Video Article

Isolation of CD4$^+$ T-cells and Analysis of Circulating T-follicular Helper (cTfh) Cell Subsets from Peripheral Blood Using 6-color Flow Cytometry

Elliot Byford[1], Matthew Carr[1], Lucia Piñon[2], Matthew J. Ahearne[1], Simon D. Wagner[1]

[1]Leicester Cancer Research Centre and Ernest and Helen Scott Haematology Research Institute, University of Leicester

Correspondence to: Simon D. Wagner at sw227@le.ac.uk

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Abstract

Aberrant T-follicular helper (Tfh) cell activity is detectable in autoimmune conditions and their presence is associated with clinical outcomes when the lymph node microenvironment in B-cell non-Hodgkin's lymphoma is analyzed. Subsets of circulating T-follicular helper cells (cTfh), the circulating memory compartment of Tfh cells in the blood, are also perturbed in disease and therefore represent potential novel predictive biomarkers. Peripheral blood-based testing is advantageous because it is relatively non-invasive and allows simple serial monitoring.This article describes a method for isolating CD4$^+$ T-cells from human blood, and further analysis by flow-cytometry to enumerate cTfh cells and the proportions of their various subsets (cTfhPD-1$^{-/+/hi}$, cTfh1,2,17 and cTfh1/17). The level of these subsets was then compared between normal subjects and patients with lymphoma. We found that the method was robust enough to obtain reliable results from routinely collected patient material. The technique we describe for the analysis can be easily adapted to cell sorting and downstream applications such as RT-PCR.

Video Link

The video component of this article can be found at https://www.jove.com/video/58431/

Introduction

T-follicular helper cells (Tfh) are a CD4$^+$ T-cell subset that was initially characterized in lymphoid tissues[1]. These cells express PD-1 and CXCR5 surface receptors, secrete IL-21 and IL-4 and show nuclear expression of the transcription factor, BCL-6[2,3]. As their name suggests, they are found in germinal centers and are essential for high affinity antibody production[1].

Dysregulated Tfh responses have been implicated in disease pathogenesis, most notably autoimmune disease, where they promote the expansion of autoreactive B cells[4]. They also play a role in the tumor microenvironment of both solid[5,6] and lymphoid cancers[7]. Conversely, genetic defects of surface proteins essential for Tfh function such as inducible T-cell costimulator (ICOS) result in human immunodeficiency syndromes[8]. CD4$^+$ CXCR5$^+$ cells in human peripheral blood are termed circulating T-follicular helper cells (cTfh) and are believed to be the memory compartment of Tfh cells in tissues[9]. The purpose of the method described here is the analysis of cTfh subsets following CD4$^+$ cell isolation from peripheral blood samples.

Several cTfh subsets have been defined and the efficiency with which they provide B-cell help differs from one subset to another[9,10,11,12]. The relative proportions of these subsets are altered in a number of diseases, most prominently autoimmune disease in which there is almost always a relative increase in the more functional PD-1$^{+/hi}$ and/or cTfh2 or cTfh17 subsets in comparison to the less functional PD-1$^-$ and/or cTfh1 subsets[12]. The extent of these changes frequently associate with clinical parameters including disease activity and autoantibody titers, indicating a potential role of cTfh subset distribution as a prognostic biomarker in disease, which may reflect the activity of Tfh in lymphoid tissues[9,12,13,14]. Additionally, taking blood samples from participants is quick, safe and acceptable, and so allows serial monitoring for the analysis of disease progression or response to therapy.

The use of isolated CD4$^+$ T-cells over traditional peripheral blood mononuclear cell (PBMNC) suspensions enables higher throughput flow cytometry experiments by reducing the time required to acquire a substantial number of cTfh cells for analysis. This is particularly helpful when sorting cells from rare cTfh subsets using flow activated cell sorting (FACS). To aid the efficiency, these suspensions can be cryopreserved to enable "batching" of samples to be used in the flow cytometry experiment. On testing, the CD4$^+$ purity was not reduced by cryopreservation.

While different laboratories used different markers to categorise cTfh cells in the early stages of their discovery, the method presented here makes use of a unified scheme of two groups of cell-surface markers as proposed by Schmidt et al.[12,15] to enable the simultaneous identification of cTfh and their nine recognized subsets in a single flow cytometry experiment.

As only cell surface markers are used, the cells do not require fixation or permeabilization, and thus can remain alive for downstream functional studies. This could be facilitated by cell sorting using FACS with the same antibody panel. This panel could be expanded to include other markers, allowing for the restrictions of the flow cytometer being used.

The analysis of multi-color flow cytometry experiments can be challenging due to the inherently subjective nature of gating on 2-dimensional dot plots, especially when cell populations do not have a clear bi-modal distribution in marker fluorescence, as is the case for cTfh cells and their subsets. For this reason, it is imperative to set up effective controls to reduce the artefacts to enable better resolution of populations and to set gating strategies confidently. As such, antibody panel design and the set-up of basic controls for a flow cytometry experiment, i.e., using compensation and FMO controls are outlined in Step 3.4.2 and 3.4.3,respectively.

All cTfh cells are defined as $CD4^+$ $CXCR5^+$ $CD45RA^-$. The level of expression of the characteristic Tfh activation marker PD-1 can then be determined to identify the subsets of PD-1^-, PD-1^+ or PD-1^{hi} cTfh cells. Then, using a combination of the chemokine receptors CXCR3 and CCR6, which are differentially expressed by traditional Th1,2 or 17 cells, cTfh can be characterized as cTfh1,2 or 17-like by a profile of $CXCR3^+$ $CCR6^-$, $CXCR3^-$ $CCR6^-$, and $CXCR3^-$ $CCR6^+$, respectively.

The antibody panel used by our laboratory is displayed in **Table 1**. The user may have to adapt their fluorophore selection to account for the laser and light filter configuration available on their local flow cytometer.

The following considerations influence the choice of fluorophores. Use bright fluorophores where possible. In particular, use the brightest available fluorophores on the dimmest (less highly expressed) markers. Dimmer markers include PD-1, CXCR3 and CCR6, and to a lesser extent, CXCR5. We specifically made use of the newer BB, BV and BUV fluorophores which provide excellent brightness and thus enable easier resolution of distinct populations of the cells.

Spread the fluorophore selection across the emission spectra as much as possible to minimize spectral overlap and thus the level of compensation required. A free, online tool that can be used to assist designing a flow cytometry panel can be found here: http://www.bdbioscienes.com/us/s/spectrumviewer. To save space on the emission spectrum, we employed a "dump channel" by using a viability dye with an emission wavelength that overlaps with that of APC-H7 (conjugated to CD45RA) to enable the detection (and exclusion) of both dead and/or $CD45RA^+$ using a single detector.

Here, a protocol is presented for the isolation of peripheral blood $CD4^+$ T-cells and their subsequent analysis by flow cytometry to determine the proportions of the different and recently described circulating subsets.

Protocol

Blood samples were obtained from normal subjects (NS) (n = 12) as well as patients with marginal zone lymphoma (MZL) (n = 7) and other types of B-cell non-Hodgkin's lymphoma (BNHL) (6 FL patients, 2 lymphoplasmacytic lymphoma patients and 1 low-grade B-cell non-Hodgkin's lymphoma not otherwise specified patient). Patients were recruited from the hematology clinics at Leicester Royal Infirmary after having given informed, written consent, with ethical approval in place for all studies. Ethical approval was obtained by Leicestershire, Northamptonshire and Rutland Research Ethics Committee 1, reference 06/Q2501/122 for patient samples and the Health Research Authority (HRA) NRES Committee East Midlands-Derby, reference 14/EM/1176 for normal subjects.

1. Isolation of $CD4^+$ T-cells from Whole Peripheral Blood

1. Take fresh peripheral blood (2 to 15 mL) into K_2EDTA tubes by standard venipuncture and process as soon as possible to maintain maximum viability.
   NOTE: Use standard laboratory personal protective equipment (coat, gloves, glasses) and carry out the work in a class II Biosafety Cabinet.
2. Bring the blood and all necessary reagents ($CD4^+$ enrichment cocktail, 2% fetal bovine serum/phosphate buffered saline (FBS/PBS), density gradient media, and freezing medium if cryopreserving cells) to room temperature.
3. Mix the blood with a commercially available cocktail of antibodies for the enrichment of human $CD4^+$ T Cells. Use 50 µL of the reagent for every 1 mL of blood. Use a 50 mL conical polypropylene tube for 5 to 15 mL of blood.
   NOTE: If using 2 to 4 mL of blood, perform this step in a 14 mL conical polypropylene tube.
4. Incubate the mixture at room temperature for 20 min.
5. Dilute the mixture with an equal volume of 2% FBS/PBS
6. **Layer this diluted mixture on top of the density gradient media slowly to avoid disturbing the interface and proceed to centrifugation immediately to avoid diffusion of the blood into the density gradient medium.**
   1. For 2 to 3 mL of blood, use 3 mL of density gradient medium in a 14 mL conical polypropylene tube, but for 4 mL of blood, use 4 mL of density gradient medium in a 14 mL conical polypropylene tube and for 5 to 15 mL blood, use 15 mL of density gradient medium in a 50 mL conical polypropylene tube.
7. Centrifuge the layered mixture for 20 min at 1,200 x *g* with the brake off at 20 °C.
   NOTE: A temperature below ambient may result in the contamination with red blood cells and granulocytes. A lower speed will cause the $CD4^+$ isolation process to fail. Leaving the break engaged will decrease cell yield. Use a bench top centrifuge with rotors that can be closed to avoid aerosols.
8. Remove the enriched $CD4^+$ cell layer from the interface using a Pasteur pipette.
   NOTE: The enriched cell layer will resemble a standard "buffy coat" of white cloudy cells, but as only $CD4^+$ cells are present, this will naturally be smaller and more difficult to see.
9. Add 2% FBS/PBS to the $CD4^+$ cells up to a total volume of 10 mL and then wash twice with 2% FBS/PBS by centrifuging for 10 min at 400 x *g* during each wash.
10. Resuspend the pellet in 2% FBS/PBS and count the cells stained with a vital dye (trypan blue) to determine the cell numbers and viability.

11. Optional step: resuspend 5 x 10^5 to 1 x 10^6 cells in freezing medium (10% dimethyl sulfoxide in FBS) in 1 mL aliquots.
NOTE: Levels of some surface markers may be altered by cryo-preservation and thawing. It is, therefore, very important that all samples are processed consistently. In order to minimize the differences in analytical variables, the samples were cryo-preserved and batched for analysis in this study.

2. Flow Cytometry

1. Thaw the cryopreserved $CD4^+$ cells quickly in a water bath at 37 °C and wash once in pre-warmed medium (RPMI 1640 + L-glutamine supplemented with 10% FBS and 1x penicillin-streptomycin).
2. Add the cells to 9 mL of medium, centrifuge for 5 min at 400 x *g* and remove the supernatant.
3. Resuspend the cells in 1% bovine serum albumin (BSA) in PBS (50 µL) so that each condition to be tested uses between 5 x 10^5 and 1 x 10^6 cells.
4. Mix the cells with Staining Buffer (50 µL).
NOTE: Brilliant Stain Buffer prevents various staining artefacts that may interfere with data analysis when two or more BV or BUV stains are used simultaneously owing to inherent chemical properties of these dyes. In single or unstained conditions, the 50 µL of brilliant stain buffer can be substituted for 50 µL of 1% BSA/PBS.
5. Add the antibodies to cells (as per the "volume used" column **in Table 1**) and incubate on ice in the dark for 30 min.
6. Wash the cells twice in 1% BSA/PBS by centrifuging for 3 min at 600 x *g* during each wash.
7. Resuspend in 1% BSA/PBS (400 µL) and transfer to a flow cytometry acquisition tube.
8. Vortex the cells gently before acquiring data on a flow cytometer:

3. Flow Cytometry Controls and Set-up

1. Using an unstained sample, set photodiode voltages so that lymphocytes can be separated from obvious debris and dead cells (events with high side scatter (SSC) area and low forward scatter (FSC) area)[16]. Using single stained samples, set photomultiplier (PMT) voltages so that positive fluorescence can be discerned from background fluorescence while making sure that all events fall within the detectable scale.
NOTE: An improvement would be to plot CV against PMT voltage for each PMT using dimly fluorescent beads to find the minimum voltage for optimum resolution (The "Peak 2" method[19]).
2. Account for spectral overlap by generating a compensation matrix using single stains with capture beads. Add commercially available compensation beads (60 µL), and negative control beads (60 µL) to 1% BSA/PBS (100 µL), and vortex.
NOTE: The capture beads used must match the host species and IgG isotype of the antibody being used. The compensation matrix should be re-calculated if the lot number of any tandem dyes changes, as they can display considerable variability in their emission spectra between lots.
3. Add a single antibody (20 µL) and vortex.
4. Incubate at room temperature in the dark for 30 min.
5. Centrifuge at 200 x *g* for 10 min and discard the supernatant.
6. Resuspend in 1%BSA/PBS (500 µL) and vortex.
7. Acquire the sample with a flow cytometer using the designated compensation matrix generator in the acquisition software according to the manufacturer's instructions.
8. Use FMO controls to guide the placement of gates for positive marker fluorescence to account for dye spill over, which is the main source of background fluorescence in experiments using ≥4 colours[16]. Follow the general cell-surface staining protocol as described in Step 3, but for each condition, omit **one** of the fluorophores from the staining step (where CD45RA is omitted, also omit the live/dead stain). Acquire 100,000 lymphocytes for each condition.
9. Set the gate positivity at ≤0.5% of cells. See **Figure 2** for an illustrated example of FMO controls.

4. Data Analysis

1. Employ the flow cytometer's acquisition software to set a threshold of 5,000 units on the FSC parameter to exclude very small debris.
2. Use a stopping gate to acquire 10,000 cTfh cells ($CD4^+$ $CD45RA^-$ $CXCR5^+$).
NOTE: The individual user can collect more than 10,000 cells. This number was a compromise between collecting enough cells to provide meaningful results and the time taken for collection.
3. Using an FSC-Area / SSC-Area dot plot, draw an ellipse or polygon gate to select the population of lymphocytes whilst excluding debris and overtly dead cells (events with a high SSC-Area and low FSC-Area).
4. Using an FSC-Area / FSC-Width dot plot, draw a polygon gate to select single cells while excluding doublets (doublets have an increased area but similar width to single cells).
5. Using an FSC-Area / CD45RA and viability marker dot plot, draw a rectangular gate to select live, $CD45RA^-$ cells (cells with a low fluorescence for this marker).
6. Using an FSC-Area / CD4 dot plot, draw a rectangular gate to select $CD4^+$ cells (cells with a high fluorescence for this marker).
7. Using a CD4 / CXCR5 dot plot, draw a rectangular gate to select $CXCR5^+$ (i.e., cTfh) cells (cells with a high fluorescence for this marker).
8. Using a CXCR3 / CCR6 dot plot, place a quad gate to subdivide cTfh cells into cTfh1,2 and 17 cells (cells that are high and low for each marker).
NOTE: Cells with the phenotype $CD4^+$ $CXCR5^+$ $CXCR3^+$ $CCR6^+$ are poorly characterized. We refer to these cells as cTfh1/17[18] because conventional helper T-cells ($CD4^+$ $CXCR5^-$) that are transitioning between Th17 and Th1 show expression of CXCR3 and CCR6 and have been described as cTfh1/17[19].
9. Using a PD-1 histogram, use the range tool to subdivide cTfh cells (or if preferred, the individual cTfh1,2,17 or 1/17 subsets) into PD-$1^{-/+}$ or ^{hi} populations.

NOTE: The distinction between PD-1^{+} and PD-1^{hi} is not well defined in the literature. The threshold was set as the same PD-1 intensity required to detect bona-fide Tfh in human tonsil lymphocyte suspensions using flow cytometry with the same antibody panel. Alternatively, a marker for ICOS can be added to the antibody panel, as only PD1^{hi} cells are ICOS^{+}.

Representative Results

High CD4^{+} purity was achieved using the CD4^{+} isolation protocol, which was reliable across all blood samples tested by us (mean: 96.6%, SD: 2.38, n=31) (**Figure 3**).

Identification of cTfh (CD4^{+} CXCR5^{+} cells) in a representative normal subject is presented (**Figure 4A**). The proportion of total cTfh cells within CD4^{+} cells had a median value of 29.4% (inter-quartile range (IQR) = 10.8) across 12 normal subjects. Multiple studies across several different diseases including hepatocellular carcinoma[17,20], systemic lupus erythematous[11,21], and rheumatoid arthritis[11,14] have been conflicting in the ability to detect a difference in overall cTfh between heathy controls and patients. No significant differences were found in overall cTfh between normal subjects and MZL or BNHL patients (**Figure 4B**)[21].

Identification of PD-1 expression within cTfh cells from a representative normal subject and BNHL patient for comparison is shown in **Figure 5A**. PD-1 expression was significantly higher in MZL and BNHL patients than normal subjects (**Figure 5B**)[21]. Similar increases in PD-1 expression have been demonstrated in multiple autoimmune disorders[11,12,13,14].

Identification of cTfh1,2,17 and 1/17 within the population of cTfh cells using CXCR3 and CCR6 expression from a representative normal subject is shown in **Figure 6A**. The proportion of cTfh1 cells was significantly higher in MZL and BNHL patients than normal subjects (**Figure 6B**)[21].

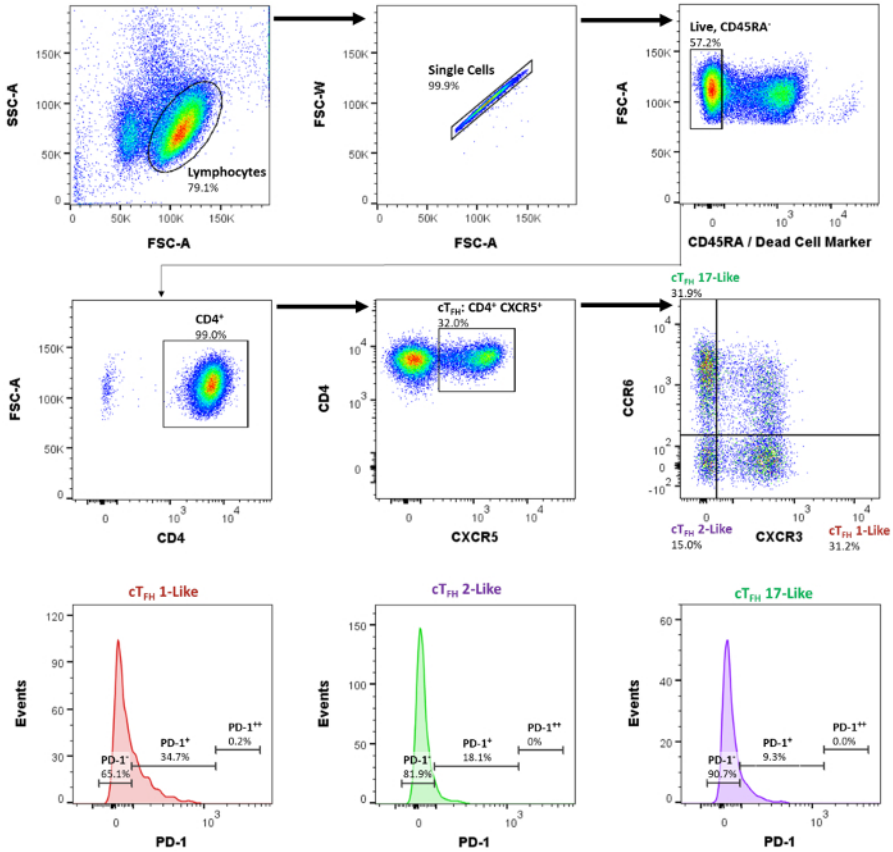

Figure 1: Illustration of overall gating strategy used to identify cTfh cells and their subsets. From top left, a population of lymphocytes are distinguished, and the doublets are excluded. Live CD45RA^{-} cells are selected using a "dump channel". CD4^{+} cells are gated and CXCR5^{+} cells are identified as cTfh. cTfh are divided into cTfh1, 2 or 17-like using CXCR3 and CCR6 expression. PD-1 expression within these subsets is then distinguished. Figures taken from a representative normal subject blood sample. FSC-A, FSC Area; SSC-A SSC Area; FSC-W, FSC Width. Please click here to view a larger version of this figure.

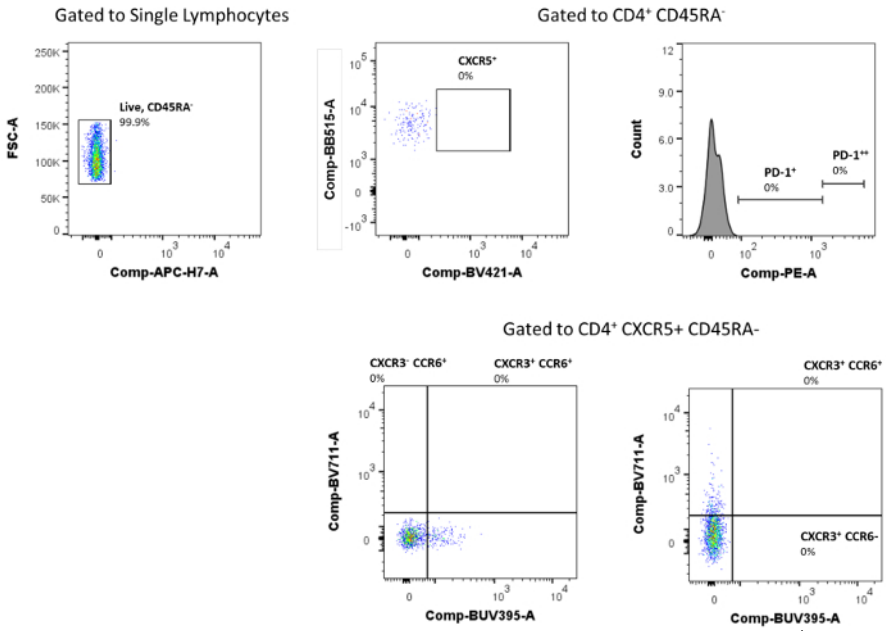

Figure 2: Illustration of FMO controls. Each biaxial flow cytometry plot shows CD4$^+$ cells stained with all fluorophores except the one of interest to demonstrate the minimum at which gates for fluorescence positivity can be set. Please click here to view a larger version of this figure.

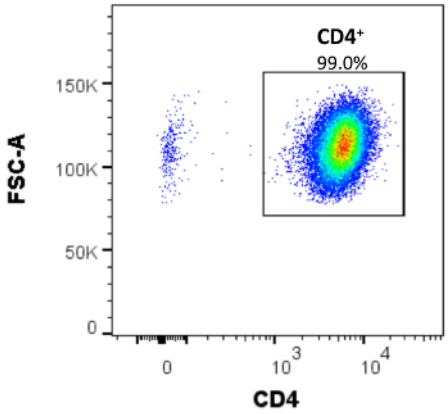

Figure 3: Biaxial flow cytometry plot showing the identification of CD4$^+$ cells after gating to live lymphocytes. Taken from a representative normal subject blood sample. Please click here to view a larger version of this figure.

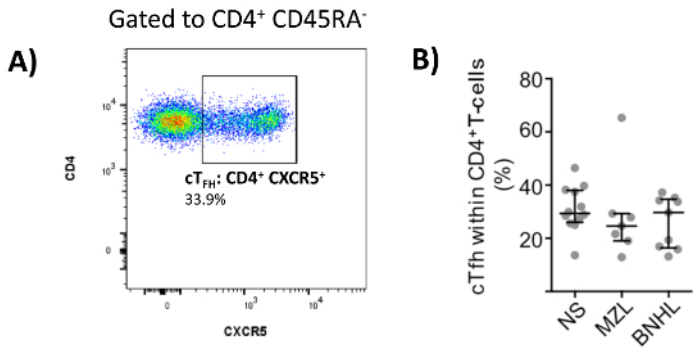

Figure 4: Identification of cTfh cells. (**A**) Biaxial flow cytometry plot showing CXCR5$^+$ expression on cells gated for CD4$^+$ CD45RA$^-$. Taken from a representative normal subject. (**B**) Relative percentages of cTfh within total CD4$^+$ cells in normal subjects (n = 12), MZL (n = 7), BNHL (n = 9). Horizontal lines represent the median, and error bars represent inter-quartile range. No significant differences were found between groups using the Mann-Whitney U test. This figure has been modified from Byford et al.[21]. Please click here to view a larger version of this figure.

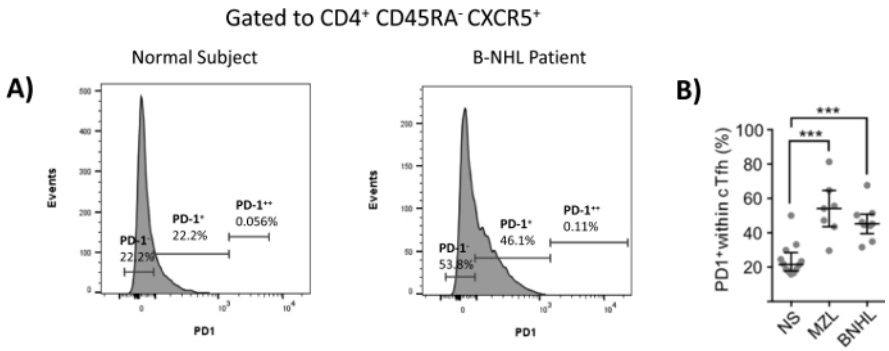

Figure 5: PD-1 expression and relationship to cTfh cells. (**A**) Flow cytometry histogram used to determine PD-1 expression within total cTfh cells. Taken from a representative normal subject and BNHL patient. (**B**) PD-1^{+} cells as a proportion of total cTfh cells. Horizontal lines represent the median, and error bars represent inter-quartile range. Medians are significantly (Mann-Whitney U-test) different between normal subjects (21.5%, IQR = 10.8, n = 12) and lymphoma patients (MZL 54.1%, IQR = 21.2, n = 7, p = 0.0008 and BNHL 45.2%, IQR =11.4, n = 9, p = 0.0003). This figure has been modified from Byford et al.[21]. Please click here to view a larger version of this figure.

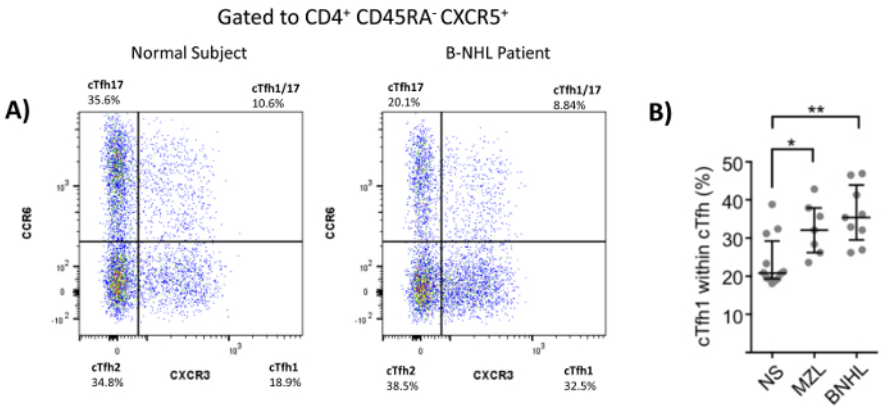

Figure 6: CXCR3 and CCR6 expression and their relationship to cTfh1 numbers. (**A**) Biaxial flow cytometry plot showing the expression of CXCR3 and CCR6 on CD4^{+} CD45RA^{-} CXCR5^{+} cells. Taken from a representative normal subject and BNHL patient. (**B**) cTfh1 cells as a percentage of total cTfh cells. Horizontal lines represent the median, and error bars represent inter-quartile range. Medians are significantly (Mann-Whitney U-test) different between normal subjects (20.8%, IQR = 6.7, n = 12) and lymphoma patients (MZL 32.1%, IQR = 6.8, n = 7, p = 0.013, and BNHL 35.4%, IQR = 7.6%, n = 9, p = 0.0056). This figure has been modified from Byford et al.[21]. Please click here to view a larger version of this figure.

Antibody Target	Conjugated Fluorophore	Species	Isotype	Clone	Volume used	Excitation laser source	Detection Filter
CXCR3	BUV395	Mouse	IgG1, κ	IC6	3 µL	UV (355 nm)	379/28
CCR6	BV711	Mouse	IgG1, κ	IIA9	3 µL	Violet (405 nm)	710/50
CXCR5	BV421	Rat	IgG2b, κ	RF8B2	3 µL	Violet (405 nm)	450/50
CD4	BB515	Mouse	IgG1, κ	RPA-T4	3 µL	Blue (488 nm)	530/30
PD-1	PE	Mouse	IgG1, κ	MIH4	3 µL	Yellow/ Green (561 nm)	582/15
CD45RA	APC-H7	Mouse	IgG2b, κ	HI100	3 µL	Red (640 nm)	780/60
Viability Marker		--	--	--	0.5 µL	Red Red (640 nm)	780/60

Table 1: Flow cytometry antibody panel.

Discussion

This protocol represents a simple and efficient way to analyze peripheral blood cTfh cells, enabling the detection of all relevant subsets identified in the literature thus far. Blood samples can be easily and efficiently obtained as part of standard out-patient clinics and serial samples can be collected in parallel with clinical data. In turn, this enables prospective studies evaluating cTfh subsets as biomarkers for disease progression or response to treatment. These studies would be particularly warranted in disease where Tfh dysregulation is implicated in pathogenesis such as autoimmunity and certain types of solid and haematological cancer. In addition, the changes in cTfh function in disease could be investigated by sorting cTfh cells using flow cytometry as only cell surface markers are used in this protocol. Sorting cTfh cells in MZL patients enabled us to find differences in gene expression profiles when compared to normal subjects[21].

We found that efficient $CD4^+$ T-cell isolation improved the data analysis. We describe the steps involved in detail because minor issues such as centrifuge braking and speed were critical to the isolation procedure. Another important step, as for all flow cytometry analysis, is to set the compensation and FMO controls.

PD-1 expression varies on cTfh cells and those cells with the highest PD-1 might be the most functional and therefore relevant, especially for the study of autoimmune disease[11,12]. One important issue was to decide the gate for the identification of $PD\text{-}1^{hi}$ cells, the activated cTfh subset, as there is no standard limit defined in the literature. This important but minor subset probably reflects active Tfh differentiation in lymphoid tissue[11]. To overcome this challenge, the threshold was set such that it was the same as that required to detect bona-fide Tfh cells from human tonsil with the same antibody panel. We recognize that obtaining tonsils might be problematic for some users. An alternative, would be to expand the antibody panel used in this protocol by the addition of anti-ICOS, as only $PD\text{-}1^{hi}$ cells are $ICOS^+$ [12].

Here, we present an antibody panel to detect surface markers characteristic of circulating $CD4^+$ T-cells. Circulating T-follicular regulatory cells (cTfr) are the blood memory compartment of T-follicular regulatory cells (Tfr) that are resident in lymphoid tissue and have important roles in regulating the germinal center reaction[22,23]. cTfr are blood $CD4^+$ $CXCR5^+$ cells that co-express T-regulatory cell markers including the transcription factor FoxP3. Measuring cTfr alongside cTfh may provide a more complete picture of activity in the germinal center, and could present a biomarker in disease in its own right[24]. Although cTfr numbers are inherently low (mean: 1.82%, SD: 1.40, n = 24 of total $CD4^+$ cells in our own experiments), separating cTfr from cTfh would also increase the specificity of cTfh analysis. Adding a combination of specific T-regulatory cell-surface markers to our panel such as CD25 and CD127[25] would enable the detection of cTfr in addition to all cTfh subsets in the same experiment using a very similar protocol. Alternatively, the more classical intracellular regulatory marker FoxP3 could be used, though this requires fixation and permeabilization preventing downstream functional assays. In concentrating on cTfh analysis, however, there are also advantages to defining a minimum panel that can be employed in conjunction with a standard laboratory flow cytometer to obtain clinically or experimentally useful results.

Disclosures

The authors have nothing to disclose.

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