Chapter 16

High-Dimensional Single-Cell Profiling of Tumor-Infiltrating CD4$^+$ Regulatory T Cells

Giorgia Alvisi, Simone Puccio, Rahul Roychoudhuri, Caterina Scirgolea, and Enrico Lugli

Abstract

CD4$^+$ T regulatory cells (Tregs) are a specialized subset of T lymphocytes, which promote immune homeostasis and tumor immunosuppression by restricting effector T cell immune responses. The characterization of context-specific Treg phenotypic heterogeneity is pivotal to determine their potential contributions to diseases. In the recent years, high-dimensional single-cell technologies, such as single-cell RNA sequencing, mass cytometry, or polychromatic flow cytometry, have played a central role in elucidating the heterogeneity of the Treg compartment at the cellular and molecular levels. Here we describe an example of high-dimensional flow cytometry analysis capable of defining an effector Treg subpopulation that positively correlates with cancer progression. Moreover, we provide a workflow template of high-dimensional single-cell analysis that is readily applicable to any leukocyte subpopulation.

Key words Treg, Single-cell, Polychromatic flow cytometry, High-dimensional data, Profiling, UMAP, Phenograph, Clustering, Cancer, Autoimmunity

1 Introduction

Immunotherapy with adoptive T cell transfer and immune checkpoint blockade has revolutionized the treatment of several types of cancers. However, durable responses are limited to a subset of patients and suppression of the antitumor immune response in the tumor microenvironment (TME) is a major obstacle to tumor regression [1]. While immune cells, such as T and NK cells, normally infiltrate tumors and their abundance correlates with better prognosis in certain cancers, the presence of inhibitory subpopulations, such as regulatory T cells (Tregs), counteracts protection [1]. Tregs are a subset of CD4+ T cells whose differentiation depends on the transcription factor (TF) FOXP3 [2] and that are indispensable to limit autoimmunity and immunopathology. Although several subsets of Treg can be delineated according to

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the different technologies that are employed, Tregs are broadly subdivided into two major populations according to their immunophenotypic landscape, activation status, and localization in the body [3]. Resting Tregs (rTregs) are FOXP3^{low}CD45RA^{hi}CD25^{int}, represent the majority of Treg cells in the circulation and secondary lymphoid organs, and share phenotypic features with naïve and memory conventional T cells (Tconv). Effector Tregs (eTregs) are FOXP3^{hi}CD45RA^{low}CD25^{hi}, share phenotypic features with activated Tconv, and are empowered by enhanced migration capacity to nonlymphoid tissues [3].

In tumors, Tregs are specifically recruited, become hyperactive, and exert their immunosuppressive functions via multiple mechanisms, which include secretion of soluble factors, cytolysis of effector cells, and contact-dependent inhibition [1]. The detrimental effect on antitumor immunity is demonstrated by a plethora of preclinical data where Treg depletion, or inhibition of their function, promotes antitumor immunity. The molecular mechanisms at the basis of Treg hyperactivity in the TME are just begun to be elucidated, and involve metabolic reprogramming and genome-wide transcriptional changes, among others, thereby resulting in chronic immune activation and enhanced suppressive activity. We have recently shown that Treg hyperactivity is not shared by all intratumoral Tregs, rather by a subset of CCR8^{+} ICOS^{+} effector Tregs (eTregs), present in several human tumors. These eTregs overexpress the transcription factor (TF) IRF4, which orchestrate a molecular program leading to superior immunosuppression and driving disease progression [4, 5]. Importantly, selective targeting of the hyperactive eTregs promotes tumor regression and synergizes with Programmed Death-1 (PD-1) checkpoint blockade without severe, systemic autoimmunity, that is otherwise observed with nonspecific Treg depletion [6], demonstrating that therapeutic targeting of molecular mechanisms that are specifically active in intratumoral Treg is pivotal to achieve tumor control.

The considerable heterogeneity of the immune responses can now be dissected by several, improved single-cell technologies that are capable to measure dozens to thousands of parameters in millions of single cells and with a diverse throughput. Fluorescent-based polychromatic flow cytometry is one of the most used and versatile tools to define cellular heterogeneity at the protein level [7, 8]. User-dependent variability in the analysis of the data due to subjective manual gating can be overcome by different computational approaches that take advantage of unsupervised algorithms for the identification of cell populations in high-dimensional data.

Here, we provide a step-by-step protocol for the generation and analysis of high-dimensional flow cytometry data. Specifically, we offer a workflow for high-dimensional single-cell analysis from data processing to dimensionality reduction, clustering, and

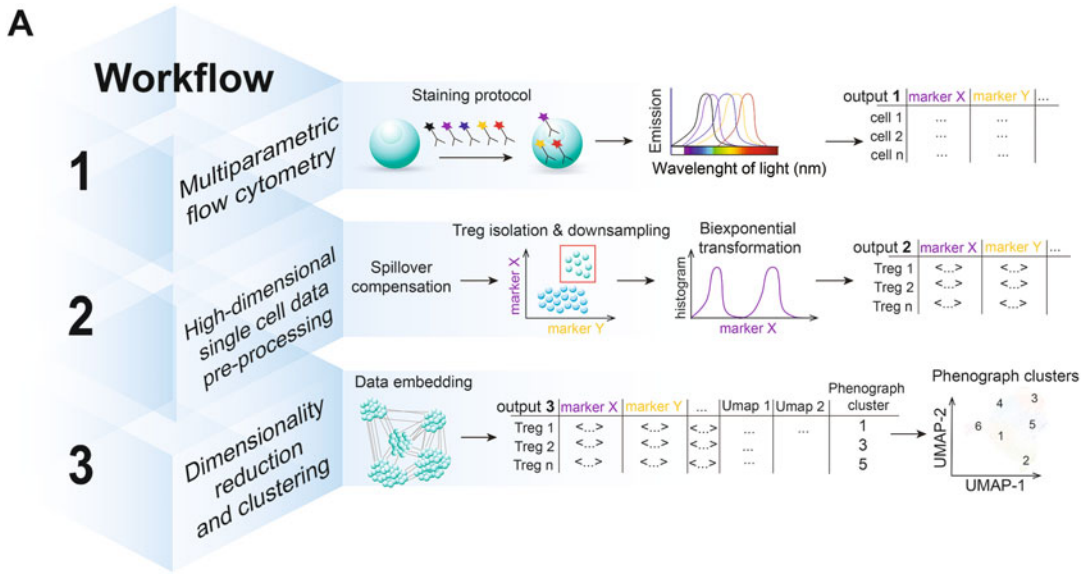

Fig. 1 High-dimensional flow cytometry. (**a**) Experimental workflow

visualization (Fig. 1). Data preprocessing and quality control can be achieved by the use of a dedicated software, for example, FlowJo, followed by dimensionality reduction and clustering analysis by dedicated algorithms, such as Uniform Manifold Approximation and Projection (UMAP) and Phenograph, respectively. For the latter part, we have compiled a comprehensive workflow illustrated with a readily applicable Python code accessible to immunologist with basic computational background. The entire procedure is exemplified with a high-dimensional panel suitable for the characterization of Treg subsets infiltrating human tumors. Nevertheless, the same approach can be exploited to interrogate any leukocyte population of interest.

2 Materials

2.1 Cells

1. Human peripheral blood mononuclear cells (PBMCs) or tissue-derived single cell suspensions.

2.2 Reagents

1. FBS (*see* **Note 1**).
2. Dimethylsulfoxide (DMSO).
3. RPMI 1640 medium.
4. Penicillin–streptomycin.
5. Ultraglutamine (Lonza, cat. no. BE17-605E/U1).
6. Deoxyribonuclease I from bovine pancreas (DNase I; Sigma-Aldrich, cat. no. D25).

7. PBS and HBSS without calcium and magnesium (hereafter referred to as $PBS^{-/-}$ and $HBSS^{-/-}$).
8. Trypan blue solution for cell counting: make a ten-fold dilution (from the 0.4% stock solution to 0.04% working solution) in $PBS^{-/-}$.
9. dH2O.
10. EDTA.
11. Viability dye such as Zombie Aqua (BioLegend, cat. no. 423102).
12. Brilliant Stain buffer (BD Biosciences, cat. no. 563794).
13. Fluorescently conjugated monoclonal antibodies (Abs) (*see* **Note 2**).
14. Formalin, 1% (wt/vol).
15. Intracellular staining buffer such as FOXP3/Transcription Factor Staining Buffer Set (eBioscience, cat. no. 00-5523-00) (*see* **Note 3**).
16. BD CompBeads Set, Antimouse Ig, κ (BD Biosciences, cat. no. 552843).
17. ArC Amine Reactive Compensation Bead Kit (Thermo Fisher Scientific, cat. no. A10346).

2.3 Plastic Components

1. Polystyrene round-bottom tubes (5 mL; BD Falcon, cat. no. 352054).
2. Conical tubes (BD Falcon, cat. no. 352096).
3. Microcentrifuge tubes (1.5 mL; Eppendorf, cat. no. 022363204).

2.4 Instruments

1. Microcentrifuge.
2. Incubator (set to 37 ° C).
3. 28-color FACS Symphony A5 flow cytometer equipped with five lasers (UV, 350 nm; violet, 405 nm; blue, 488 nm; yellow/green, 561 nm; red, 640 nm). Details on the instrument configuration are depicted in Fig. 2a. Machine quality control (QC) was performed according to the protocol designed by Perfetto et al. [9].

2.5 Software

1. FlowJo (https://www.flowjo.com/).
2. BD FACSDiva™ (https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software).
3. Anaconda (https://www.anaconda.com).
4. Jupyter (https://jupyter.org).

A

Laser	WL	Power	LP filter	BPfilter	Fluorochrome
Blue	488	100	750	780/60	BB790
			685	710/50	Per-CP-eF710
			635	670/25	BB660
			600	610/20	BB630
			505	530/30	AlexaFluor488
Red	640	100	750	780/60	APC-Cy7
			690	730/45	AlexaFluor700
				670/30	APC
Violet	405	100	770	800/30	BV786
			685	710/20	BV711
			640	675/20	BV650
			595	605/20	BV605
			550	575/25	BV570
			505	515/20	Aqua
			450	470/15	BV480
				450/50	BV421
UV	355	60	770	820/60	BUV805
			690	740/35	BUV737
			630	670/25	BUV661
			600	615/24	BUV615
			550	580/20	BUV563
			450	515/30	BUV496
				379/28	BUV395
YG	561	100	750	780/60	PE-CY7
			685	710/50	PE-CY5.5
			635	670/30	PE-CY5
			600	610/20	PE-eF610
				586/15	PE

B

Specificity	Fluorochrome	Clone	Manufacturer	Cat.no.
Viability staining mix (RT)				
Dead cells	Aqua	-	BioLegend	423102
Chemokine receptor staining mix (37°C)				
CCR8	BV421	433H	BD	566380
CCR5	APC-Cy7	2DT/CCR5	BD	557755
CXCR6	PE-CY7	K041E5	BioLegend	356012
Surface staining mix (RT)				
TIGIT	PerCP-eF710	MBSA43	eBioscience	46-9500-42
CD25	BV711	BC96	BioLegend	302636
OX4O	BV650	ACT35	BD	563658
TIM3	BV605	7D3	BioLegend	345018
CD27	BV570	O323	BioLegend	356936
PD-1	BV480	EH12.1	BD	566112
CD8	BUV805	SK1	BD	564312
CD28	BUV737	Cd28.2	BD	564438
CD4	BUV615	SK3	BD	624297
CD45RA	BUV563	HI100	BD	565702
ICOS	BUV395	DX29	BD	564777
CD127	PE-CY5	eBioRDR5	eBioscience	15-1278-42
Intracellular staining mix (4°C)				
CD3	BUV496	UCHT1	BD	564809
FOXP3	PE-CY5.5	PCH101	eBioscience	35-4776-42
EOMES	PE-eF610	WD1928	eBioscience	61-4877-42
CXCL13	APC	53610	ThermoFisher	MA5-23629
GZMK	PE	GM6C3	SantaCruz	sc-56125
GZMB	AlexaFluor700	GB11	BD	561016
Ki67	BB780	B56	BD	624269
GNLY	AlexaFluor488	RB1(RUO)	BD	558254

Figure 2. Multi-parametric flow cytometry panel development. **A.** Configuration of the FACSymphony A5 flow cytometer. WL: Wavelength (nm); Power: mW; LP FILTER: Dichroic Filter; BP: Bandpass Filter; BUV: Brilliant Ultraviolet; BV: Brilliant Violet; BB: Brilliant Blue; **B.** Example panel for Treg characterization in tumors.

Fig. 2 Multiparametric flow cytometry panel development. (**a**) Configuration of FACSymphony A5 flow cytometer. WL: Wavelength (nm); Power: mW; LP FILTER: Dichroic Filter;BP: Bandpass Filter; BUV: Brilliant Ultraviolet; BV: Brilliant Violet; AF: AlexaFluor; BB: Brilliant Blue; (**b**) Example panel for Treg characterization in tumors

5. Python packages: Pandas, Numpy, Sklearn, UMAP, Seaborn, Matplotlib.
6. R packages: ggplot2, pca3d.
7. GraphPad (https://www.graphpad.com).

3 Methods

3.1 Multicolor Flow Cytometric Panel Design

With the development of new cytometers and fluorescent dyes, it is possible nowadays to simultaneously measure up to 40 antigens in each single cell by flow cytometry [10]. In order to succeed in

multiparametric panel design, first of all, rank markers that you want to inspect according to the biological hypothesis need to be addressed. Then, follow the technical advices about (i) selection of antigen-fluorochrome combinations, (ii) reagent titration and validation, (iii) limiting spreading error (SE), as previously described [11].

In the example shown here, we took advantage of a 27-parameter panel (Fig. 2b) that we designed to compare T cells infiltrating non-small cell lung cancer (NSCLC) to those isolated from the cancer-free adjacent lung tissue and peripheral blood of the same patients. In total, we stained 50 tumor samples, 25 adjacent cancer-free lung tissues, and 25 peripheral blood samples. We performed the wet experiments in two different days. Each day we stained blood cells from the same healthy donor (HD) as a technical control to determine reproducibility. This allowed us to investigate biological intersample variation and exclude the contribution of interexperimental variability.

3.2 Staining Protocol

1. Obtain single-cell suspensions from circulating blood or tissues by standard Ficoll density gradient isolation or appropriate tissue digestion protocols (*see* **Note 4**). The human samples used here were obtained as previously described [12, 13].
2. Wash cells with 10 mL $HBSS^{-/-}$ or $PBS^{-/-}$ (*see* **Note 5**) and then count viable cells with Trypan blue. Up to 10^7 cells can be stained with the following instructions. Centrifuge at 515 × *g* for 10 minutes (min) at room temperature (24 °C, RT).
3. During centrifugation, prepare viability dye mix by resuspending 0.5 μL of Zombie Aqua viability dye in 19.5 μL of H_2O, and then add 380 μL of $HBSS^{-/-}$ or $PBS^{-/-}$.
4. Remove supernatant from the pelleted cells and add 100 μL/sample viability dye mix. Mix thoroughly and incubate 15 min in the dark at RT.
5. During incubation, prepare FACS buffer: 2% (vol/vol) FBS in $HBSS^{-/-}$ or $PBS^{-/-}$.
6. Prepare the staining buffer containing 50% (vol/vol) FACS buffer and 50% Brilliant stain buffer (*see* **Note 6**).
7. Prepare the chemokine receptor Ab staining mix: combine optimal antibody concentration in an appropriate volume of staining buffer so that the final volume of antibody mix is 100 μL.
8. Wash the cells by diluting the stain with 2 mL FACS buffer (20-times the staining volume) and centrifuge at 515 × *g* for 5 min at RT.

9. Add the chemokine receptor Ab staining mix to cells. Pulse vortex gently to mix. Incubate for 20 min at 37 ° C (*see* **Note 7**). Protect from light.
10. During incubation, prepare the Ab mix to stain for surface proteins.
11. Wash the cells by diluting the stain with 2 mL FACS buffer (20-times the staining volume) and centrifuge at 515 × *g* for 5 min at RT.
12. Add 100 μL of Ab mix to each sample and incubate for 20 min at RT in the dark.
13. During incubation, prepare FOXP3 Fixation/Permeabilization working solution and 1× Permeabilization Buffer according to the manufacturer's instructions. The solutions must be prepared immediately prior to use (*see* **Note 8**).
14. Wash the cells by diluting the stain with 2 mL FACS buffer (20-times the staining volume) and centrifuge at 515 × *g* for 5 min at RT.
15. Add 250 μL of FOXP3 Fixation/Permeabilization working solution to each tube and pulse vortex.
16. Incubate for 30 min, at 4 °C, in the dark.
17. During incubation, prepare the Ab mix for detection of intracellular proteins.
18. Without washing, add 2 mL of 1× Permeabilization Buffer to each tube.
19. Centrifuge at 515 × *g* for 5 min, and then discard the supernatant.
20. Add intracellular Ab mix buffer to cells and incubate for 30 min, at RT, in the dark.
21. During incubation, prepare compensation controls as follows (*see* **Note 9**):
    - Gently vortex beads.
    - Add 30 μL of beads in polystyrene round-bottom tubes and add the same amount of fluorochrome-conjugated Abs used for the staining. For the Zombie Aqua compensation control, mix 2 drops of ArC Amine Reactive Compensation Beads with 2.5 μL of Zombie Aqua. Use unstained beads as a negative control.
    - Gently vortex tubes and incubate for 15 min at RT in the dark.
    - Wash beads by diluting the stain with 2 mL FACS buffer (20-times the staining volume) and centrifuge at 515 × *g* for 5 min at RT.

- Fix samples with 120 μL of 1% formaldehyde in $HBSS^{-/-}$ or 1× $PBS^{-/-}$ under the chemical hood for 10 min at RT. Protect samples from light (*see* **Note 8**).
- Wash by diluting the stain with 2 mL FACS buffer (20-times the staining volume) and centrifuge at 515 × *g* for 5 min at RT.
- Remove the supernatant under the chemical hood and resuspend the pellet in 200 μL of FACS washing buffer. Keep compensation controls in the dark at 4 °C till the acquisition by flow cytometer.

22. Add 2 mL of 1× Permeabilization Buffer to each tube.
23. Centrifuge at 515 × *g* for 5 min at RT, and then discard the supernatant.
24. Add 2 mL of 1× FACS Buffer to each tube. Centrifuge at 515 × g for 5 min at RT, and then discard the supernatant.
25. Resuspend stained cells in an appropriate volume of FACS Buffer and acquire samples at the flow cytometer.
26. Export Flow Cytometry Standard (FCS) 3.0 files (*see* **Note 10**).

3.3 High-Dimensional Single-Cell Data Preprocessing

1. Upload all FCS files into FlowJo v9.4 and correct for spill-over of fluorochromes by applying compensation, as previously described [14]. FlowJo v10 can be also used for this purpose.
2. The gating strategy to identify Treg cells can be performed as follows (Fig. 3a):
   - First gate on the time parameter versus at least one fluorescence parameters per laser to check that fluorescence is stable during acquisition as in the example provided. If you notice interruptions of event acquisition, exclude the interested area by gating (*see* **Note 11**).

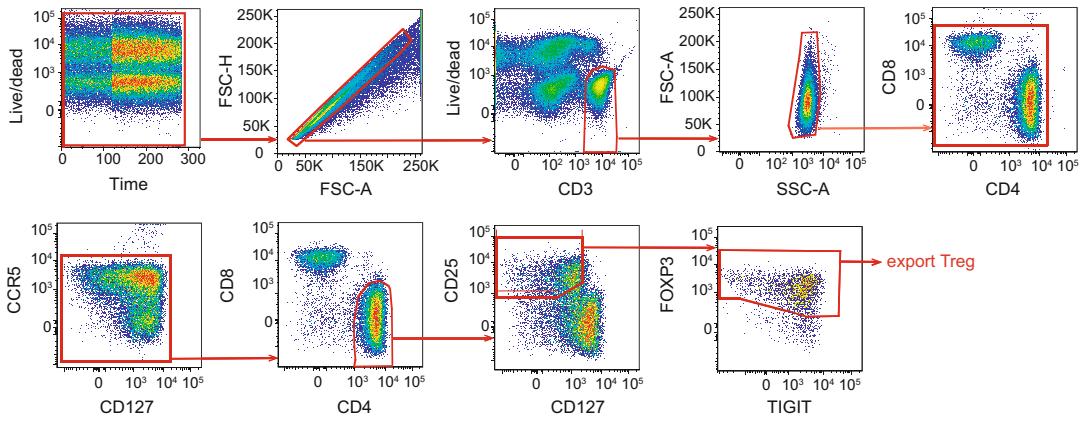

Fig. 3 Data processing before computational analysis. (**a**) Data cleaning and gating strategy applied to isolate live Treg (CD3 + CD4 + CD25 + CD127lowFoxp3+) cells. One representative tumor sample is depicted

- Gate singlets that lie on the diagonal of forward scatter area (FSC-A) versus forward scatter height (FSC-H).
- Gate viable $CD3^+$ (Aqua vs CD3) T cells.
- Gate lymphocytes according to physical parameter: FSC-A versus side scatter area (SSC-A). FSC is proportional to the diameter of the cell while SSC is an indicator of intracellular complexity (granules, the nucleus, etc.).
- Exclude fluorochrome aggregates. These appear brighter than the fluorescence patterns that are generally conserved among the different stains.
- Gate on $CD4^+$ cells.
- Gate on $CD25^+CD127^-$ cells.
- Gate on $FOXP3^+$ cells. Refer to a protocol previously published to set the threshold of positivity for a given antigen [11].

3. Export the same number of Treg cells per sample:
   - Click on the $FOXP3^+$ gate of one sample in the workspace → Select Equivalent Analyses (this command will select the same gate for all samples in the group).
   - Click on the Workspace menu → Export → Select all compensated parameters → Add the number of events (e.g., 200 cells in the example provided). Cells will be selected randomly: the more cells are exported, the slower the subsequent computational analysis will be. Subsequently, → Keep the data type on FCS Files → Click Export and select the folder in which you want to save the files.
4. Open FlowJo version 10 (v10.4) and import the newly exported FCS files.
5. Perform the biexponential transformation:
   - Click on the "T" button → "Customize Axis" → select all parameters → change Scale to "Biex" → Apply.
   - Select one parameter at a time and optimize the biexponential transformation by changing the Width Basis until a correct distribution of the peaks is observed. Avoid the generation of false-negative or false-positive signals by keeping the negative population at the same spot in the axis for all samples.
   - Repeat this step for all compensation matrices (*see* **Note 12**).
6. Export the samples:
   - Select all the samples in the workspace → "File" → "Export/Concatenate" → "Export/Concatenate Group" → Select "CSV – Channel values" in order to export the compensated, transformed files (by mistakenly selecting

"CSV-Scaled values" instead, you will export raw files without compensation or transformation applied. This may generate technical artifacts in the subsequent computational analysis). Subsequently, → Include header: "Stain" and "Parameter" → select all compensated parameters.

- At this point, you can change the names of the exported files. For example, you can choose your own keywords (i.e., tissue origin, stimulation type, clinical information, etc.) that you can add by selecting "Custom" → "Edit" → select your keywords. Make sure to label your samples with a unique computational barcode for further identification in the computational analysis (*see* **Note 10**).

3.4 Computational Analysis: Phenograph Clustering and UMAP Analysis

1. Analyze the single-cell data in Python (3.7.3) taking advantage of our custom-made pipeline called "Cytophenograph" freely available in our GitHub webpage: https://github.com/luglilab/Cytophenograph. Cytophenograph is based on Phenograph package and exploits the k-nearest neighbors (KNN) technique by creating a graph ("network") representing phenotypic similarities between cells and by subsequently identifying communities in this graph. To install the pipeline, you should create a *conda* environment and install one of the freely available text-editors (*Jupyter*, *Visual Studio Code*, or *TextWrangler*) for the execution. In particular, the pipeline is composed of two files: "Phenofunctions.py," containing all functions, and "Cytophenograph.py," executing these functions and enabling to set the variables and indicating where to save the output files.

In the specific example provided here, blood ($n = 25$), adjacent cancer-free lung tissues ($n = 24$), and tumor ($n = 53$) samples were concatenated in a single matrix by using the merge function of *pandas* package. The "k" value, indicating the number of nearest neighbors identified in the first iteration of the algorithm, was set at 500 (*see* **Note 13**). Parameters not useful for separating cell subsets such as physical parameters, "Time" parameter, viability staining, lineage markers (CD3, CD4, CD8), and other markers not expressed by Treg cells (e.g., GNLY, GZMK, CXCR5, EOMES) were excluded from the analysis .

2. Once the pipeline has run, different types of files will be exported (*see* **Note 14**):
   - *Images*: overlays of Phenograph clusters on UMAP plots: name_UMAP.pdf (Fig. 4a).
   - *csv* file with all cells (name_concatenated.csv) ready to be uploaded in FlowJo v10 for visualization.

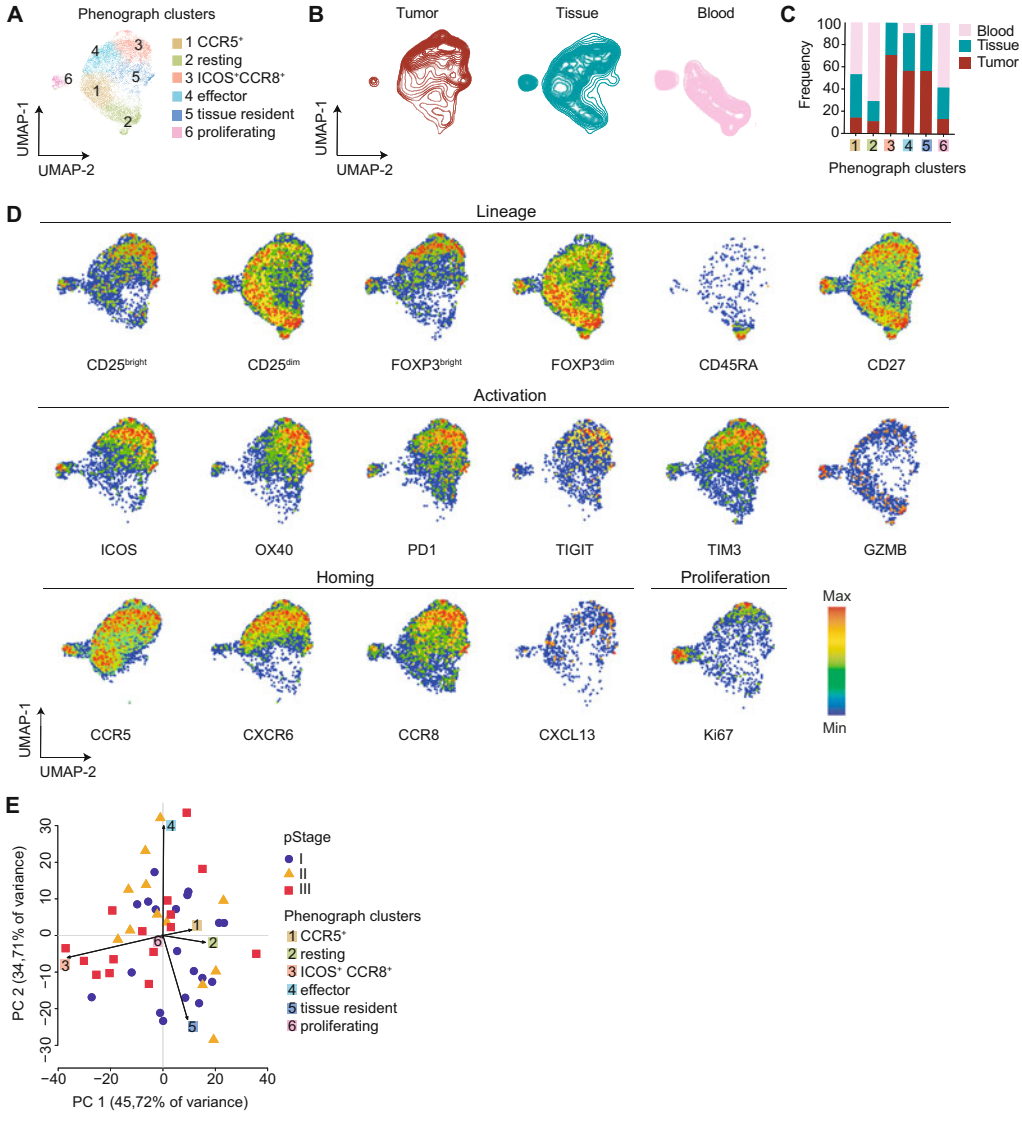

Fig. 4 Dimensionality reduction and clustering output. (**a**) Color-coded Umap depicting clusters identified by Phenograph. (**b**) Umap analysis of concatenated Treg cells from tumor (n = 50, dark red), adjacent cancer-free lung tissues (n = 25, light blue), and peripheral blood (n = 25, pink) samples from NSCLC patients. (**c**) Bar graph depicting the proportion of each Phenograph cluster in different compartments. (**d**) Umap of relative marker expression by concatenate Treg from the same samples in B. (**e**) Principal component analysis (PCA) plot showing the distribution of patients (n = 46) according to the frequency of Treg PhenoGraph clusters in each patient. Patients were classified according to pathological stage (pStage) I, II, or III of the International TNM classification

- *Folder CSV/TSV_sample*: csv/tsv files for each sample. Using FlowJo10, you can concatenate all files that belong to the same category (i.e., tissue of origin) and explore their distribution in the UMAP projection (Fig. 4b).
- A text file with the number of cells per cluster for each sample (Tot_counts.txt) and a text file with the frequency per cluster for each sample (Tot_percentages.txt). You can visualize the content of this .txt file by generating a bar plot in R (the CRAN package *ggplot2*) or GraphPad. Here, we show the proportion of each Treg Phenograph cluster in the different compartments: tumor, adjacent cancer-free lung tissues, and peripheral blood (Fig. 4c).
- *Folder CSV/TSV_cluster*: csv/tsv files for each Phenograph cluster, containing all cells that belong to the given cluster. Draw a gate for each marker and apply it to each cluster from all fcs files. You can take advantage of the concatenate file (name_concatenated.csv) to define a threshold of positivity. If a specific marker is particularly responsible for high SE into the channel of interest, draw the gate by plotting the first versus the second. Finally, you can investigate the relative marker expression in the UMAP (Fig. 4d).
- *txt* file with all cells (name_concatenated.txt) and keywords added by the user (i.e., tissue of origin, stimulation type, clinical information). You can exploit this file to correlate phenotypes and/or frequencies of the clusters with the data of interest (e.g., clinical data). Here, for instance, we performed in R (the CRAN package *pca3d*) a principal component analysis (PCA) showing the distribution of patients according to the frequency of Treg PhenoGraph clusters in each tumor sample. Patients were classified according to pathological stage (pStage I, II, or III) of the International TNM classification. PCA loadings identified Phenograph clusters contributing the most to sample distribution. In particular, $CCR8^{+}ICOS^{+}$ Tregs [4] (Cluster 3) tend to be more abundant in patients with advanced pathological stages II and III (Fig. 4e).

4 Notes

1. Heat FBS in a water bath at 56 °C for 1 h in order to inactivate complement. Prepare FBS in advance. It can be stored at −20 °C until expiration date.
2. For optimal performance of fluorochrome-conjugated antibodies, store vials at 2–8 °C in the dark. Do not freeze.
3. Fixation and permeabilization buffers can influence the staining of both intracellular and cell surface markers by decreasing

the dye fluorescence intensity or altering epitope density, hence resulting in a diminished separation of positive and negative population. It is therefore recommended to titrate all the monoclonal Abs included in the panel by using the same buffer chosen for the staining.

4. All human samples should be always considered potentially infectious and therefore processed following BSL-2 safety precautions. If required, you can freeze the single-cell suspension by resuspending it in 1 mL cold (4 °C) freezing medium mix (10% DMSO in heat-inactivated FBS). The freezing medium mix can be stored at 4 °C for up to 2 weeks. Keep vials for 24/48 h in a cryobox at −80 °C, and then move to liquid Nitrogen container. Thaw the vials in a 37 °C water bath until ice melting. Resuspend cells in prewarmed (37 °C) R10 complete medium implemented with DNase (20 μg/mL). DNase reduces cells aggregates.
5. This step enables to remove serum proteins and thus avoid undesired reaction with the viability dye.
6. The Brilliant stain buffer prevents fluorochrome aggregation and nonspecific binding of antibodies.
7. Many chemokine receptors recycle continuously through early endosomes. Recycling is favored by incubating cells at 37 °C, so more receptors are accessible for staining, thereby resulting in enhanced fluorescent signals.
8. Fixation buffers contain toxic components such as paraformaldehyde: prepare the solution under the chemical hood.
9. Compensation controls are required for each fluorochrome and should contain a positive and a negative unstained population with the same autofluorescence. Generally, beads allow a uniform binding of monoclonal Abs, resulting in brighter fluorescent signals compared to cells. However, for some fluorochromes such as BV711, cells are recommended in order to avoid overcompensation, as previously described [11].
10. In order to avoid problems during the subsequent computational analysis, make sure to only use "_" to separate samples ID names. Don't add spaces or other special characters (punctuations and nonalphanumeric characters) that are not compatible with naming conventions of programming languages (e.g., R/Python). Remember that R/Python is case sensitive (i.e., "name" ≠ "Name").
11. Interruptions in the flow stream and variation in fluorescence during the acquisition can be avoided by filtering samples before acquisition with the flow cytometer. Filtering indeed will help avoiding clogs that prevent a stable flow stream, especially if you work with single cell suspensions derived from tissues, in which cells tend to aggregate.

12. The biexponential transformation is linked to the compensation matrix. In turn compensation values depend on the fluorescence signals in the primary and secondary detectors that can be slightly different between independent experiments. N-by-N overlay between control samples, showing all possible biplots, facilitates the identification of combinations in which biexponential transformation may be different between experiments.
13. Lower "k" values take into account subtle structures in the data and are optimal for detecting small subpopulations. Be aware that they can also identify cell subsets with negligible differences in protein expression that may not describe differences that are biologically relevant. On the other hand, higher k values reveal the most prevalent populations but can prevent the identification of rare populations. It is recommended to always compare PhenoGraph clustering with at least one low and one high k value.
14. All csv files contain UMAP and Phenograph coordinates, shown as parameters in Flowjo v10 (in addition to all those added before launching the "cytophenograph" pipeline in Python). When importing the csv files into FlowJo v10, you need to customize the axes by pressing the "T" bottom next to the marker for optimal visualization: *SSC-A*: log scale (adjust Min and Max values); *UMAP1 and UMAP 2*: linear scale (adjust Min and Max values, so that all cells are in the plot); *Phenograph*: linear scale; (Min = 0 and Max = number of clusters+1); *all markers*: Biex).

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