

Chapter 17

Flow Cytometric Detection of Human Regulatory T Cells

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Abstract

Tregs are absolutely required for the maintenance of self tolerance in mouse and man. Major abnormalities in Treg number or function cause rare but fatal syndromes with autoimmune, allergic and inflammatory features. Whether subtle Treg abnormalities contribute to the pathogenesis of sporadic autoimmune, allergic and immunoinflammatory diseases in man remains controversial. Robust methods for identifying and isolating human Tregs in patients and healthy controls are essential if we are to understand their role in these increasingly common diseases. We have outlined below a flow cytometric technique to detect and isolate the entire human Treg population based on expression of CD4, CD25, and CD127. Use of a number of additional antibodies for defining subsets within the Treg compartment is described. For analysis, anti-Foxp3 can be added to the cocktail, but the necessity for fixation and permeabilisation may reduce the signal from other antibodies.

Key words: Treg, Human, Flow cytometry, CD127, Foxp3

1. Introduction

Tregs were originally identified as a distinct population of CD4⁺ T cells expressing the alpha chain of the IL-2 receptor, CD25 (1). Laboratories attempting to replicate the murine studies in humans encountered an unexpected difficulty, namely that CD25 is also expressed by a significant proportion of CD45RO⁺CD45RA⁻ effector/memory CD4⁺ T cells. A number of strategies were employed in an attempt to isolate a pure population of human Tregs. Using an in vitro suppression assay as readout, Baecher-Allen et al. first showed that when peripheral blood CD4⁺ T cells were sorted according to CD25 expression, only the fraction expressing the highest level of CD25 could reliably suppress in vitro (2).

The human CD25^{hi} phenotype was then defined as equivalent to the mouse CD25⁺, even though only 1–3% of circulating human CD4⁺ T cells were identified as Tregs (2), compared with 6–7% in the mouse. The placement of the CD25 gate was difficult to standardize between laboratories, since human CD25 expression is essentially unimodal with a long tail (Fig. 1a), unlike the essentially bimodal mouse CD25 distribution. Indeed, in the 5 years after the initial publication describing the CD25^{hi} phenotype, the range for Treg in the blood of healthy controls varied between 1.4 and 20%, depending on where the CD25 gate was placed. This problem was particularly apparent in studies aimed at testing whether Treg numbers were altered in patients with autoimmune and inflammatory diseases, since “contamination” of the CD25^{hi} Treg gate by CD25-expressing effector/memory CD4⁺ T cells involved in disease pathogenesis could not be ruled out. Disease-related changes in Tregs in the early studies showed no consistent trend, no doubt in part due to these technical issues.

A number of labs, our own included, subsequently showed that CD25 was expressed bimodally within human CD45RA⁺CD45RO⁻

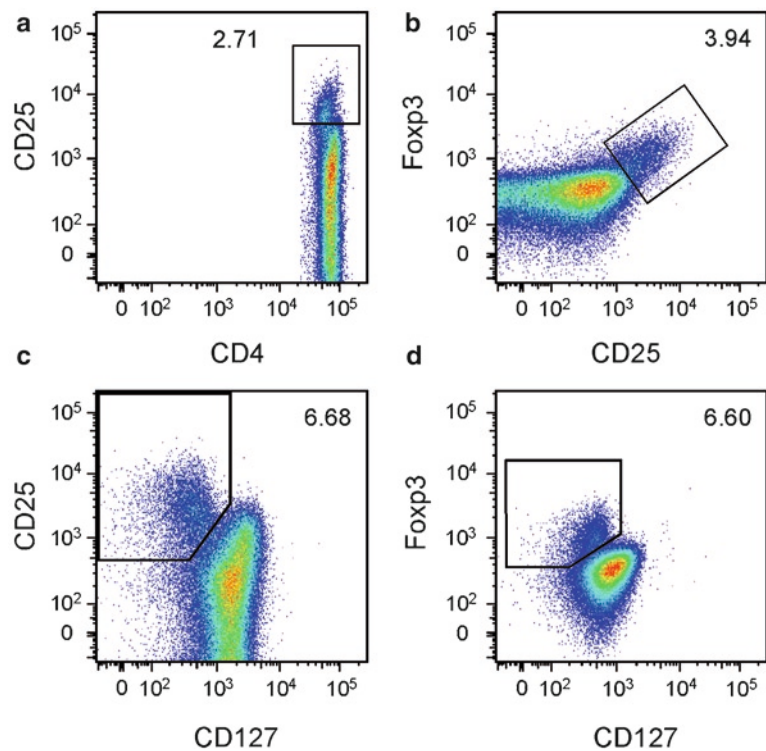


Fig. 1. Gating strategies for human Tregs. Plots are gated for peripheral blood CD4⁺ lymphocytes. (a) CD25^{hi} gate. (b) CD25/Fopx3 gate. (c) CD25/CD127 gate. (d) CD127/Fopx3 gate. The use of CD127 allows nonorthogonal gating for CD127^{lo} cells expressing intermediate levels of CD25 and/or Fopx3, identifying more Treg cells than can be detected with the CD25/Fopx3 gate.

naïve CD4⁺ T cells, allowing identification of a well-defined subpopulation of naïve Tregs (3, 4). This population declined with age, as would be expected for a naïve, thymically derived T cell subset.

Within the CD45RO⁺RA⁻ subset, we subsequently discovered that costaining for CD25 and CD127, the alpha chain of the IL-7 receptor, separated a distinct CD25⁺CD127^{lo} population from the bulk of the CD25^{lo-hi}CD127^{hi} cells (5, 6) (Fig. 1c). The CD25⁺CD127^{lo} population represented 6–7% of human peripheral blood CD4⁺ T cells and expressed very high levels of *FOXP3* mRNA as assessed by qPCR of flow-sorted cells. When Foxp3 antibodies became available, we were able to show for the first time the correlation between Foxp3 and the subsets to which Treg function had been attributed (5).

Foxp3 is often regarded as the definitive marker for Treg cells, based on murine data. However, in humans, Foxp3 can also be expressed by activated cells (7). Currently no single marker can provide an unequivocal identification of all human Treg cells. In addition, Foxp3 detection requires an intracellular stain that is incompatible with the recovery of viable cells. Thus both the identification and sorting of human Tregs still requires the detection of small changes in the expression of multiple surface markers, one of the most challenging applications of flow cytometry.

2. Materials

2.1. Cell Preparation

1. Phosphate Buffered Saline (PBS), pH 7.2: Milli-Q filtered H₂O containing 8 g/L NaCl, 0.2 g/L KCL, 1.15 g/L Na₂HPO₄ prepared at the Centenary Institute, kept sterile and stored at room temperature.
2. Tissue Culture Medium (TCM): RPMI Medium 1640 (Gibco) with L-glutamine and HEPES buffer (Invitrogen) supplemented with 10% heat activated fetal calf serum (FCS) (JRH Bioscience), 2 mM L-Glutamine, 0.05 mM 2-Mercaptoethanol (Invitrogen), 1,000 units/L of Penicillin (Invitrogen) and 1,000 µg/L Streptomycin (Invitrogen).
3. 9 ml Lithium Heparin tubes (Greiner bio-one) for blood collection.
4. Ficoll-Paque Plus (GE Health).
5. Freezing medium: 10% DMSO (Sigma), 20% FCS (JRH Bioscience) in RPMI1640 (Gibco).

- 6. Tissue digestion enzymes: Collagenase Type III and DNase Type I (Sigma).
- 7. 14 ml round bottom tubes (BD) for tissue digestion.
- 8. 15 and 50 ml sterile conical tubes (Falcon) for cell processing.
- 9. 70 µm nylon cell strainer (BD Falcon).
- 10. 0.2% Eosin in phosphate-buffered saline (PBS), haemocytometer chamber.
- 11. 2 ml cryovials (Sardtedt).
- 12. Cryo-1°C/min Freezing Container (Nalgene).

**2.2. Cell Staining:
Basic Protocol**

- 1. 96-well U bottom plate (Greiner bio-one).
- 2. Antibodies for basic staining cocktail.
 - (a) Our-preferred 2-laser combination – chosen on the basis of comparison of a number of anti-CD25 antibodies and conjugates.

mAb/Stain	Clone	Fluorochrome	Source	Cat#
Mouse IgG1 κ, antihuman Fxp3	259D	AF488	BioLegend	320212
Mouse IgG1 κ, antihuman CD127	hIL-7R-M21	PE	BD Pharmingen	557938
Mouse IgG2b κ, antihuman CD4	OKT4 (alternative)	PerCP-Cy5.5	BD Pharmingen	341654
Mouse IgG2b κ, antihuman CD4	OKT4 (alternative)	PE-Cy7	BioLegend	317414
Mouse IgG1 κ, antihuman CD25	M-A251 (alternative)	APC	BD Pharmingen	555434
Mouse IgG1 κ, antihuman CD25	2A3 (alternative)	APC	BD	340939
LIVE-DEAD® Fixable Near-IR	–	–	Invitrogen	L10119

(b) Our-preferred 3-laser combination.

mAb/Stain	Clone	Fluorochrome	Source	Cat#
Mouse IgG1 κ , antihuman Foxp3	259D	AF488	BioLegend	320212
Mouse IgG1, antihuman CD127	eBioRDR5	Pacific Blue	eBioscience	57-1278-73
Mouse IgG2b κ , antihuman CD4	OKT4 (alternative)	PerCP-Cy5.5	BD Pharmingen	341654
Mouse IgG2b κ , antihuman CD4	OKT4 (alternative)	PE-Cy7	BioLegend	317414
Mouse IgG1 κ , antihuman CD25	M-A251 (alternative)	APC	BD Pharmingen	555434
Mouse IgG1 κ , antihuman CD25	2A3 (alternative)	APC	BD	340939
LIVE- DEAD® Fixable Near-IR	–	–	Invitrogen	L10119

3. Medium for cell staining (FACS wash): 5% FCS (JRH Bioscience) in PBS plus 0.05% Na Azide (Sigma), 0.22 μ m filtered to remove particulates.
4. Fixative for biohazard reduction prior to running human samples on flow cytometer (FACS fix): 1% paraformaldehyde (BDH Laboratory Supplies) in PBS.
5. Foxp3 staining kit: our preferred kit contains anti-Foxp3 conjugated with AF 488 (Biolegend, 320212, 100 tests), plus Fix/Perm Buffer set (Biolegend, 421403, 100 tests). Many kits are marketed and we have not tested them all in parallel to determine the best kit.
6. 1.5 ml microcentrifuge tubes (Greiner bio-one).
7. 70 μ m cell strainers (BD).
8. 5 ml round-bottom tubes (BD).

2.3. Data Collection, Analysis, and Sorting

- 1. Flow cytometer. Many flow cytometers are suitable for this procedure. We use a FACSCanto II from BD.
- 2. Analysis software. We use FlowJo from Treestar.

2.4. Variations of Basic Protocol

Additional antibodies

mAb/Stain	Clone	Fluorochrome	Source	Cat#
Antihuman CD45RO	UCHL1	Biotinylated	Conjugated in house	–
Streptavidin		Pacific Orange	Invitrogen	S32365
Mouse IgG1 κ, antihuman CD45RA	L48	PE-Cy7	BD Pharmingen	337167
Rat IgM, antihuman CLA	HECA-452	PE	Miltenyi Biotec	130-091-635
Rat IgG2a κ, antihuman β7	FIB504	PE	BD Pharmingen	555945
Mouse IgG2b κ, antihuman CCR6	TG7/CCR6	PerCP/Cy5.5	Biolegend	335505

3. Methods

This is a basic protocol for identifying Treg cells, using a minimal cocktail of antibodies to CD4, CD25, and CD127, with or without addition of anti-Foxp3. The use of anti-CD127 is important as it is the combination of 2 Treg markers, one expressed at a higher level than conventional cells (CD25 and/or Foxp3) and one at a lower level (CD127) that allows a relatively clean gate to be set even though neither marker is adequate on its own (Fig. 1c,d). In the commonly used combination of CD25 and Foxp3, cells generally co-express low levels of each marker and so the combination is only marginally more discriminating than use of either as a single marker (Fig. 1b).

3.1. Cell Preparation

3.1.1. Isolation of Mononuclear Cells from Peripheral Blood

This protocol is designed for a single venous blood sample collected in a 9 ml Lithium Heparin tube. All blood samples are handled in a Class 2 biosafety hood and under sterile conditions.

- 1. First take an aliquot (usually 50–100 μl is required) for determining the concentration of leucocytes and lymphocytes using an automated hematology analyser such as the Sysmex KX-21.
- 2. Dilute the remaining blood 1:2 in PBS and transfer to a 50-ml tube.
- 3. Layer 12 ml of Ficoll-Paque Plus solution under the blood.
- 4. Centrifuge for 30 min at 1,600 rpm, 22°C with the break disengaged.

5. Collect peripheral blood mononuclear cells (PBMCs) from the interface between the Ficoll-Paque and the diluted plasma and transfer into sterile 15 ml tubes.
6. Wash twice with PBS (10 min, 1,000 rpm, 22°C).
7. Resuspend cells in PBS and take an aliquot for counting.
8. Dilute the counting aliquot in 0.2% eosin and perform a viable cell count using a haemocytometer.
9. Centrifuge the remaining cells and resuspend in FACS wash for staining.

OR

10. Resuspend in ice cold freezing medium at a concentration of $2-4 \times 10^6$ cells per ml for freezing. Freeze 1 ml aliquots in -70°C freezer at -1°C/min using a Cryo-1°C/min Freezing Container and then store in liquid NO₂ until use (see Note 4.1).

3.1.2. Isolation of Mononuclear Cells from Tissue Biopsies

This protocol is designed for a tissue biopsy up to 125 mm³.

1. Collect specimens in cold TCM and store on ice until processing.
2. Cut the tissue into <0.5 mm³ cubes.
3. Suspend in 1 ml TCM containing a final concentration of 100 U/ml Collagenase III and 0.1 mg/ml DNase I in 14 ml round bottom tubes. Incubate 60–90 mins at 37°C with shaking – for example at 200 rpm in an orbital mixer incubator (Ratek).
4. Pour the digested tissue through a 70 µm nylon cell strainer to remove debris. Rinse the strainer through with PBS to wash any trapped cells from the strainer.
5. Wash twice with PBS (1,500 rpm, 10 mins, 4°C).
6. Resuspend cells in PBS and take an aliquot for counting.
7. Dilute the aliquot in 0.2% eosin and perform a viable cell count using a haemocytometer.
8. Centrifuge the remaining cells and resuspend in FACS wash for staining.

OR

9. Resuspend in ice cold freezing medium at a concentration of $2-4 \times 10^6$ cells per ml for freezing. Freeze 1 ml aliquots in -70°C freezer at -1°C/min using a Cryo-1°C/min Freezing Container and then store in liquid NO₂ until use.

3.2. Cell Staining

1. Thaw the frozen mononuclear cells rapidly in a 37°C water bath and transfer to tubes containing 9 ml of prewarmed TCM (37°C).
2. Centrifuge at 1,000 rpm, 10 min, 22°C.

3. Resuspend in 2 ml cold FACS wash.
4. Take an aliquot, dilute in 0.2% eosin and perform a viable cell count using a haemocytometer.
5. Transfer 1×10^6 viable cells from each sample to a 96-well U-bottom plate.
6. Centrifuge the plate at 1,500 rpm, 4°C for 3 min to pellet cells. Carefully remove the supernatant with a pipette.
7. Add 100 µl of prediluted antibody mixture to all samples and mix by pipetting. All antibodies used have been titrated to obtain the best profile for the staining (see Note 4.2).
8. Incubate on ice for 45 min.
9. Wash cells twice with FACS wash (centrifuge at 1,500 rpm for 3 min). Remove supernatant carefully and discard.
10. *For analysis without Foxp3 staining:* Resuspend cells in 150 µl FACS fix and incubate 15 mins on ice. Wash cells (centrifuge 1,500 rpm, 4°C, 5 mins), resuspend in 200 µl.
11. *For Foxp3 staining:*
 - (a) Transfer cells to 1.5 ml microcentrifuge tubes.
 - (b) Fix cells in 1 ml 1× Biolegend Foxp3 Fix/Perm solution for 20 min, 22°C in the dark.
 - (c) Centrifuge cells at 1,100 rpm, 5 min, 22°C and remove supernatant.
 - (d) Wash cells once with 200 µl FACS wash and discard supernatant.
 - (e) Wash cells again with 1 ml of Foxp3 Perm buffer at 1,100 rpm, 5 min, 22°C and discard supernatant.
 - (f) Resuspend cells in 1 ml Foxp3 Perm buffer and incubate for 15 min in the dark (22°C).
 - (g) Spin cells at 1,500 rpm, 5 min, 22°C and remove supernatant.
 - (h) Resuspend in 100 µl Foxp3 perm buffer and add 5 µl of anti-Foxp3 antibody.
 - (i) Incubate for 30 min in the dark (22°C).
 - (j) Wash cells twice with 200 µl FACS wash (1,500 rpm, 5 mins, 22°C). resuspend in 200 µl FW and keep at 4°C in the dark until data acquisition.
12. Filter cells with 50 µm nylon mesh into 5 ml FACS tubes before data acquisition.

3.3. Data Collection and Analysis

1. Prepare compensation controls and collect at least 10^4 events of each. We use antibody capture beads for convenience but PBMCs can also be used. For samples, collect at least $1\text{--}2 \times 10^5$ events (excluding dead cells and fragments with low FSC) on

flow cytometer. For tissue samples in which CD4⁺ T cells may comprise only 5–10% of total, it may be necessary to collect up to $1\text{--}2 \times 10^6$ events. We generally run samples uncompensated and perform compensation only during data analysis with FlowJo. This prevents errors due to overcompensation, which cannot be reversed after data collection.

2. Data analysis is a crucial step in Treg detection. Figure 2 outlines our gating strategy for standard CD4/25/127 staining of PBMCs. The first three gates are designed to remove artifacts due to fluidics instability and cell doublets, after which dead cells are excluded. After gating for CD4⁺ T cells using a size parameter, the standard Treg CD25/127 gate is applied (5).

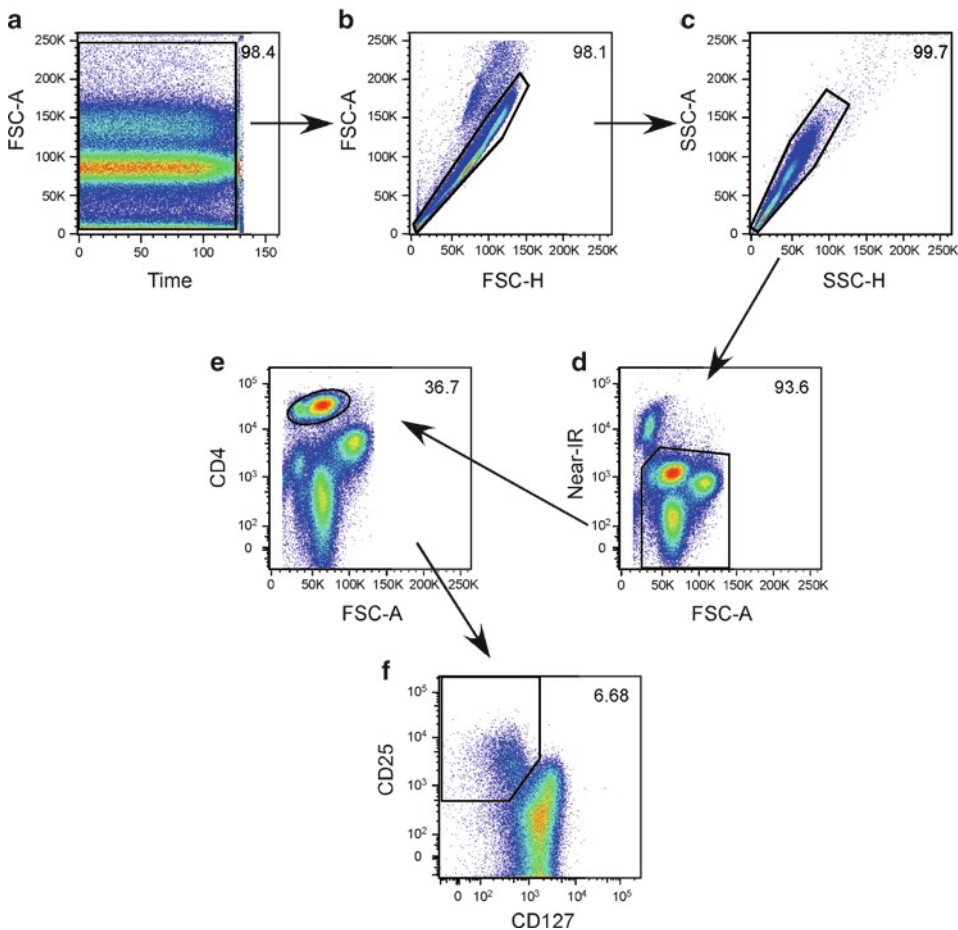


Fig. 2. Standard gating for Treg cells in human peripheral blood after staining for CD4, CD25, and CD127. (a) Cells are gated on the basis of time to exclude artifacts due to fluidics instability at the start and end of the sample. (b) and (c) Use of sequential pulse height vs. area gates for forward and side scatter allows doublets to be identified and excluded. (d) Use of the fixable dead cell exclusion dye LIVE-DEAD[®] Fixable Near-IR allows dead cells to be excluded from fixed human samples. (e) CD4^{hi} cells with low forward scatter are lymphocytes. The cells with higher FSC-A and lower CD4 expression are monocytes. (f) Gating for Tregs using the nonorthogonal CD25/CD127 gate.

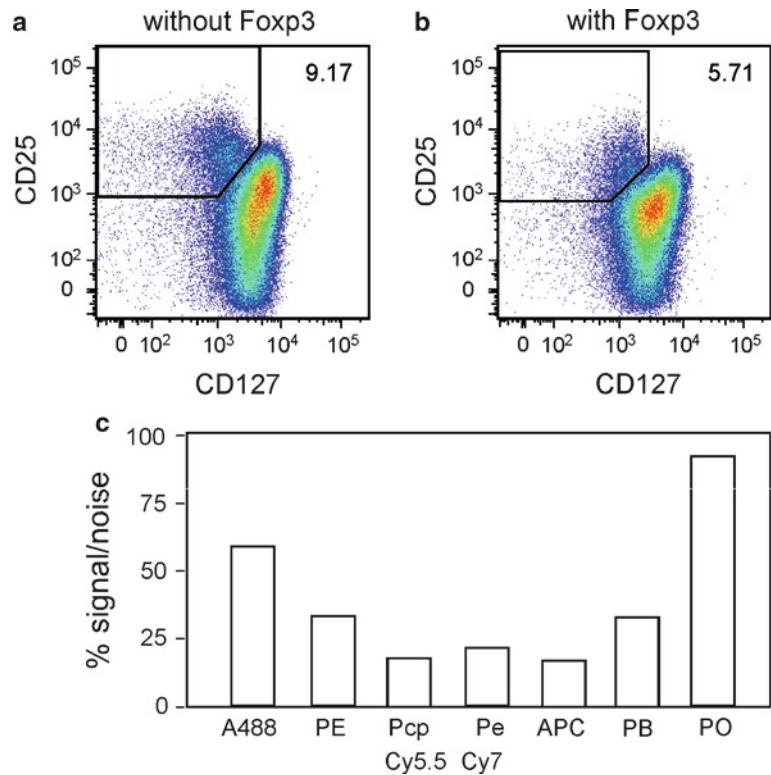


Fig. 3. Effect of Foxp3 fix/perm treatment on fluorescence of the conjugated antibodies used for Treg identification. (a) Expression of CD25 and CD127 by peripheral blood leukocytes gated for CD4⁺ T cells, after staining with CD4/CD25/CD127, Near-IR and formaldehyde fixation. (b) The same sample, with additional staining for Foxp3 according to the manufacturer's instructions. (c) Effect of Foxp3 fix/perm treatment on fluorescence. Single color compensation samples were stained and formaldehyde fixed. Half of each sample was then treated with fix/perm buffers as per the manufacturer's instructions for Foxp3 staining. This treatment reduced the signal and increased the background of unstained cells for each fluorochrome tested. Signal:noise ratio was calculated for all samples and the reduction in signal:noise after fix/perm was expressed as a percentage of signal:noise without fix/perm treatment. As expected, fluorescence of tandems such as PCP-Cy5.5 and PE-Cy7 was reduced, but the unexpected drop in the APC signal is problematical when anti-CD25-APC is part of the staining cocktail.

3. The addition of Foxp3 to staining cocktails may have unanticipated consequences, as illustrated in Fig. 3. In our hands, sensitive Foxp3 detection is inevitably accompanied by a reduction in sensitivity for other parameters. The fixation-dependent change in forward and side scatter prevents the use of FSC/SSC gating to exclude dead cells, but this problem can be overcome by including a fixable dead cell stain. However the reduction in fluorescence in the other channels is a major problem, particularly as it affects detection of CD25 via an APC conjugate and prevents the use of tandem fluorochrome conjugates to detect further markers on Treg subsets (Fig. 3).

4. To overcome the problems associated with Foxp3 staining, we have adopted the following strategy. We stain 2×10^6 cells from each sample with 200 μ l CD4/25/127 cocktail. When staining is complete, we remove half the sample for additional Foxp3 staining. The matched samples are run in parallel and the number of CD25⁺CD127^{lo} cells is calculated from the first sample and the number of Foxp3⁺CD127^{lo} cells from the second sample (in which CD25 signal is too low for reliable CD25⁺CD127^{lo} gating, as shown in Fig. 3b). The estimates of Treg numbers from the 2 samples should be within 10% of each other.
5. Because the staining and gating procedure has such a profound effect on the detection sensitivity for Tregs, we believe that it is important to include full methodological details in publications. Flow data plots showing how gates have been placed, in addition to calculated values for each individual, are vital if data from different laboratories are to be usefully compared.
6. Treg numbers can be expressed either as concentration in venous blood or as a percentage of another parameter such as CD4⁺ T cells. In our hands, lymphocyte concentrations are far more variable than percentages. In particular, the percentage of CD4⁺ T cells within a lymphocyte FSC-A/SSC-A gate is stable in healthy subjects across a wide range of ages. We therefore routinely present Treg data expressed as a percentage of CD4⁺ T cells. With essentially unlimited capacity to include supplementary data in most publications, it is also very useful to include concentrations in addition to percentages. For conditions such as HIV infection in which changes in CD4⁺ T cell concentrations are a crucial and highly variable parameter, we suggest that both measures should be routinely presented.

3.4. Variations on the Basic Protocol

1. *Staining for CD45RA and/or CD45RO*: Include appropriate antibodies in the staining cocktail (see Subheading 3.2, step 7 above).
 - (a) Gating for Treg subsets must take into account the lower expression of both CD25 and Foxp3 by CD45RA⁺RO⁻ Treg cells compared with CD45RA⁻RO⁺ Tregs. However the “background” expression of CD25 by CD45RA⁺RO⁻ non-Tregs is also lower. Hence the precise positioning of the Treg gate is often slightly different within the two subpopulations.
 - (b) Figure 4 illustrates the use of CD45RO to subset Tregs. When the CD25/127 gate appropriate for total CD4⁺ T cells (Fig. 4a) is applied to the CD45RO⁻ subset, it underestimates Treg numbers (Fig. 4e) whereas for the

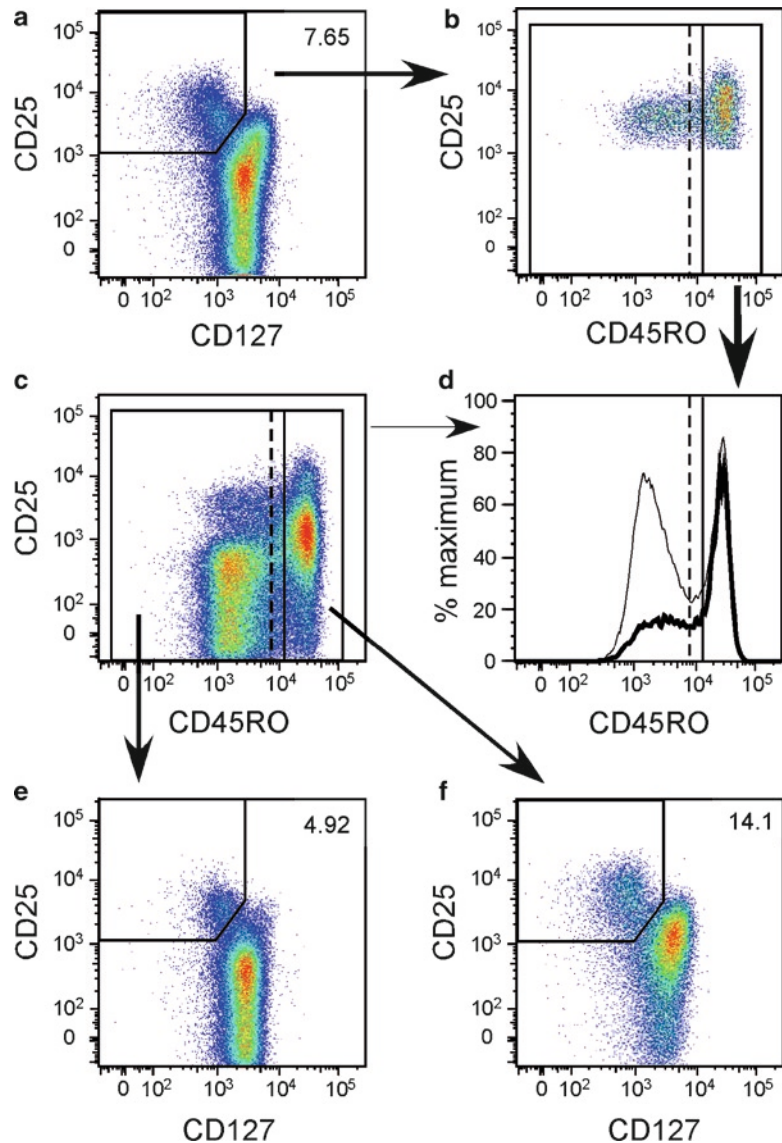


Fig. 4. Use of CD45RO staining to subset Tregs and total CD4⁺ T cells in human peripheral blood after staining for CD4, CD25, CD127, and CD45RO. **(a)** Standard gating of CD4⁺ T cells for Tregs, as shown in Fig. 2. **(b)** Expression of CD45RO by Tregs gated as in **(a)**. Optimal gating of Tregs into CD45RO⁻ and CD45RO⁺ subpopulations, based on expression of CD25 and CD45RO, is shown by the solid black lines. The *dotted line* is derived from optimal gating of conventional CD4⁺ T cells as in **(c)**. **(c)** Expression of CD45RO by CD4⁺ T cells. Optimal gating into CD45RO⁻ and CD45RO⁺ subpopulations is shown by the *dotted line*. **(d)** Comparison of CD45RO expression by Tregs (**bold line with solid black gate**) and total CD4⁺ T cells (**fine line with dotted black gate**). This overlay shows that the solid black gate suitable for CD45RO⁺ cells within total CD4⁺ T cells includes a subpopulation of Tregs that are phenotypically identical to CD45RO⁻ Tregs in terms of CD25 expression. Thus the position of the CD45RO gate must be adjusted according to whether conventional or Treg cells are gated. **(e)** and **(f)** Gating for Tregs within CD45RO⁻ **(e)** and CD45RO⁺ **(f)** CD4⁺ T cells, using the Treg gate shown in **(a)**. These plots show that the gate is not optimal for either population, illustrating the necessity for modifying gates according to the differential expression of CD25 and CD127 by CD45RO⁻ and CD45RO⁺ CD4⁺ T cells respectively.

- CD45RO⁺ subset, it overestimates numbers (Fig. 4f). The most accurate analysis requires the percentage in each subset to be determined separately (5).
- (c) Gating for CD45RO may also present a challenge. Comparison of CD45RO expression within Tregs (Fig. 4b) vs. total CD4⁺ T cells (Fig. 4c, overlays in Fig. 4d) shows that the optimal CD45RO⁺ gate for Tregs is placed at a significantly higher channel than for total CD4⁺ T cells. Thus it is often necessary to set a preliminary Treg CD25/127 gate, then set the CD45RO gate appropriate for Tregs, and then apply that CD45RO gate to total CD4⁺ T cells, after which the Treg CD25/127 gate can be adjusted to achieve the best accuracy within each subset.
2. *Staining for further Treg subsets.* Include appropriate antibodies in the staining cocktail (see 3.2.7 above). As noted above, use of Foxp3 staining is generally incompatible with cocktails containing multiple subset markers, especially if they rely on tandem dyes.
- (a) A gating strategy for possible Treg subsets based on expression of tissue-homing integrins is illustrated in Fig. 5.

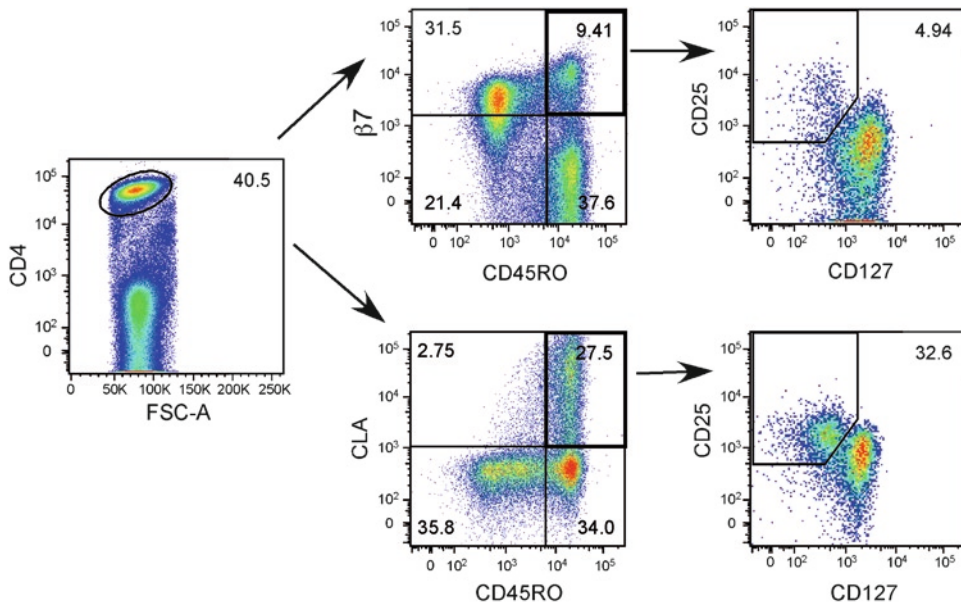


Fig. 5. Use of additional surface markers to subset human Tregs. Expression of integrin $\beta 7$ and CLA by CD45RO⁺ CD4⁺ T cells is shown in the *bold rectangular gate*. Differential expression between conventional and Treg cells is indicated by the relative decrease in Treg cells within the integrin $\beta 7$ -expressing population and the marked increase in Treg cells within the CLA-expressing population. Use of an alternative gating strategy with sequential gates for Tregs, CD45RO and then integrin $\beta 7$ or CLA should yield identical final populations, excluding unanticipated gating artifacts.

4. Notes

1. *Use of frozen cells*: Our comparison of results from fresh vs. frozen samples has indicated that virtually identical fluorescence values and population percentages are obtained for all the markers discussed here. Expression of a subset of chemokine receptors, including CCR5, may however be affected by freeze/thaw procedures.
2. *Titering antibodies and testing cocktails*: Reagents conjugated with different fluorochromes and/or from different companies can give vastly different signal levels.
 - (a) The signal: noise ratio for detecting CD25, CD127 and Foxp3 expression by Tregs is in the mid to low range (unlike CD4, for which even a suboptimal signal is usually adequate), so it is vital that the antibody cocktail be optimised.
 - (b) In general, highly expressed molecules or those for which very high affinity antibodies are available can be detected with the dimmer fluorochromes such as Pacific Blue and Pacific Orange (we detect CD4 and CD45RO respectively in those channels in our 6–8 color panels). Of the remaining fluorochromes, PE and APC are the brightest and so they can be reserved for CD25 and/or Foxp3. Substituting AF488 for FITC usually increases the signal in that channel, as the AF488 does not undergo intermolecular quenching, unlike FITC.
 - (c) Avoid detecting highly expressed molecules in a fluorescence channel with significant cross-over into neighboring channels, especially when the molecule is expressed by only a subset of Tregs. For example, the signal from CD45RO-APC-Cy7 may interfere significantly with CD25-APC, leading to difficulty in detecting CD25 expression by CD45RO⁺ but not CD45RO⁻ T cells.
 - (d) Most commercial suppliers market anti-human antibody conjugates as a number of tests of given volume, to be used in a final volume of 100 µl per sample. In many cases (for example, CD25-APC) these concentrations are not saturating, as indicated by the readily apparent drop in fluorescence with only twofold dilution (Fig. 6b, c). For an antibody such as CD25-APC, even a small decrease in fluorescence can cause gating problems, and the recommended amount must be used. For other antibodies such as CD4-PE-Cy7, the signal:noise ratio is so high that nonsaturating concentrations can be diluted even further without diminishing the accuracy of gating

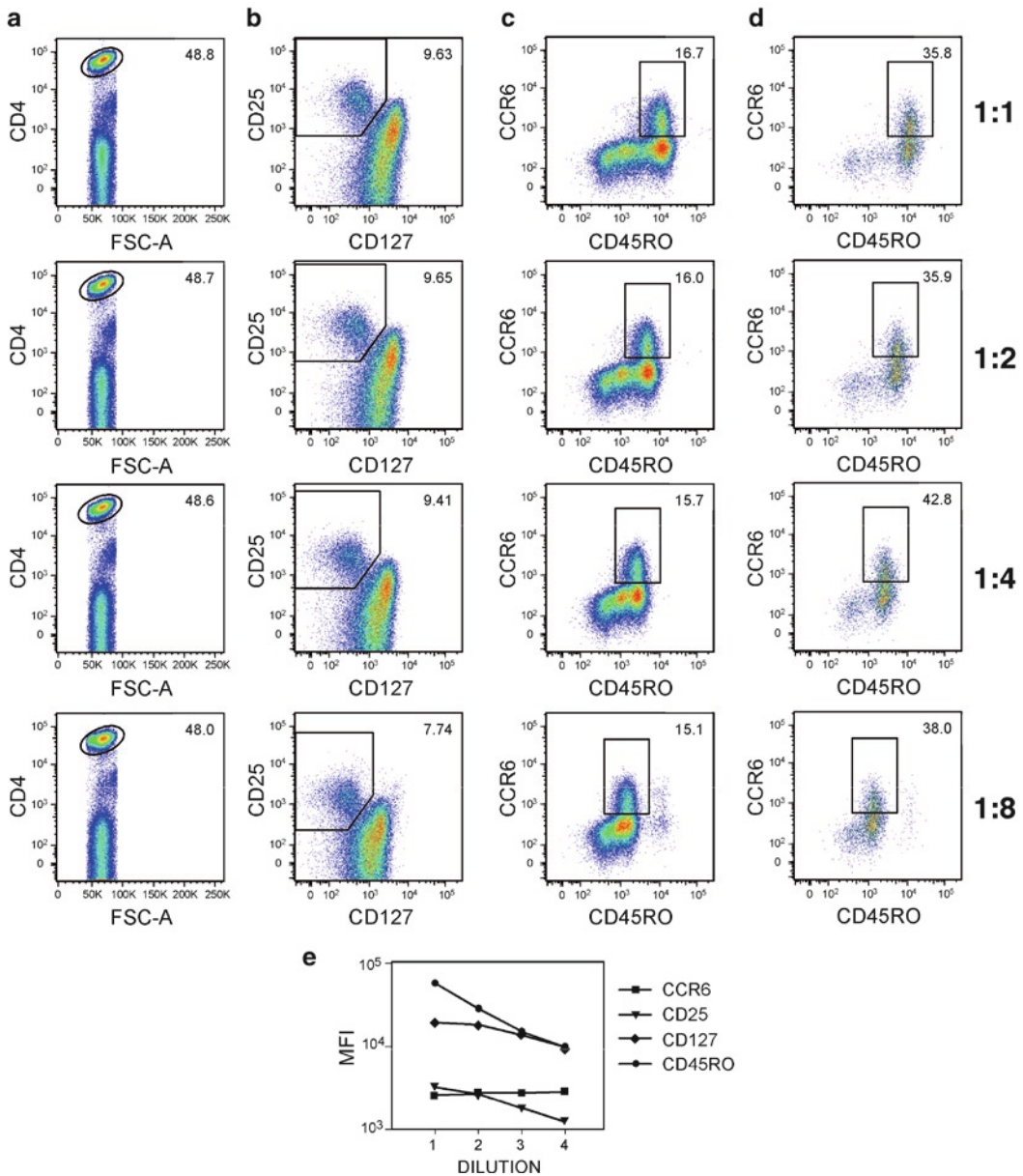


Fig. 6. Strategy for titrating antibodies for Treg subsetting. In this example, a mixture of antibodies to CD4, CD25, CD127, CD45RO and CCR6 at the concentrations recommended by the manufacturer were diluted in a two-fold series for staining PBMCs. Each row is from a single sample stained at the indicated dilution and gated as indicated for CD4⁺ T cells (**a**), CD25⁺CD127^{lo} Treg cells within CD4⁺ T cells (**b**), CCR6⁺CD45RO⁺ cells within CD4⁺ T cells (**c**) and CCR6⁺CD45RO⁺ cells within Tregs (**d**). (**e**) MFI of cells within the indicated gates is shown as a function of dilution. Although the signal from CD25, CD127 and CD45RO declines with each dilution, it is still possible to gate on Treg cells in each sample, in order to measure the signal from the test antibody. In this case, the recommended concentration of the CCR6 antibody can be reduced at least eightfold with no loss of signal.

(Fig. 6a). Lastly, some antibodies, such as the anti-CCR6 shown in Fig. 6c–e, can be diluted at least eightfold without any change in fluorescence intensity.

- (e) Titering each antibody individually can be very expensive in terms of time and reagents, because each sample must also be stained for CD4/CD25/CD127 so that expression of the new marker by Tregs can be determined. We recently tested a new strategy in which the complete cocktail, containing CD4/CD25/CD127 as well as the test antibody or antibodies, is titered (Fig. 6). The cocktail is prepared using the recommended concentrations in a final volume of 200 μ l, and then a twofold dilution series is performed to give final volumes of 100 μ l. Although this reduces the signal from the CD4/25/127 cocktail simultaneously with that of the test Ab, the Treg population is clearly gateable out to a dilution of 1:8 (Fig. 6b). In our experience, the antibody volume calculated from titration can be as little as 1:20 of the manufacturer's recommended volume.
 - (f) Signal cross-over from channel to channel can vary widely, particularly with tandem dyes, so it is also important to pretest each of the cocktails to check for unanticipated interference in any of the channels.
3. *Validating gating strategies*: we have presented several of our “standard” gating strategies in the Figures, but we always check the multiple strategies with each new experiment to make sure that the same cells are included in (and excluded from) the final population.
 4. *Validating staining reproducibility*: we always include control samples in each experiment. This requires freezing of multiple vials prepared from a single donor on a single occasion.
 5. *Tissue-specific effects*: CD25 expression can vary widely in different tissues, presumably due to availability of IL-2 which, in the mouse at least, upregulates the expression of CD25. CD25 expression in synovium of some patients with rheumatoid arthritis is high enough to define a clear CD25⁺ population without requiring either CD127 or Foxp3 staining (8). In bowel mucosa, on the other hand, our unpublished results show that a significant number of Foxp3⁺ cells express undetectable levels of CD25. This, together with the significant number of CD127^{lo} activated conventional Foxp3[−]CD4⁺ T cells in bowel mucosa, makes the use of anti-Foxp3 staining essential for identification of Treg cells.

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