notes

1. *The use of fresh vs. frozen samples.* The expression of many chemokine receptors, particularly CXCR5, is affected by the freeze/thaw procedure, based on our experience. We therefore highly recommend analyzing fresh samples. If a freeze/thaw procedure is unavoidable, post-thaw viability >90 % is required to produce reliable results. For the comparability among

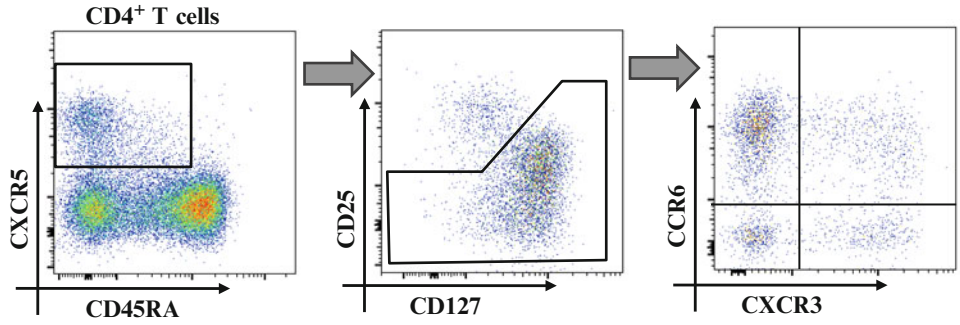

Fig. 4 The standard gating for Th1-type ($CXCR3^+$), Th2-type ($CXCR3^-CCR6^-$), Th17-type ($CCR6^+$), and Th1-/Th17-type ($CXCR3^+CCR6^+$) Tfh cells

samples, procedures among samples should be kept consistent within a single experiment.

2. *Antibody selection, titration, and panel design.* The design and validation of an antibody panel is essential for the reliability and consistency of the experiment. The antibodies selected need to be titrated to determine the optimal working concentration. Among the markers in the panel (Table 1), we recommend to choose bright fluorochromes for CXCR5, CD25, CD127, CCR7, and PD-1. This is because the expression of CXCR5 and PD-1 is relatively low on $CD4^+$ T cells and optimal staining is required to stratify subpopulations of circulating Tfh and Tfr cells. The brightness index or stain index of a fluorochrome conjugation is determined by its fluorescence intensity above the background. PE, PE-CF594, PE-Cy5, PE-Cy7, APC, Alexa Fluor 647, Brilliant Violet 421, Brilliant Violet 650, and Brilliant Violet 711 are commonly considered bright fluorochromes.
3. *A minimum antibody panel for circulating Tfh and Tfr populations.* Here we present a standard protocol with an antibody panel occupying 14 fluorescent channels. If the number of fluorescent channels is limited, we recommend a minimum of five markers composed of CD3, CD4, CXCR5, CD25, and CD127 to determine circulating Tfh and Tfr populations in human blood samples.
4. *Choosing PD-1 vs. ICOS.* Both PD-1 and ICOS are commonly used markers for mature Tfh cells in secondary lymphoid organs [1] and circulating Tfh cells in blood [5, 11, 13, 14]. Both PD-1 and ICOS expression can be used as an indicator for active Tfh cell differentiation (Fig. 3). $PD\text{-}1^+$ $CXCR5^+$ $CD4^+$ T cells are composed of $ICOS^-$and $ICOS^+$ cells [5]. There is a suggestion that the $ICOS^+$ subset is more activated than the $ICOS^-$subset [17], but this needs to be confirmed by further investigation.

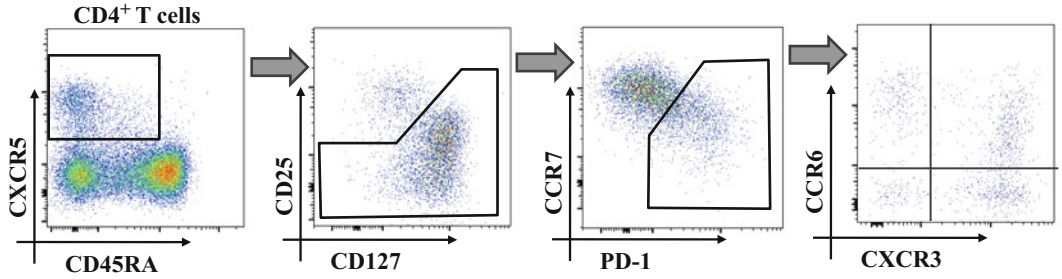

Fig. 5 The standard gating for Th1-type (CXCR3$^+$), Th2-type (CXCR3$^-$CCR6$^-$), Th17-type (CCR6$^+$), and Th1-/Th17-type (CXCR3$^+$CCR6$^+$) subsets within precursor/effector memory Tfh cells

5. *Using the "Change Axis" function in FlowJo*. We find that the "Change Axis" function in FlowJo (v10) is a very useful tool to enhance separation. By adjusting "maximum rage" and "transforms" of an axis, the separation between populations can be optimized.
6. *Combined measurement of both activity and polarization of Tfh differentiation*. High percentages of CCR7^{low}PD-1^{high} precursor Tfh cells (or effector memory Tfh cells) (Fig. 3) indicate active Tfh cell differentiation [5], whereas the expression of CXCR3 and CCR6 (Fig. 4) indicates the polarization of Tfh cell differentiation [12] (please see more discussion in this review [6]). A combined measurement of both activity and polarization of Tfh cell differentiation has been used in several studies [13, 14]. The gating strategy for the combined analysis is shown in Fig. 5.

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Chapter 18

Quantifying Helper Cell Function of Human TFH Cells In Vitro

Nina Chevalier

Abstract

Blood-circulating CXCR5$^+$ CD4$^+$ T cells and T follicular helper (TFH) cells, which participate in germinal center (GC) reactions within secondary lymphoid organs, are specialized in providing help to B cells. This chapter describes ways to isolate TFH-like cells out of peripheral blood or tonsils and to quantify their B cell helper function. This comprises different co-culture approaches of TFH-like cells and B cells and the evaluation of their capacity to induce immunoglobulin secretion and plasma cell differentiation. In addition, B cell helper function of CD4 T cells can be estimated indirectly by quantifying the expression of B cell helper cytokines and co-stimulatory and TFH-associated molecules.

Key words Antibody secretion, B cell helper cytokines, Co-stimulatory molecules, CXCR5, ELISA, Human TFH-like cells, Human TFH cells, Immunoglobulin secretion, Plasma cell differentiation, T:B co-culture

1 Introduction

Blood TFH-like cells share some phenotypic and functional properties with *bona fide* TFH cells found in secondary lymphoid organs [1–10].

Human TFH cells within secondary lymphoid tissues are identified by high expression of CXCR5 (C-X-C motif receptor 5), ICOS (inducible T cell co-stimulator), and PD-1 (programmed cell death 1) [11–13], while blood-circulating memory TFH-like cells display only moderate but stable expression of the TFH signature molecules CXCR5 and PD-1 [1, 3, 4, 7–10]. Most circulating CXCR5$^+$ TFH cells are identified as CCR7$^+$CD45RO$^+$ central memory CD4$^+$ T cells [1, 9]. Circulating TFH-like cells can be further divided into functionally distinct subsets based on the differential expression of the chemokine receptors CXCR3 and CCR6 [5] as well as PD-1 and ICOS [3, 4].

To quantitate the B cell helper function of TFH-like cells or other CD4$^+$ T cell subsets, their ability to trigger immunoglobulin

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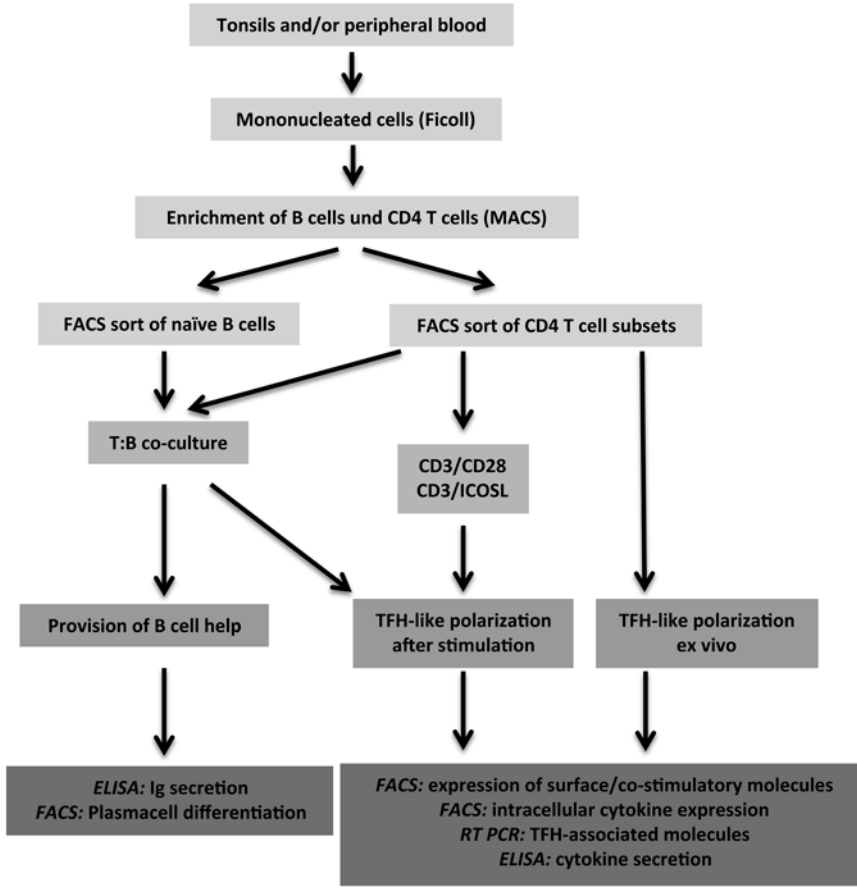

Fig. 1 Flow chart and overview of experiments to quantify B cell helper function of human TFH-like cells in vitro

secretion and plasma cell differentiation when cultured with B cells can be assessed in vitro. In addition, the expression of B cell helper cytokines, co-stimulatory molecules, and/or TFH-like characteristics can be used as indirect means to estimate the B cell helper function of $CD4^+$ T cell subsets (Fig. 1). Since most of the described methods require highly purified cell subsets, this chapter also comprises strategies for isolation of TFH-like cells and B cells from both tonsils and peripheral blood.

The isolation of highly pure TFH-like cells or $CD4^+$ T cell subsets and B cells is typically achieved in three steps that comprise (1) the extraction of mononucleated cells from blood or tonsils using density gradient centrifugation (preceded by tissue digestion for tonsils), (2) the enrichment of $CD4^+$ T or B cells by magnetic cell separation, and (3) the purification of TFH-like cells or subsets and (naïve) B cells by FACS (fluorescence-activated cell sorter). Generally, for magnetic separation of cells, different commercially

available kits can be used, following the manufacturer's instructions. The protocol described in this chapter uses the MACS® technology and is largely adapted from the manufacturer's instructions (Miltenyi Biotec).

There are different ways to co-culture B and T cells, and this chapter details three different approaches. In allogeneic co-cultures, B and T cells to be cultured are isolated from different donors. In these cultures no additional stimulators are needed as the foreign histocompatibility antigen stimulates T cells. When autologous B and T cells from the same donor are cultured, additional stimuli are required. This chapter describes (a) nonspecific T cell activation by T cell receptor (TCR) cross-linking through addition of bead particles loaded with antibodies against human CD2/CD3/CD28 and (b) high-avidity T cell activation through stimulation of superantigen-reactive T cells by bacterial toxins. Bacterial toxins that are often referred to as superantigens can bind to MHC (major histocompatibility complex) class II molecules and induce a vigorous stimulation of T cells via particular variable (V) TCR gene segments. This chapter describes the isolation and stimulation of $Vb2^+$ T cells that are responsive to TSST-1 (toxic shock syndrome toxin 1).

For the quantification of the B cell helper function, B and T cells are usually cultured for 5–7 days. Thereafter, culture supernatants are collected for detection of immunoglobulin secretion by ELISA (enzyme-linked immunosorbent assay), and cultured B cells can be examined by flow cytometry for plasma cell differentiation.

The expression of B cell helper cytokines or TFH-associated molecules can be used as indirect means to quantify the helper function of TFH-like cells or other CD4 T cell subsets. Such TFH-like characteristics can typically comprise the expression of B helper cytokines IL-21, IL-10, and IL-4 or the expression of co-stimulatory CD40L or ICOS that is important for B cell activation [11–13]. Further characteristics displayed by human TFH cells are high expression of the transcription factor Bcl-6, the chemokine CXCL13, and the surface molecule CD57 [11–13].

Generally, T cells can be examined in different activation states—resting versus activated—for the preferential expression of these B cell helper or TFH-associated molecules. When examined in a resting state, increased expression of TFH-like molecules may indicate a potential polarization or commitment of a T cell subset towards the TFH lineage. On the other hand it needs to be considered that the true relevance of a T cell subset may only become evident after activation and differentiation and with adoption of effector functions. It is therefore meaningful to compare the B cell helper phenotype of a T cell subset in a resting state and after activation. Activation of CD4 T cell subsets in vitro can be achieved in different ways. The most common way is $CD4^+$ T cell stimulation

using plate-bound CD3/CD28. Considering the importance of ICOS co-stimulation in GC reactions and high-affinity antibody responses, it is also interesting and useful to examine the expression of TFH-like molecules after stimulation of $CD4^+$ T cells on plate-bound CD3/ICOL and/or after co-culture with B cells with which TFH-like cells are thought to preferentially interact. The length of stimulation should depend on the physiological question to be answered. Generally, if starting from naïve T cells, stimulation for a period of 5 days or more may be necessary to allow for a differentiation into one or the other Th lineage. When starting from memory T cells, a more rapid development of the effector response is to be expected due to a polarization to a defined effector lineage. In this case it may be meaningful to examine expression of TFH-like molecules already as early as 24 or 48 h after the initiation of stimulation. Generally, expression of TFH-like molecules can be detected by different means including flow cytometry, ELISA, and real-time PCR.

2 Materials

2.1 Isolation of Mononucleated Cells from Blood or Tonsils

1. Density gradient solution, for example, Ficoll-Paque. Store at room temperature and protected from light.
2. RPMI 1640 (HEPES, no glutamine): Store at 4 °C and protected from light.
3. Digestion buffer: Complete medium (please *see* Subheading 2.4), 2 mg/ml collagenase D, 0.05 mg/ml DNAse. Prepare freshly before use; do not store.
4. Cell strainers.
5. 50 ml conical tubes for cell processing.
6. Sterile compresses.
7. 0.9 % sterile saline solution.
8. Petri dishes.
9. 3 ml syringes.

2.2 Magnetic Cell Separation of $CD4^+$ T Cells and B Cells

1. MACS buffer: PBS, 2 % fetal bovine serum (FBS), 2 mM EDTA. Store at 4 °C and keep sterile.
2. $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). Store at 4 °C and protected from light.
3. CD19 microbeads (Miltenyi Biotec). Store at 4 °C and protected from light.
4. MACS LS columns (Miltenyi Biotec).
5. MACS separator (Miltenyi Biotec).

2.3 Flow Cytometric Sorting of TFH-Like Cells and B Cells

1. Antibodies used for B cell sorting: CD19, CD20, CD27, CD3, CD14.
2. Antibodies used for CD4 T cell sorting: CD4, CD45RO or CD45RA, CXCR5, CCR7, ICOS, CCR6, CXCR3, Vb2, CD19, CD14, CD8, CD25.
3. Polychromatic flow sorter.

2.4 Co-culture of B and T Cells

1. Complete medium: RPMI 1640 with HEPES, without glutamine, 2 mM glutamine, 1 % (v/v) nonessential amino acids, 1 % (v/v) sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml kanamycin, and 10 % (v/v) FBS. Store at 4 °C and protected from light. For cell culture, we usually do not use medium older than 2 weeks.
2. 96 well U-bottom plates.
3. TSST-1 (Toxin Technology): Reconstitute in sterile PBS and store at −80 °C.
4. CD2/CD3/CD28 T cell activation and expansion beads. Store at 4 °C and protected from light.
5. Phorbol 12,13-dibutyrate.

2.5 ELISA from Culture Supernatants

1. 96 or 384 well flat bottom plates with high protein-binding capacity.
2. Coating buffer: 2.86 g/l Na_2CO_3, 7.56 g/l $NaHCO_3$, pH 9.5. Prepare using ultrapure water and store at 4 °C.
3. Washing buffer: PBS, 0.05 % (v/v) Tween. Store at room temperature.
4. Blocking buffer: PBS, 10 % (v/v) FBS. Store at 4 °C and keep sterile.
5. Dilution buffer for immunoglobulin ELISA: PBS, 1 % (w/v) BSA. Store at 4 °C and keep sterile.
6. Coating antibodies for immunoglobulin ELISA: Goat antihuman IgG-UNLB, goat antihuman IgM-UNLB, goat antihuman IgA-UNLB. Concentrations may vary between suppliers; refer to their recommendation. Store at 4 °C.
7. Antibodies for detection: Goat antihuman IgG-Biotin, goat antihuman IgM-biotin, goat antihuman IgA-biotin. Concentrations may vary between suppliers; refer to their recommendation. Store at 4 °C.
8. Human immunoglobulin standard: Reconstitute in sterile PBS and store at −20 °C.
9. Cytokine ELISA kits for CXCL13, IL-10, IFN-γ, IL-17, and IL-4.
10. Streptavidin-HRP (horseradish peroxidase) conjugate.

11. 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate solution. Store at 4 °C.
12. 2 M sulfuric acid as stop solution. Prepare using ultrapure water and store at room temperature.
13. ELISA reader.

2.6 FACS Analysis

1. 96-Well U-bottom plates.
2. FACS buffer: PBS, 2 % FBS, 0.05 % sodium azide. Store at 4 °C.
3. Medium for restimulation of cells for intracellular cytokine staining: Complete medium, 50 ng/ml PMA (phorbol-12-myristat-13-acetate), 1 μg/ml ionomycin, brefeldin A (diluted to 1×, from 1,000), monensin (diluted to 1×, from 1,000). Prepare fresh prior to use.
4. Antibodies used for the detection of plasma cell differentiation: CD20, CD38.
5. Antibodies used for the detection of intracellular cytokines: IL-10, IL-4, IL-21, IL-17, IFN-γ.
6. Antibodies used for the detection of co-stimulatory molecules and TFH-associated surface molecules: ICOS, CD40L, PD1, CCR7, CD57.
7. Fixation/permeabilization kit of choice: This protocol describes the use of the BD Cytofix/Cytoperm kit from BD Biosciences.

2.7 Plate-Bound Stimulation of CD4 T Cells

1. 96 well flat bottom plates with high protein-binding capacity.
2. Anti-CD3 (clone TR66, 2 μg/ml). Store at 4 °C and keep sterile.
3. Anti-CD28 (2 μg/ml; clone 28.2). Store at 4 °C and keep sterile.
4. Recombinant human ICOS ligand (ICOS-L) (B7-H2/Fc chimera (5 μg/ml). Reconstitute in sterile PBS and store at −80 °C.

3 Methods

Cell isolation and culture are performed under sterile conditions.

3.1 Purification of TFH-Like Cells and B Cells

3.1.1 Isolation of Mononucleated Cells out of Peripheral Blood or Buffy Coats

1. Collect anticoagulated blood (or buffy coats) and store at room temperature until further processing (*see* **Notes 1** and **2**).
2. Before Ficoll-Paque gradient preparation, bring reagents to room temperature and mix peripheral blood 1: 1 with RPMI1640 (*see* **Note 3**).
3. Carefully layer 30 ml of diluted blood onto 15 ml Ficoll-Paque in a 50 ml conical tube.

4. Spin the gradient for 30 min at 20 °C, 400 × *g* and with no brake (*see* **Note 4**).
5. After centrifugation remove the upper plasma and platelets containing layer using a clean Pasteur pipette and leaving the lymphocyte layer undisturbed.
6. Collect the lymphocyte layer using a clean Pasteur pipette and transfer into a clean 50 ml conical tube (*see* **Note 5**).
7. Wash cells in 3 volumes of RPMI1640 and spin at 400 × *g* and room temperature for 10 min.
8. Wash cells in 3 volumes of MACS buffer and spin at 400 × *g* for 5 min at 4 °C.
9. Resuspend in MACS buffer, filter over 70 μm cell strainer, and store on ice for magnetic cell separation (*see* **Notes 6** and 7).

3.1.2 Isolation of Mononucleated Cells out of Tonsils

1. Wrap surgically removed tonsils into sterile compresses soaked with 0.9 % sterile saline and transfer into a sterile 50 ml conical tube which is immediately stored on ice.
2. Rinse tonsils with sterile PBS and transfer on a sterile Petri dish.
3. Mince tonsils into little pieces and transfer into a fresh 50 ml conical tube containing 10 ml of pre-warmed digestion buffer.
4. Incubate minced tonsil pieces for around 45 min at 37 °C under continuous rotation (e.g., in a MACSmix Tube Rotator).
5. After incubation, pour minced tonsil pieces in digestion medium into a cell strainer on a sterile Petri Dish.
6. Gently dissociate tonsil pieces with the stamp of a sterile 3 ml syringe to retrieve further cells.
7. Remove cell strainer containing organ debris from the Petri dish and rinse with complete medium to collect adherent cells.
8. Break clustered cells and generate single-cell suspension by pipetting digested cells up and down.
9. Transfer over a sterile 70 μm filter mesh or 70 μm cell strainer into a fresh 50 ml conical tube.
10. Wash single-cell suspension by addition of one volume of complete medium and centrifugation at 400 × *g* for 10 min at room temperature.
11. Resuspend cells in 30 ml of RPMI1640 and carefully layer onto 15 ml Ficoll-Paque in a 50 ml conical tube.
12. Centrifuge gradient for 30 min at 20 °C, 400 × *g* and with no brake (*see* **Note 4**).
13. After centrifugation remove the upper layer using a clean Pasteur pipette and leaving the lymphocyte layer undisturbed.
14. Collect the lymphocyte layer using a clean Pasteur pipette and transfer into a clean centrifuge tube.

15. Wash cells twice in 3 volumes of MACS buffer and spin at 400 × *g* for 5 min at 4 °C.
16. Resuspend in MACS buffer, filter over 70 μm mesh, and store on ice for magnetic cell separation (*see* **Notes 6** and **7**).

3.1.3 Magnetic Cell Separation and FACS Sort: Preparations and Strategy

1. Precool reagents and buffers (*see* **Note 8**).
2. Conditions for magnetic cell separation are largely adapted from the manufacturer's instructions ($CD4^+$ T Cell Isolation Kit II and CD19 microbeads, Miltenyi Biotec) and are outlined for 10^7 cells. When working with more cells, reagents and MACS buffer need to be scaled up accordingly (*see* **Note 9**).
3. *Strategy*: If B and T cells are isolated from the same donor, decide on one of the following two strategies (Fig. 2):
   (a) With a sufficient amount of mononucleated cells, part of the cells can be used for isolation of T cells and the other part for simultaneous isolation of B cells, which saves time (Fig. 2a).
   (b) Alternatively, B and T cells can be isolated consecutively in two steps—positive selection of B cells followed by negative selection of T cells—which takes more time, but requires less mononucleated cells to start with (Fig. 2b).

3.1.4 Magnetic Cell Separation of CD4 T Cells and Sort of TFH-Like Cell Subsets

1. Resuspend 10^7 cells in 15 ml conical tube in 40 μl of MACS buffer.
2. Add 10 μl of biotin-antibody cocktail.

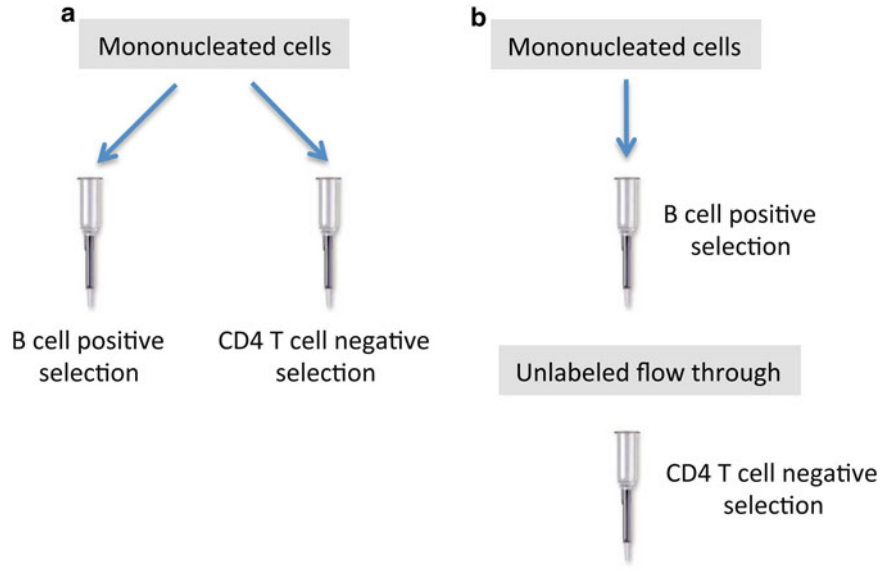

Fig. 2 Strategies of magnetic bead separation for isolation of B or T cells from one donor. (**a**) Simultaneous isolation of B and T cells. (**b**) Consecutive isolation of B and T cells

3. Mix well by pipetting up and down and incubate for 20 min on ice.
4. Add 30 µl of MACS buffer.
5. Add 20 µl of anti-biotin microbeads.
6. Mix well by pipetting up and down and incubate for an additional 30 min on ice.
7. Wash cells by adding 10× labeling volume of MACS buffer, centrifuge at 400 × *g* for 5 min at 4 °C, and remove supernatant completely.
8. Resuspend up to 10^8 cells in 500 µl of MACS buffer.
9. Magnetic separation: Place LS column in the magnetic field of a suitable MACS separator and rinse column with 3 ml MACS buffer. Apply resuspended cells over filter onto the column that has been equilibrated with MACS buffer. Allow the cells to pass through the column by gravity and collect the flow-through fraction containing the unlabeled enriched $CD4^+$ T cell fraction (*see* **Note 10**). Wash column 3× with 3 ml of cooled MACS buffer and collect the entire effluent in the same tube containing the unlabeled enriched $CD4^+$ T cell fraction.
10. Count cells.
11. Spin cells at 400 × *g* for 5 min at 4 °C.
12. Resuspend 10^7 cells in 500 µl MACS buffer.
13. For FACS sorting stain cells with the respective antibody combinations for 30 min on ice (*see* Table 1). If possible, stain

Table 1
Typical staining used for isolation of TFH-like cells and subsets

	Typically sorted populations	Ref.
Bona fide TFH cells (tonsil)	$CD4^+CD45RO^+CXCR5hiICOShi$	[2]
$CXCR5^+$ central memory T cells (blood)	$CD4^+CD45RO^+CCR7^+CXCR5^+$	[1]
$Vb2^+$ $CXCR5^+$ central memory T cells (blood)	$Vb2^+CD4^+CD45RO^+CCR7^+CXCR5^+$	[1]
TFH-like cell subsets based on CXCR3 and CCR6 expression (blood)	$CD4^+CXCR5^+CCR6^+CXCR3^+$ $CD4^+CXCR5^+CCR6^-CXCR3^+$ $CD4^+CXCR5^+CCR6^+CXCR3^-$ $CD4^+CXCR5^+CCR6^-CXCR3^-$	[5]
TFH-like cell subsets based on CXCR3 and PD1 expression (blood)	$CD4^+CD45RA^-CXCR5^+CXCR3^+PD1^+$ $CD4^+CD45RA^-CXCR5^+CXCR3^-PD1^+$ $CD4^+CD45RA^-CXCR5^+CXCR3^+PD1^-$ $CD4^+CD45RA^-CXCR5^+CXCR3^-PD1^-$	[4]
TFH-like cell subsets based on CCR7 and PD1 expression (blood)	$CD4^+CD45RA^-CXCR5^+CCR7hiPD1lo$ $CD4^+CD45RA^-CXCR5^+CCR7loPD1hi$	[3]

CD19, CD14, CD8, and CD25 to exclude B cells, monocytes, CD8 T cells, and regulatory T cells (cells to be depleted can in principle be stained with the same fluorochrome).

14. Wash cells twice between the stains by adding 10–20× labeling volume of MACS buffer and centrifugation at 400 × *g* for 5 min at 4 °C.
15. For FACS sorting, resuspend cells in MACS buffer at a concentration of 10^7 cells per ml.
16. Sort cells under sterile conditions and collect sorted cells in complete medium (*see* **Note 11** and Table 1).

3.1.5 Magnetic Cell Separation and Flow Cytometric Sorting of B Cells for Culture with CD4 T Cells

1. Resuspend 10^7 cells in a 15 ml conical tube in 80 µl of MACS buffer.
2. Add 20 µl of CD19 microbeads.
3. Mix well by pipetting up and down and incubate for 20 min on ice.
4. Wash cells by adding 10× labeling volume and centrifuge at 400 × *g* for 5 min at 4 °C and remove supernatant completely.
5. Magnetic separation: Place LS column in the magnetic field of a suitable MACS separator and rinse column with 3 ml of MACS buffer. Apply resuspended cells onto the prepared column. Allow the cells to pass through the column by gravity. Wash column 3× with 3 ml of cooled MACS buffer. Remove column from the separator and place it on a 15 ml conical collection tube. Pipette 5 ml of MACS buffer onto the column and immediately flush out the magnetically labeled $CD19^+$ cells by firmly pushing the plunger into the column.
6. For flow cytometric cell sorting, proceed as outlined above. Naïve B cells are sorted as $CD3^-CD14^-CD19^+CD20^+CD27^-$ cells (*see* **Note 11**).

3.2 Quantification of the Provision of B Cell Help by CD4 T Cells

3.2.1 Co-culture of B and T Cells

1. Incubate T and B cells at a ratio of 1:1 (usually 50,000 cells each) in complete medium at a final volume of 200 µl, in 96 well U-bottom plates (*see* **Notes 12–14**). Culture in triplicate.
2. For allogeneic T:B co-cultures, no addition of stimuli is required (*see* **Note 15**).
3. For autologous T:B co-cultures, additional stimuli need to be added to induce activation of T cells: (1) bead particles loaded with antibodies against human CD2/CD3/CD28 (bead-to-cell ratio 2:1) or (2) superantigen TSST-1 (between 10 and 100 ng/ml) when TSST-1-responsive $Vb2^+$ T cells are cultured with B cells (*see* **Note 15** and Table 2).
4. Culture cells for a minimum of 5–7 days at 37 °C, 5 % CO_2, and 100 % humidity (*see* **Note 16**).

Table 2
Overview of the different T:B co-culture conditions

	B cells	CD4 T cells	Stimulus
Allogeneic	Naïve B cells (donor 1)	TFH-like subsets (donor 2)	Not required
Autologous	Naïve B cells (donor 1)	TFH-like subsets (donor 1)	CD3/CD28/CD2-loaded beads
Autologous high-avidity stimulation	Naïve B cells (donor 1)	$Vb2^+$ TFH-like subsets (donor 1)	TSST-1

Table 3
Minimal volumes used when working with 96 or 384 well ELISA plates

	96 well plate (μl)	384well plate (μl)
Coating antibodies	50	10
Blocking	250	100
(Diluted) substrate/standards	50	10
Detection antibodies	50	10
Detection with TMB	50	20
Stop solution	50	20

5. Thereafter, collect culture supernatants for quantification of secreted Ig by ELISA (*see* **Note 17**).
6. Collect cultured cells for further readouts (plasma cell differentiation of B cells and/or phenotype of T cells).

3.2.2 Immunoglobulin ELISA from Culture Supernatants

1. Coat high protein-binding 96 or 384 well ELISA plates with the respective coating antibodies in coating buffer (*see* **Note 18** and Table 3).
2. Seal and incubate over night at 4 °C.
3. Remove coating antibodies in buffer from the ELISA plate.
4. Add blocking solution directly (no washing required at this step).
5. Seal and incubate for 1 h at room temperature.
6. Wash plates 3× with ELISA washing buffer.
7. Add samples and standards, made up in the respective concentrations in dilution buffer, to the plates (*see* **Note 19**).
8. Seal and incubate for 2 h at room temperature.
9. Wash plates 3× with ELISA washing buffer.

10. Add biotinylated detection antibodies in the respective dilution. Incubate for 2 h at room temperature.
11. Wash plates 3× with ELISA washing buffer.
12. Add streptavidin-HRP conjugate in the respective dilution. Incubate for 1 h at room temperature.
13. Wash plates 5× with ELISA washing buffer.
14. Add 100 µl TMB solution and observe color change.
15. When solution turns dark blue, add 50 µl 2 M sulfuric acid stop solution to quench the enzymatic reaction.
16. Read the plate on an ELISA plate reader at 450 nm (Table 3).

3.2.3 FACS Analysis for Plasma Cell Differentiation

1. After cell collection, perform all steps on ice in U-bottom culture plates.
2. Wash cells 2× with FACS buffer: Add 200 µl of FACS buffer, spin for 2 min at 4 °C and 350 × *g*, and remove supernatants after cell sedimentation.
3. For staining, resuspend cells in 50 µl FACS buffer containing antibodies against CD19, CD38, CD20, and CD3.
4. Stain on ice for 30 min.
5. Wash cells three times as described in **step 2** above.
6. Resuspend cells in around 200 µl FACS buffer for FACS analysis.
7. Plasma cells are detected as $CD3^{-}CD19^{+}CD20^{-}CD38^{hi}$ cells.

3.3 Examination of CD4 T Cells Ex Vivo for Expression of a TFH-Like Phenotype

3.3.1 Expression of Surface Molecules by FACS

1. Seed 100,000–250,000 purified cells in U-bottom plates (*see* **Note 20**).
2. Wash cells 2× with FACS buffer as outlined in Subheading 3.2.
3. Resuspend cells in 50 µl of FACS buffer containing the respective antibody combinations (Table 4).
4. Incubate on ice for 30 min.
5. Wash cells 3× with FACS buffer as outlined in Subheading 3.2.
6. Resuspend cells in 200–300 µl of FACS buffer and perform flow cytometry analysis.

3.3.2 Intracellular Cytokine Expression by FACS

1. Seed 100,000–250,000 purified cells in U-bottom plates.
2. Wash cells 2× with complete medium as outlined in Subheading 3.2. (*see* **Note 21**).
3. Resuspend cells in 200 µl complete medium containing PMA (50 ng/ml), ionomycin (1 µg/ml), brefeldin A (1×, stock 1,000×), and monensin (1×, stock 1,000×).
4. Incubate for 4–5 h at 37 °C.
5. Wash cells 2× with FACS buffer as outlined in Subheading 3.2.

Table 4
Overview on read outs and typical TFH-associated molecules

Read out	TFH-associated molecules
Co-stimulatory and TFH-associated surface molecules *(FACS)*	ICOS, CD40L, PD1, CCR7, PD1
Intra-cellular cytokines *(FACS)*	IL-4, IL-10, IL-21
TFH-associated molecules *(RT PCR)*	BcL6, CXCL13, c-Maf, IL-21
Cytokine secretion *(ELISA)*	IL-4, IL-10, IL-21

6. Perform surface stains as outlined before (Subheading 3.3. expression of surface molecules by flow cytometry).
7. Wash cells 3× with FACS buffer as outlined in Subheading 3.2.
8. Fix cells by resuspension in 100 µl of fixation buffer of choice, for example, BD Cytofix/Cytoperm.
9. Incubate on ice for at least 30 min (*see* **Note 22**).
10. Spin for 2 min at 400 × *g* and 4 °C and flick supernatants.
11. Permeabilize cells by washing twice with permeabilization buffer of choice, for example, BD Perm/Wash buffer.
12. Resuspend cells in 50 µl 1× BD Perm/Wash buffer containing the respectively diluted antibodies for detection of intracellular cytokines (Table 4).
13. Incubate on ice for 30 min.
14. Wash cells three times in 1× BD Perm/Wash buffer.
15. Wash cells one time with FACS buffer.
16. Resuspend cells in 200–300 µl of FACS buffer and perform flow cytometric analysis.

3.3.3 Secretion of B Cell Helper Cytokines by ELISA

1. Coat high protein-binding 96 well flat bottom plates with anti-CD3 (2 µg/ml) antibodies in 50 µl PBS.
2. Incubate at 4 °C over night.
3. Remove the anti-CD3-containing PBS.
4. Seed 100,000 purified $CD4^+$ T cells in 100 µl complete medium containing phorbol 12,13-dibutyrate (PdBu) (50nM).
5. Incubate 24 h at 37 °C and collect supernatant for cytokine ELISA (Table 4).
6. Perform cytokine ELISA according to the manufacturer's instructions (*see* **Note 23**).

3.3.4 Expression of TFH associated molecules by RT PCR

- Subject purified cells to RNA extraction, cDNA synthesis and quantitative RT PCR for *mRNA* expression of TFH-associated molecules (Table 4).

3.4 Examination of CD4 T Cells After Activation for Expression of a TFH-Like Phenotype

3.4.1 Cell Stimulation

- *T:B co-culture:* Follow the instructions as outlined in Subheading 3.2.
- *Plate-bound stimulation*
  - Coat high protein-binding 96-well flat bottom plates with anti-CD3 (2 μg/ml) and/or anti-CD28 (2 μg/ml) and/or anti-ICOS-L (5 μg/ml) antibodies in 50 μl PBS.
  - Incubate at 4 °C over night (*see* **Note 24**).
  - Remove antibody containing PBS from the tissue culture plate.
  - Seed 50,000–100,000 purified CD4 T cells in 200 μl complete medium.
  - Incubate 48–72 h at 37 °C (*see* **Note 25**).
  - To analyze the expression of surface molecules or intracellular cytokines by flow cytometry (Table 4), transfer cells from plate-bound stimulation into U-bottom plate and proceed as outlined under Subheading 3.3. Transfer is not necessary when starting from T:B co-cultures (*see* **Note 26**).

3.4.2 Secretion of B Cell Helper Cytokines by ELISA

- Collect culture supernatant and use for cytokine ELISA as outlined under Subheading 3.3 (Table 4).

3.4.3 Expression of TFH associated molecules by RT PCR

- Collect cells after culture and subject to RNA extraction, cDNA synthesis and quantitative RT PCR for *mRNA* expression of TFH-associated molecules (Table 4).
- When starting from T:B co-cultured cells, CD4 T cells need to be re-isolated as $CD4^+CD19^-$ cells by FACS sorting beforehand.

4 Notes

1. There are several types of anticoagulants, which differ in their mechanism of action. Generally, we found that use of heparin or EDTA produces equivalent results for the described assays. Nevertheless, for consistency it is advisable to stick to one anticoagulant in all experiments.
2. For reasons of practicability, blood or buffy coats may be stored over night at room temperature and processed the next day. For consistency, time between blood withdrawal and processing should be the same in all experiments.
3. Dilution of blood samples reduces trapping and sedimentation of lymphocytes in erythrocyte aggregates and increases lymphocyte yields.

4. A temperature of 18–20 °C gives optimal results in terms of lymphocyte yield; generally, higher temperatures enhance erythrocyte aggregation and lymphocyte sedimentation, whereas lower temperatures increase the time of separation.
5. To retrieve the maximum number of cells, it is critical to remove all the material at the interface but in a minimum volume. Excess supernatant results in platelet contamination and excess Ficoll-Paque in granulocyte contamination.
6. For reasons of practicability, cells may be stored over night until further processing. For overnight storage, resuspend cells in complete medium and store at 4 °C in the fridge.
7. Ideally the described experiments are performed with freshly isolated cells. However, at this stage it is also possible to freeze cells, which may result in a loss of cell viability and reduce the overall cell yield. We have successfully isolated and cultured T and B cells after freezing. To freeze cells, resuspend 10×10^6 cells in 10 % dimethyl sulfoxide/FBS and aliquot into cryovials. Viability was best when cells were frozen using a freezing container (e.g., Nalgene, Mr. Frosty) and transferred into liquid nitrogen 24–48 h later.
8. Cells should be kept cold on ice and reagents and buffers precooled to avoid unspecific staining as well as modulation and internalization of surface antigens resulting in a possible loss of fluorescence.
9. According to our experience, scaling down the amount of reagents used per 10^7 cells is possible. For negative selection this may go along with a reduced purity of enriched cells, for positive selection with a decreased cell yield. Depending on our needs we often used only ½ or ¼ of the proposed amount of buffer and reagents.
10. Make sure no more than 2×10^9 cells are applied per LS column, as cell overload or column saturation slows down or blocks the flow through of further cells, which leaves them uncooled and reduces the purity of enriched cells.
11. Both cell sorting and magnetic cell separation are stressful to cells. Although not mandatory, it is recommended to rest cells overnight before subjecting them to downstream experiments. To that end, purified cells are resuspended in complete medium at a concentration of 10^6 cells/ml and rested at 37 °C over night.
12. If cell numbers are limiting, it is possible to scale down cell numbers, e.g., to a minimum of 30,000 cells per well.
13. As negative control, culture B cells without T cells.
14. If possible spare the outer wells and fill with PBS since their evaporation is most pronounced which can influence quantification of immunoglobulin levels in culture supernatants.

15. The different co-culture conditions differ in the stringency of T cell stimulation, which can be exploited for different purposes. In our experiments, a mostly moderate stimulation was achieved in allogeneic co-cultures or in autologous co-cultures supplemented with CD2/CD3/CD28-loaded bead particles. To the contrary, a stringent T cell proliferation was achieved using TSST-1 in combination with TSST-1-responsive $Vb2^+$ T cells. In our hands, stimulation of T cells in T:B co-cultures on plate-bound CD3/CD28 mostly resulted in a too strong T cell activation often resulting in an out-proliferation of B cells by co-cultured T cells. For allogeneic co-cultures, we observed the highest degree of variability between the different donors due to differences in the allogeneic effects. Since TFH cell differentiation and B cell selection in the GC reaction involve high-affinity interactions between antigen-specific B and T cells, high-avidity stimulation of $Vb2^+$ cells by TSST-1 may reflect the most physiologic approach. However, in experiments that aim at evaluating the effects of, e.g., blocking reagents, moderate stimulation may be preferred.
16. To determine plasma cell differentiation, 6–7 days post culture is a good time point for cell harvest. Moreover, after 6–7 days of culture, B cells still display a reasonable level of fitness. Since plasma cells need additional niche factors for their long-term survival (e.g., from bone marrow stromal cells), they will soon succumb to cell death after their differentiation in vitro. If the only aim is to collect supernatant for detection of immunoglobulin secretion, 14 days is a good time point for harvest, since at this stage immunoglobulin levels are usually higher than at 7 days.
17. For practical reasons it is possible to freeze culture supernatants and to perform immunoglobulin ELISA at a later time point. If stored at room temperature or 4 °C, it is advisable to keep supernatants sterile, since bacterial contamination can lower the actual immunoglobulin amount.
18. Use of 96 and 384 well plates works equally well. 384 well plates are preferred when multiples dilutions are compared to each other and the amount of supernatants is limiting.
19. Sample dilutions usually depend on the culture conditions and need to be evaluated individually. If possible it is advisable to compare different dilutions. For the outlined co-cultures, we usually had good results with the following dilutions: IgM, 1:10–1:20; IgG, 1:3–1:6; IgA, 1:2–1:4.
20. For CD40L detection of resting cells, a short stimulation is required. We usually tend to restimulate cells with PMA and ionomycin for 1 h in the concentrations used for the detection of intracellular cytokines (*see* Subheading 2.6).

21. Do not wash cells with azide-containing FACS buffer before restimulation with PMA/ionomycin, since this requires viable and healthy cells.
22. Cells fixed in BD Cytofix/Cytoperm can be stored at 4 °C for up to 2 days until further processing. In rare cases we observed fading intensity of surface stains when using less stable fluorophores.
23. As for immunoglobulin ELISA, sample dilutions usually depend on the culture conditions and need to be evaluated individually. Therefore, if possible it is advisable to compare different dilutions. We were unable to reliably detect IL-21 by ELISA in culture supernatants of cultured T cells.
24. As an alternative to overnight incubation at 4 °C, coating can be performed for 2 h at 37 °C with equivalent outcome.
25. When culturing cells for 4–5 days or more, start from no more than 30,000 $CD4^+$ T cells since plate-bound stimulation induces strong cell proliferation, which may result in overgrowth and may require cell splitting.
26. Regardless of whether cells have been stimulated in T:B cocultures or with plate-bound CD3/CD28/ICOS-L, restimulation with a cocktail containing PMA, ionomycin, brefeldin A, and monensin is required for detection of intracellular cytokine expression.

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