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Eight-color panel for immune phenotype monitoring by flow cytometry

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ABSTRACT

Flow cytometry (FC) is a fast and highly informative technology that has gained prominence in immune phenotype monitoring. FC standardization is crucial to obtain reliable results that are comparable among laboratories and immune monitoring studies, as this method is influenced by several variables, including equipment, reagents, staining procedures, and pre-analytical and analytical factors. Recent studies have standardized antibody panels and analytical procedures to analyze circulating immune cells in peripheral blood (PB). However, these panels cannot be adapted for laboratories that perform eight-color FC with liquid reagents. The aim of this study was to design and test an eight-color panel, intended to phenotype the main immune cell subsets in PB using liquid reagents and fresh whole blood samples. Samples were collected from healthy individuals recruited from staff and students and from six chemotherapy patients with leukopenia. The antibody panel was designed on the basis of previous studies. Quality controls comprised antibody titration, fluorescence minus one controls, internal controls, and compensation controls. Samples were analyzed by two operators using an eight-color three-laser FACSCanto II flow cytometer (BD Biosciences, USA) and Infinicyt software (Cytognos, Spain). The proposed eight-color panel is composed of six tubes. Analysis of these tubes allowed evaluation of frequencies and classification of various immune cells, such as naïve T, central memory T, effector memory T, CDRA$^+$ effector memory T, activated T, and regulatory T cells; class-switched B, non-switched B, memory B, regulatory B cells, and plasmablasts; myeloid and plasmacytoid dendritic cells, classical and non-classical monocytes; and immature neutrophils. Immunophenotyping of leukocytes using the proposed panel was efficient to correctly differentiate the majority of immune cell subtypes. It is a promising tool to determine the immunological profile of patients in clinical trials and establish associations with disease prognosis, complications, and outcomes.

1. Introduction

The immune system is responsible for defending the organism against pathogens, healing wounds, and clearing neoplastic cells. To be efficient, these multiple responses depend on the balanced and coordinated distribution of a great number of cells with different functions (Proserpio and Mahata, 2016).

Flow cytometry (FC) has become one of the most important tools for immune analysis, especially at a cellular level (Proserpio and Mahata, 2016). Over the past years, FC has gained prominence in studies of primary immunodeficiency diseases (Abraham and Aubert, 2016; Kanegane et al., 2018), solid organ transplantation (Maguire et al., 2014; Sindhi et al., 2016), sepsis (Venet et al., 2011; Monneret and Venet, 2016), and chronic diseases (Kotake et al., 2016; Arregui et al., 2017).

Because of the advantages of FC, such as high speed, sensitivity, and specificity, research groups have focused on standardizing antibody panels and procedures to analyze and classify immune cell subtypes for clinical trials. The ONE Study consortium and the Human ImmunoPhenotyping consortium are examples of such efforts (Maecker et al., 2012; Streitz et al., 2013; Finak et al., 2016; Burel et al., 2017). These studies have analyzed immune cells circulating in peripheral blood (PB), as this tissue is considered a window for a comprehensive analysis of the immune system (Brodin and Davis, 2017).

Standardization of FC is crucial to control the several variables that affect result reliability (Maecker et al., 2010; Maecker et al., 2012). The diversity of equipment, reagents, staining procedures, pre-analytical and analytical factors, and the need of a trained operator (expertise) are among the main issues that increase variability and cause divergent results within and between laboratories (Kalina et al., 2012; Streitz

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et al., 2013).

Antibody panels previously standardized for immune phenotype monitoring were successfully designed to identify subsets of B cells, T cells, dendritic cells (DC), monocytes, and natural killer (NK) cells (Maecker et al., 2012; Streitz et al., 2013; Wingender and Kronenberg, 2015; Finak et al., 2016; Burel et al., 2017). However, these panels and procedures cannot be adapted for laboratories that do not perform FC with more than eight colors or do not work with cryopreserved peripheral blood mononuclear cells (PBMC), lyophilized reagent plates, or dry tubes.

In view of the above, the aim of this study was to design and test an eight-color panel, intended to phenotype the main immune cell subsets by FC in PB using liquid reagents and fresh whole blood samples.

2. Materials and methods

2.1. Samples

For antibody panel design, the study included ten samples collected from healthy individuals recruited from staff and students and from six chemotherapy patients with leukopenia attended at the University Hospital of the Federal University of Santa Catarina, Brazil. Furthermore, the complete procedure was tested in 30 samples collected from healthy individuals recruited from staff and students. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Federal University of Santa Catarina (CEPSH UFSC 1822/2011).

Blood was drawn into vacutainers (Vacuette® Greiner Bio-One, Austria) containing EDTA for anticoagulation. Samples were collected in the morning and processed within 4 h, as recommended by Streitz et al. (2013) (Streitz et al., 2013).

2.2. Antibody panel

During panel design, the fluorochromes for each antibody were selected in order to achieve high sensitivity for the detection of dim antigens, taking into account antigen size. Antibody titration was performed to choose the concentration that provided the maximal brightness of the positive cell population and the lowest signal for the negative cell population (background staining) (Perfetto et al., 2004). Fluorescence minus one (FMO) controls, internal positive and negative controls, and compensation controls were used, as recommended by the literature for the validation of FC multicolor panels (Baumgarth and Roederer, 2000; Perfetto et al., 2004; Kalina et al., 2012; Wang and Hoffman, 2017).

2.3. Sample preparation and staining

EDTA-whole blood was used for determining sample cellularity and for staining of T cells and DC, and PBMC was used for staining of B cells. Staining protocols were developed and are presented in detail in standard operating procedures (see Appendix).

2.4. Data acquisition and analysis

Samples were analyzed using an eight-color three-laser FACSCanto II flow cytometer (BD Biosciences, USA). Instrument setup and calibration were performed daily using Cytometer Setup and Tracking beads (BD Biosciences, USA). In addition, cytometer configuration and compensation were set according to EuroFlow Consortium recommendations (Kalina et al., 2012).

Data acquisition was performed using FACSDiva software (BD Biosciences, USA). For Tube 1, which defined sample cellularity (leukocyte profile), a total of 200,000 events were recorded. Ten thousand events were collected for the unstained control (UC) tube. To standardize the number of analyzed events among samples, 100,000 gated $CD3^+$ events (tubes 2, 3, and 4) and 50,000 gated $CD19^+$ events (tube 5) were collected. With this procedure, it was possible to analyze all events using a defined number of target events, for instance, $CD3^+$ events (T cells). For DC analysis, one million gated $CD45^+$ events were recorded (leukocytes).

Data analysis was performed by two different operators using Infinicyt version 1.7.0 (Cytognos S.L., Salamanca, Spain).

3. Results

3.1. Development of an eight-color FC panel to identify circulating immune cells

The antibody panel was designed on the basis of previous studies (Maecker et al., 2012; Streitz et al., 2013; Finak et al., 2016; Burel et al., 2017) and EuroFlow antibody panels, mainly the lymphoid screening tube (LST) and the panel for T-cell chronic lymphoproliferative diseases (T-CLPD) (van Dongen et al., 2012). Antibody titration, and internal negative and positive controls presented satisfactory results. In addition, the use of FMO controls ensured that the selected combination of antibodies and fluorochromes had no important effect on staining for each antigen and allowed the determination of cut-off values between positive and negative cell populations (data not shown).

3.2. Composition of the eight-color FC panel

The antibody panel is composed of six tubes (Table 1) that allowed to detect the frequencies, differentiation, and activation status of nearly all described circulating leukocyte subsets. Technical information on reagents is detailed in Table S1 of the Supplemental Material.

3.3. Cell subsets, gating strategies, and sample analysis

Figs. 1–6 show gating strategies and sample analyses, which allowed the clear discrimination of all target leukocyte populations.

UC tube allowed to determine the level of background fluorescence

Table 1
Composition of the antibody panel.

Tube	PacB	PacO	FITC	PE	PerCP Cy5-5	PECy7	APC	APCH7
1	CD20/CD4*	CD45	CD8/Igλ	CD56/Igκ	CD5	CD19/TCR γδ	CD3	CD38
2		CD45	CD62L	CD197 (CCR7)	CD4	CD45RA	CD3	CD8
3	HLA-DR	CD45	CD57	CD28	CD4	CD45RA	CD3	CD8
4	CD3	CD45	CD127	CD25	CD4	CD45RA	FoxP3 *	CD8
5	CD20	CD45	IgD	CD24	CD27	CD19	IgM	CD38
6	HLA-DR	CD45	CD16	CD123	CD11c	CD10	CD14	Lin (CD3, CD19, CD20)

Tube 1: Leukocyte profile analysis (based on the EuroFlow LST (van Dongen et al., 2012)); Tubes 2 and 3: T-cell analysis; Tube 4: regulatory T-cell analysis; Tube 5: B-cell analysis; and Tube 6: dendritic cell, monocyte, and neutrophil analysis. APC: allophycocyanin; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PacB: Pacific Blue/V450; PacO: Pacific Orange/V500; PE: phycoerythrin; PECy7: phycoerythrin Cy7; PerCP Cy5–5: peridinin chlorophyll protein; Ig: immunoglobulin; Lin: lineage. *CD4 was conjugated with brilliant violet 421 (BV421) and FoxP3 was conjugated with Alexa Fluor 647 (AF647).

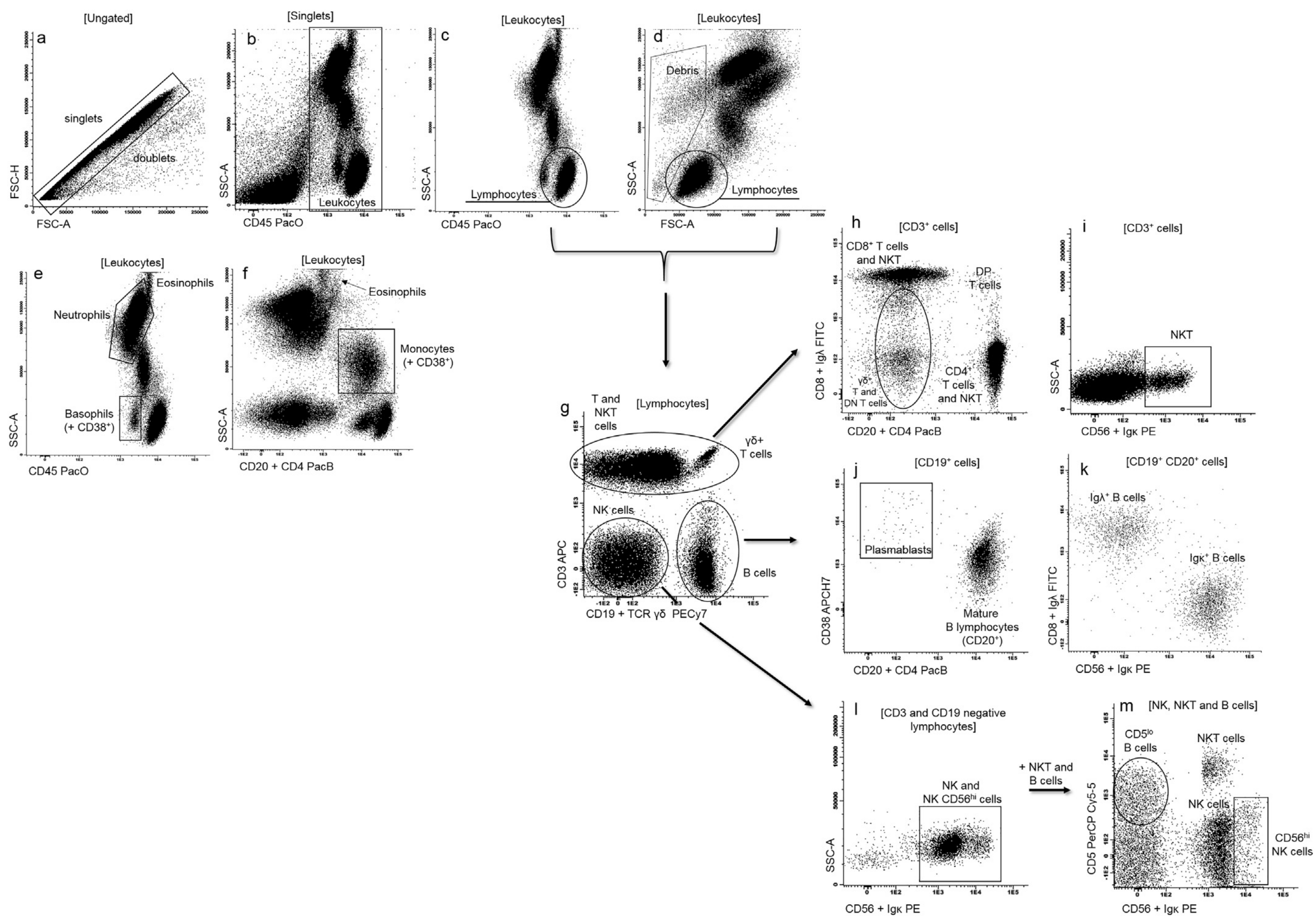

Fig. 1. Gating strategy for the analysis of cell subsets in Tube 1 (screening tube) using the sample of a healthy individual.
Subtitle: The flow cytometry data file of the stained, lysed EDTA-blood was analyzed as follows. Panel a: cell doublets were excluded using forward scatter-area (FSC-A) versus forward scatter-height (FSC-H). Panel b: leukocytes were gated using CD45 expression versus side scatter-area (SSC-A). Panel c and d: gating on lymphocytes (FSC-A versus SSC-A together with high CD45 expression) was performed and debris were excluded using FSC-A versus SSC-A. The analysis strategy shown in panels a-d were applied to all tubes. Neutrophils, basophils, monocytes, and eosinophils were separated by applying the gating strategy shown in Panels e and f. Panel g: gating on lymphocytes, which were further subdivided into T and NKT, $\gamma\delta^+$ T, B, and NK cells. Panel h: gating on $CD3^+$ lymphocytes, which were further subdivided into $CD4^+$ T, $CD8^+$ T, double-positive (DP) T, and double-negative (DN) T cells. Panel i: gating on CD3+ lymphocytes, positivity for CD56 classified NKT cells. Panel j: gating on $CD19^+$ cells, which were further subdivided into mature B cells and plasmablasts. Panel k: gating on mature B cells, which were further subdivided into $Ig\kappa^+$ and $Ig\lambda^+$ B cells to determine the Igκ/Igλ ratio. Panel l: gating on CD3 and CD19 negative lymphocytes, positivity for CD56 classified NK cells, which were further subdivided into $CD56^{dim}$ and $CD56^{hi}$ NK cells. Panel m: analysis of NK, NKT, and B cells by expression of CD5. Hi: high expression; lo: low expression; APC: allophycocyanin; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PacB: Pacific Blue/V450; PacO: Pacific Orange/V500; PE: phycoerythrin; PECy7: phycoerythrin Cy7; PerCP Cy5–5: peridinin chlorophyll protein; and Ig: immunoglobulin.

or autofluorescence of cells (data not shown).

Tube 1 is based on the EuroFlow LST Tube (van Dongen et al., 2012), and its purpose is to determine total sample cellularity (leukocyte profile), that is, relative percentages of neutrophils, monocytes, basophils, and eosinophils; lymphocyte subtypes, including B-cell clonality (assessed by Igκ and Igλ expression); γδ T-cell receptor (TCR) expression; NK cells; and NKT cells (Fig. 1). Tube 2 was designed to analyze and classify T-cell subsets into naïve (N), central memory (CM), effector memory (EM), and $CD45RA^+$ effector memory (EMRA) (Fig. 2). In this regard, we evaluated CD62L expression and CCR7 expression versus CD45RA and observed that CD62L was more efficient to separate these four T-cell subsets (Fig. S1, Supplemental Material). Tube 3 was also used to analyze T cells but taking into account the activation markers CD57, CD28, and HLA-DR (Fig. 3). Tube 4 was designed to assess regulatory T (T reg) cells (Fig. 4). In this study, the combination of markers $CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^{lo}$ was selected to enumerate T reg cells instead of $CD4^+$ $CD25^+$ $CD127^{lo}$, as shown in Fig. S2 (Supplemental Material). Various B-cell subsets can be easily separated by the antibody combination of Tube 5, especially regarding IgD, IgM, and CD27 expression (Fig. 5). Lastly, Tube 6 was designed to evaluate DC subsets as well as monocytes, neutrophils, and basophils (Fig. 6).

Median fluorescence intensity (MedFI) of Tube 1 was compared with EuroFlow LST quality assessment results (Kalina et al., 2015), which are demonstrated in Table S2 (Supplemental Material). Moreover, the results of the analysis of 30 healthy individuals´ samples, means, medians, standard deviations, and ranges of frequencies of major cell populations characterized by each tube, are detailed in Table S3 (Supplemental Material).

4. Discussion

The immune system is composed of a variety of cells that play different roles, such as phagocytosis, antigen presentation, cell toxicity, and self-tolerance regulation. The distribution and quantity of immune cells in PB can vary among individuals, even in healthy individuals, as a result of antigen exposure, cohabitation, and genetic features (Rudolf-Oliveira et al., 2015; Carr et al., 2016; Brodin and Davis, 2017).

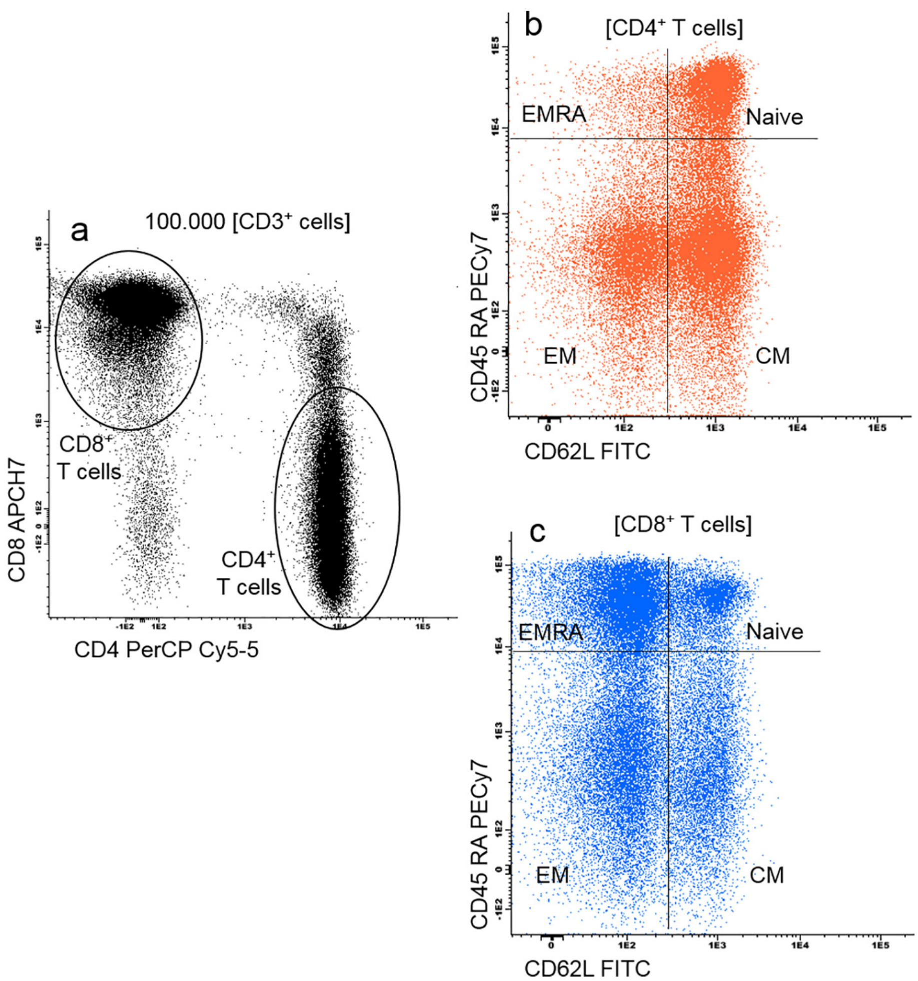

Fig. 2. Gating strategy for the analysis of T-cell subsets (Tube 2) using the sample of a healthy individual.
Subtitle: The data file of the stained, lysed EDTA-blood was analyzed as follows. Panel a: gating on CD3$^+$ lymphocytes (stopping gate set at 100,000 CD3$^+$ events), which were further subdivided into CD4$^+$ T and CD8$^+$ T cells. Panels b and c: gating on CD4$^+$ T cells (orange) and CD8$^+$ T cells (blue), respectively. For both T-cell subsets, gating on naïve (CD62L$^+$ CD45RA$^+$), central memory (CD62L$^+$ CD45RA$^-$), effector memory (CD62L$^-$ CD45RA$^-$), and EMRA (CD62L$^-$ CD45RA$^+$) subsets was performed. APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PECy7: phycoerythrin Cy7; and PerCP Cy5–5: peridinin chlorophyll protein.

Considering this variability, in order to standardize the number of cells analyzed, we defined gating strategies for each tube. For instance, the acquisition of 100,000 CD3$^+$ T lymphocytes allows a clear distinction of T-cell subsets and comparison among samples. For the analysis of samples from patients with severe leukopenia and lymphopenia, the number of recorded events must be chosen with care. In these cases, at least 40,000 CD3$^+$ T lymphocytes, 10,000 CD19$^+$ B lymphocytes, and 500,000 CD45$^+$ cells (for DC analysis) were required for further subtyping (data not shown).

Two of the objectives of this study were to perform all staining processes using only one EDTA-blood tube per individual (the same used for routine blood count) and ensure the simplest possible staining process. Thus, whole blood staining was performed for Tubes UC, 1–4 and 6, which makes the procedure faster and more practical and the results easily comparable between laboratories. PBMC isolation can alter leukocyte composition and activation (Streitz et al., 2013). Studies have shown that the expression of some antigens, such as CD62L, can be affected by density gradient separation of PBMC (Lin et al., 2002) and cryopreservation (Weinberg et al., 2009). Because of the importance of CD62L to classify T cells into N, CM, EM, or EMRA, these data support the choice of staining whole blood for T-cell analysis and classification.

Some studies opted for using CCR7 (CD197) versus CD45RA instead of CD62L versus CD45RA to classify T cells into the four subsets (N, CM, EM, or EMRA) (Maecker et al., 2012; Finak et al., 2016), whereas Streitz et al. (2013) used CD62L, CCR7, and CD45RA for this purpose (Streitz et al., 2013). According to previous studies (Sallusto et al., 2000; Streitz et al., 2013), these T-cell subsets can be distinguished by the following expression: CCR7$^+$ or CD62L$^+$ and CD45RA$^+$ (N), CCR7$^+$ or CD62L$^+$ and CD45RA$^-$ (CM), CCR7$^-$ or CD62L$^-$ and CD45RA$^-$ (EM), and CCR7$^-$ or CD62L$^-$ and CD45RA$^+$ (EMRA). CD45RO (memory and activated T cells) and CD27 (memory T cells) expression can also contribute to T-cell subtyping (Rüdiger et al., 2006; Tonaco et al., 2017). In the present study, CD62L versus CD45RA was more efficient than CCR7 versus CD45RA to separate these four T-cell subsets (Fig. S1, Supplemental Material). In addition, we tested the recommended CCR7 clones 150,503 (Streitz et al., 2013; Finak et al., 2016) and G043H7 (Burel et al., 2017); the latter gave better staining. Thus, we decided to combine CD62L, CCR7 (clone G043H7), and CD45RA (Revenfeld et al., 2016) in Tube 2.

Expression of CD57, CD28, and HLA-DR was evaluated from Tube 3. Clinical trials have focused on analyzing the loss of CD28 expression in CD4$^+$ T cells and its association with idiopathic pulmonary fibrosis (Gilani et al., 2010), end-stage renal disease (Betjes et al., 2008), and multiple sclerosis (Pinto-Medel et al., 2012). There are evidences that CD8$^+$ CD28$^-$ T cells and CD8$^+$ CD57$^+$ T cells play a significant role in various diseases and conditions (Pedroza-Seres et al., 2007; Tulunay et al., 2008; Strioga et al., 2011; Fatone et al., 2018a,b). In fact, CD57$^+$ T cells are late-differentiated cells that have high cytotoxic potential and increase in frequency during chronic immune activation (Kared et al., 2016). Studies evaluated HLA-DR expression in T lymphocytes, which is associated with cell activation, and found a heightened frequency of HLA-DR$^+$ T cells in patients with common variable immunodeficiency (Viallard et al., 2006), idiopathic dilated cardiomyopathy (Ueno et al., 2007), viral infection (Radziewicz et al., 2007), as well as in kidney transplant recipients with microcirculation inflammation (Jung et al., 2017). Taken together, these studies highlight the importance of evaluating CD57, CD28, and HLA-DR expression in T cells in clinical trials to gain a better understanding of patients'

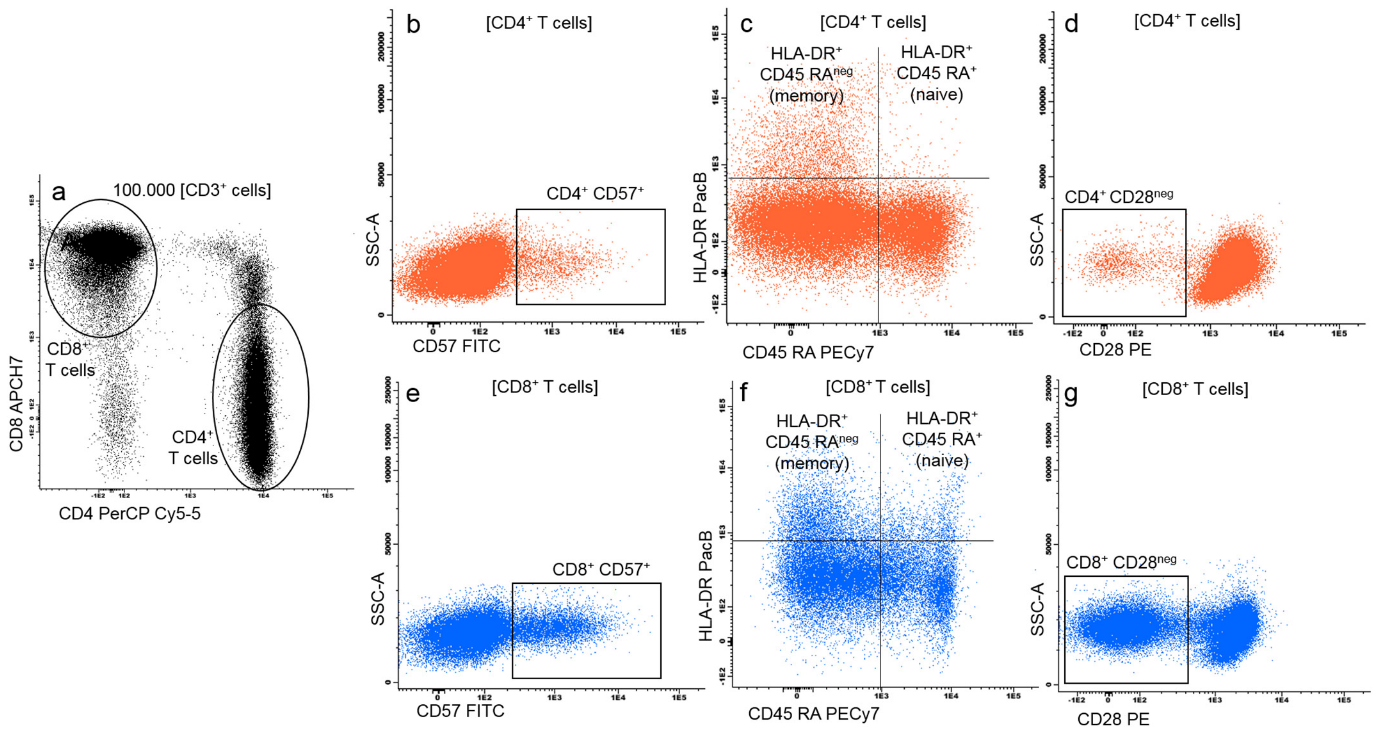

Fig. 3. Gating strategy for the analysis of T-cell activation (Tube 3) using the sample of a healthy individual.
Subtitle: The data file of the stained, lysed EDTA-blood was analyzed as follows. Panel a: gating on CD3$^+$ lymphocytes (stopping gate set at 100,000 CD3$^+$ events), which were further subdivided into CD4$^+$ T and CD8$^+$ T cells. Gating on CD4$^+$ T cells (orange), which were further classified according to the expression of CD57 (Panel b), HLA-DR and CD45RA (Panel c), and CD28 (Panel d). Gating on CD8$^+$ T cells (blue), which were further classified according to the expression of CD57 (Panel e), HLA-DR and CD45RA (Panel f), and CD28 (Panel g). Neg: negative expression; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PacB: Pacific Blue/V450; PE: phycoerythrin; PECy7: phycoerythrin Cy7; and PerCP Cy5–5: peridinin chlorophyll protein.

prognosis, complications, and outcomes.

For the detection of T reg cells (Tube 4), we used the markers CD127 and CD25 and the transcription factor FoxP3, as recommended by previous studies (Santegoets et al., 2015; Pitoiset et al., 2018), and a gating strategy also previously described (Miyara et al., 2009; Murdoch et al., 2012; Santegoets et al., 2015). Klein and colleagues found that CD4$^+$ CD25$^+$ CD127^{lo} cells do not exactly correspond to CD4$^+$ CD25$^+$ FoxP3$^+$ cells, suggesting that CD127 should not be used as an alternative to FoxP3 to identify T reg cells (Klein et al., 2010). In contrast, Liu et al. (2006) suggested that CD127, when analyzed together with CD4 and CD25, could be used as a biomarker for human T reg cells, as it is down-regulated in this cell subset (Liu et al., 2006). In the present study, as shown in Fig. S2 (Supplemental Material), the CD4$^+$ CD25$^+$ CD127^{lo} cell population comprised a higher number of cells than the CD4$^+$ CD25$^+$ CD127^{lo} FoxP3$^+$ population. On the basis of these results, we recommend the use of the four makers combined to enumerate T reg cells. Tube 4 can also provide separation between T reg subsets: FoxP3$^+$ CD45RA$^+$ (naïve or resting T reg cells) and FoxP3$^+$ CD45RA$^-$ (activated T reg cells) (Miyara et al., 2009; Santegoets et al., 2015). T reg cells are essential for self-tolerance regulation, and their frequency and function have been reported to be altered in different clinical conditions, such as autoimmune diseases and immunodeficient conditions (Santegoets et al., 2015).

PBMC isolation can cause cellular loss and activation (Hanekom et al., 2008). Despite these disadvantages, for Tube 5, which received the addition of antibodies against IgD and IgM, PBMC isolation followed by staining of the concentrated PBMC containing a low number of red blood cells was more efficient to obtain a higher number of CD19$^+$ cells than the use of multiple washing steps, as was performed with Tube 1, especially for lymphopenic samples (data not shown). An important step in the preparation of Tube 5 is the initial centrifugation of whole blood followed by plasma separation, which allows patient plasma to be stored at $-80\,^{\circ}C$ for future analysis, such as cytokine assays. Removal and substitution of plasma for PBS/FCS buffer prior to PBMC isolation improves staining of immunoglobulins (Ig), as it washes away unbound Ig molecules. Staining of surface IgD and IgM was performed successfully, resulting in a clear distinction of B-cell subsets (Fig. 5). Circulating B-cell subtypes in PB can be further classified according to their maturation state (Perez-Andres et al., 2010). Over the past decade, studies have recognized a subset of regulatory cells, the regulatory B (B reg) cells, which are linked to the inhibition of excessive inflammation (Blair et al., 2010; Flores-Borja et al., 2013; Rosser and Mauri, 2015). According to these studies, it is possible to detect B reg cells in PB by their strong expression of CD24 and CD38. In the present study, this population was detected by analyzing Tube 5. The importance of analyzing B-cell compartments, especially in diseases that compromise the immune system, such as systemic lupus erythematosus, has been highlighted by previous studies, as B-cell phenotyping can provide information on disease progression and outcome (Blair et al., 2010; Kaminski et al., 2012).

DC were clearly identified from Tube 6. In flow cytometric analysis of PB, plasmacytoid DC (pDC) and myeloid DC (mDC) are rare events, and, for this reason, a minimum of 1 million CD45$^+$ (leukocytes) events must be acquired to identify these subsets. Because DC have an important role in immune responses, both in innate and adaptive immunity (Ueno et al., 2010), studies have analyzed these cells in a variety of clinical conditions, for instance, viral infections (Della Bella et al., 2007; De Carvalho Bittencourt et al., 2012), liver transplantation (Mazariegos et al., 2003), and hepatocellular carcinomas (Han et al., 2014; Tanoue and Kaplan, 2016). DC can be separated into pDC (lineage$^-$ HLA-DR$^+$ CD123$^+$ CD11c$^-$) and mDC or conventional DC (lineage$^-$ HLA-DR$^+$ CD11c$^+$ CD123^{inter}), and mDC can be further separated into two populations according to CD16 expression (Clark et al., 2019). Interestingly, the physiological role of the CD16$^+$ mDC PB

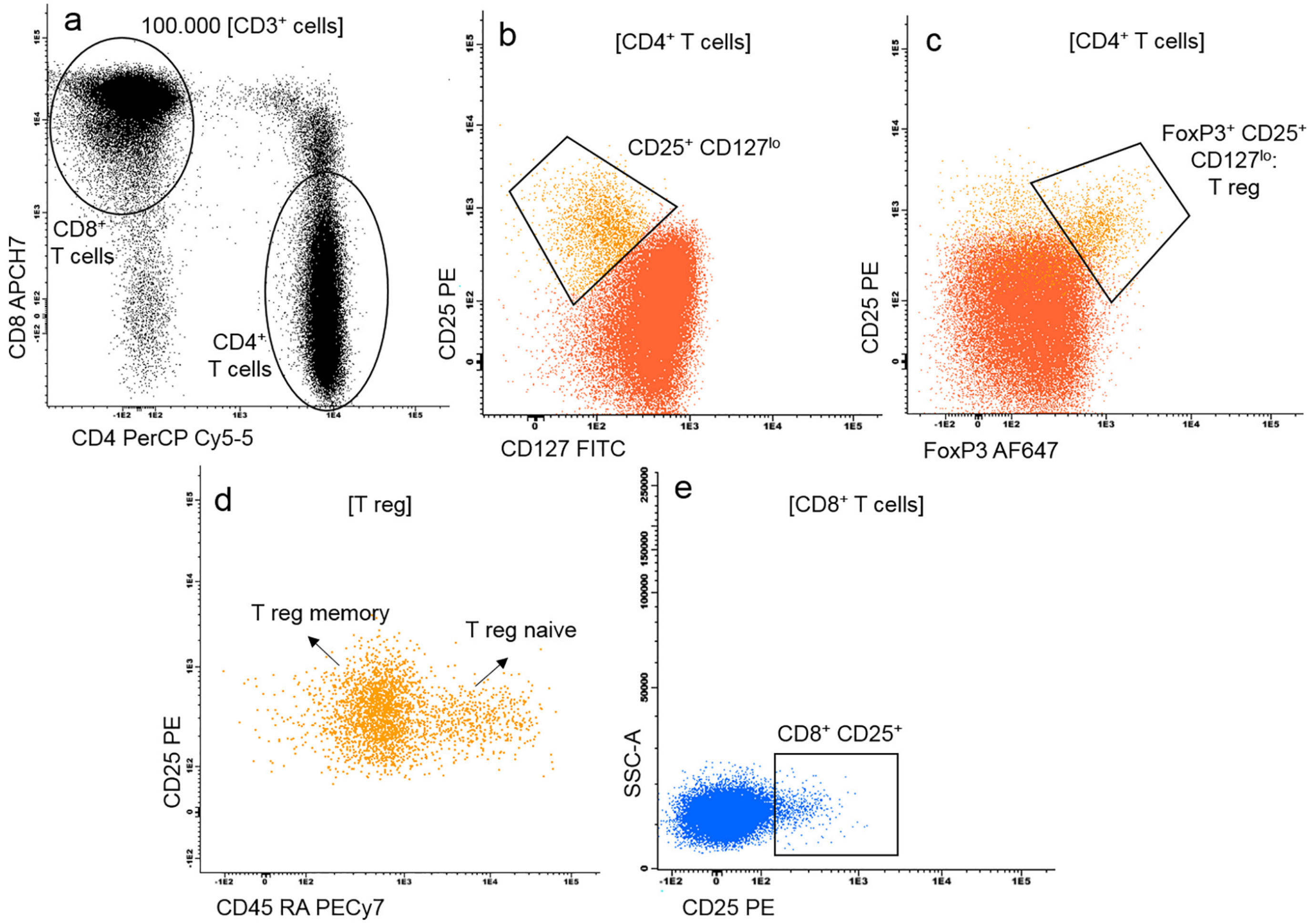

Fig. 4. Gating strategy for the analysis of regulatory T cells (Tube 4) using the sample of a healthy individual.
Subtitle: The data file of the stained, lysed EDTA-blood was analyzed as follows. Panel a: gating on $CD3^+$ lymphocytes (stopping gate set at 100,000 $CD3^+$ events), which were further subdivided into $CD4^+$ T and $CD8^+$ T cells. Gating on $CD4^+$ T cells (orange), which were further separated into $CD127^{lo}$ $CD25^+$ (Panel b) and then into $CD25^+$ $FoxP3^+$ regulatory T cells (T reg), colored in light orange (Panel c). T reg cells were further classified into naïve ($CD45RA^+$) and memory cells ($CD45RA^-$) (Panel d). Panel e: gating on $CD8^+$ T cells (blue), which were further classified into $CD8^+$ $CD25^+$ activated T cells. Lo: low expression; AF647: Alexa Fluor 647; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PE: phycoerythrin; PECy7: phycoerythrin Cy7; and PerCP Cy5–5: peridinin chlorophyll protein.

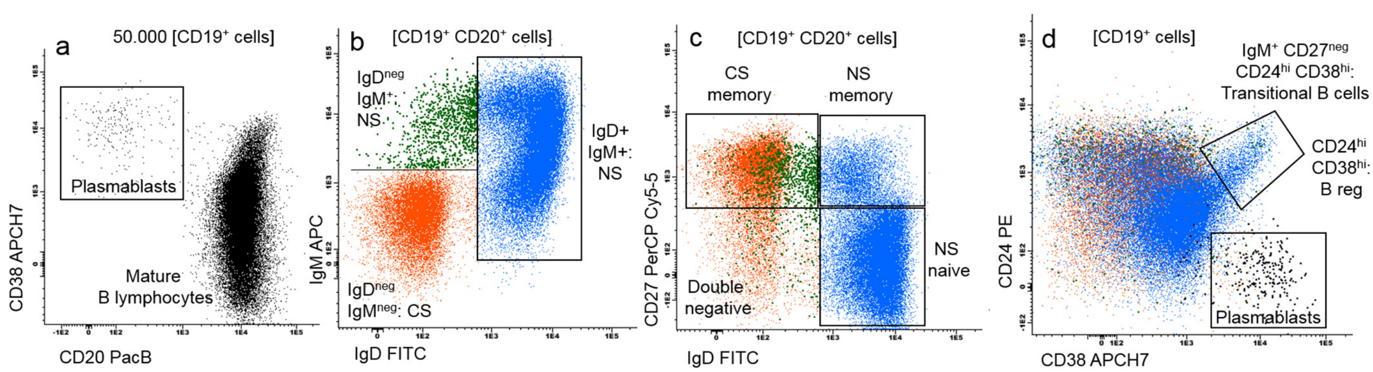

Fig. 5. Gating strategy for the analysis of B-cell subsets (Tube 5) using the sample of a healthy individual.
Subtitle: The data file of the stained peripheral blood mononuclear cells (PBMC) was analyzed as follows. Gating on $CD19^+$ lymphocytes (stopping gate set at 50,000 $CD19^+$ events), which were further subdivided into mature B cells and plasmablasts (Panel a). Mature B cells were further classified according to immunoglobulin (Ig) expression (IgD and IgM) together with CD27 expression (Panels b and c). Transitional B cells and regulatory B (B reg) cells were also enumerated (Panel d). Pre-gated IgD^+ IgM^+ cells (blue) were further used to identify non-switched (NS) naïve ($CD27^-$) and memory ($CD27^+$) B cells. Pre-gated IgD^- IgM^+ cells (green) were also used to identify NS naïve ($CD27^-$) and memory ($CD27^+$) B cells. Pre-gated IgM^- cells (orange) were used to identify class-switched (CS) memory B cells ($CD27^+$) and double-negative memory B cells ($CD27^-$) or CS memory B cells with loss of CD27. Pre-gated IgM^+ $CD27^-$ B cells were used to identify transitional B cells ($CD24^{hi}$ $CD38^{hi}$), and $CD19^+$ $CD24^{hi}$ $CD38^{hi}$ cells were classified as B reg cells (overlapping). In addition to $CD38^{hi}$, plasmablasts are $CD27^{hi}$ (not shown). Hi: high expression; neg: negative expression; APC: allophycocyanin; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PacB: pacific blue/V450; PE: phycoerythrin; PerCP Cy5–5: peridinin chlorophyll protein; and Ig: immunoglobulin.

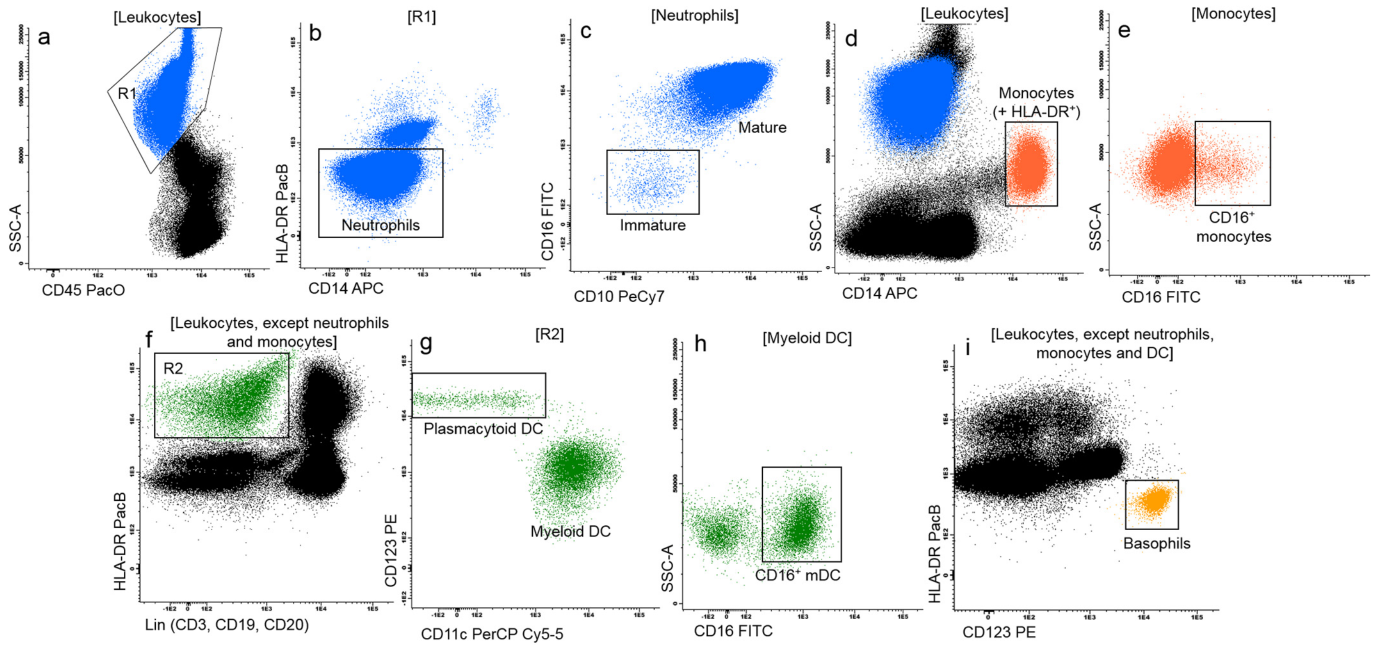

Fig. 6. Gating strategy for the analysis of dendritic cells, monocytes and neutrophils subsets (Tube 6) using the sample of a healthy individual.
Subtitle: The data file of the stained, lysed EDTA-blood was analyzed as follows. Panel a: Leukocytes were gated by CD45 expression versus side scatter-area (SSC-A), and region 1 (R1) was selected. Panel b: gating on R1 and further selection of HLA-DR$^-$ CD14$^-$ cells, which correspond to neutrophils (blue). Panel c: gating on neutrophils, which were further subdivided into mature and immature neutrophils. Panel d: gating on CD14$^+$ HLA-DR$^+$ cells, which correspond to monocytes (orange). Panel e: pre-gated monocytes were used to identify CD16$^+$ monocytes (non-classical monocytes). Panel f: gating on lineage negative cells (anti-CD3, anti-CD19, and anti-CD20) and gating on HLA-DR$^+$ cells, which were classified as region 2 (R2). Panel g: gating on R2 (green), identification of CD11c$^+$ myeloid dendritic cells (mDCs) and CD123$^+$ plasmacytoid DCs. Panel h: pre-gated mDCs were used to identify CD16$^+$ mDCs. Panel i: gating on CD123$^+$ HLA-DR$^-$ cells, which correspond to basophils. DC: dendritic cell; mDC: myeloid dendritic cell; Lin: lineage. APC: allophycocyanin; APCH7: allophycocyanin H7; FITC: fluorescein isothiocyanate; PacB: Pacific Blue/V450; PacO: Pacific Orange/V500; PE: phycoerythrin; PECy7: phycoerythrin Cy7; PerCP Cy5–5: peridinin chlorophyll protein.

subset remains unclear (Ueno et al., 2010; Jin et al., 2014). Besides DC identification and classification, Tube 6 allows the identification of mature and immature neutrophils, monocytes and the analysis of CD16 expression by these cells (the so-called non-classical monocytes), and basophils. Clinical studies (Berres et al., 2009; Zimmermann et al., 2010; Seidler et al., 2012) revealed that circulating non-classical monocytes and weaker expression of HLA-DR in circulating monocytes are associated with disease progression and prognosis.

In conclusion, this work designed and tested an eight-color panel for PB leukocyte profiling to be used in laboratories that perform eight-color FC, particularly those that use EuroFlow antibody panels, as few additional reagents would be required. Analysis of this panel, together with cytokine assays, can provide important information about the immune system. Therefore, it is a promising tool for immune profiling in clinical trials to assess, for instance, the effects of immunomodulatory treatments or the prognosis and severity of diseases that affect the immune system.

The study has the following limitations: 1) Tube 1, adapted from the EuroFlow LST, did not contain all recommended clones because of budget constraints; 2) PB lysis was not carried out using ammonium chloride, as performed by Streitz and colleagues (Streitz et al., 2013). This reagent was not purchased because of financial limitations.

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Conflict-of-interest disclosure

None of the authors has any conflict of interest to declare.

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