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Semi-automated and standardized cytometric procedures for multi-panel and multi-parametric whole blood immunophenotyping

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Abstract Immunophenotyping by multi-parametric flow cytometry is the cornerstone technology for enumeration and characterization of immune cell populations in health and disease. Standardized procedures are essential to allow for inter-individual comparisons in the context of population based or clinical studies. Herein we report the approach taken by the *Milieu Intérieur* Consortium,

Abbreviations: DC, dendritic cell; CM, central memory; CV, coefficient of variation; EM, effector memory; EMRA, effector memory expressing CD45RA; FcR, Fc receptor; FMO, fluorescence minus one; FSC, forward scatter; FVD, Fixable Viability Dye; MFI, mean fluorescence intensity; NK, natural killer cell; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear cell; PMT, photomultiplier tube; RBC, red blood cell; RT, room temperature; SOPs, standard operating procedures; SSC, side scatter; T_N, naïve T cell

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Automation;
Standardization

highlighting the standardized and automated procedures used for immunophenotyping of human whole blood samples. We optimized eight-color antibody panels and procedures for staining and lysis of whole blood samples, and implemented pre-analytic steps with a semi-automated workflow using a robotic system. We report on four panels that were designed to enumerate and phenotype major immune cell populations (PMN, T, B, NK cells, monocytes and DC). This work establishes a foundation for defining reference values in healthy donors. Our approach provides robust protocols for affordable, semi-automated eight-color cytometric immunophenotyping that can be used in population-based studies and clinical trial settings.
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1. Introduction

Multiparametric flow cytometry is widely used for phenotyping immune cell populations in human blood samples. The abundance of reagents and growing technical innovations in the field of cytometry (e.g., mass cytometry, imaging cytometry and spectral analyzers) has further enhanced the enthusiasm for applying these approaches to the management of patients and the phenotyping of healthy individuals. Flow cytometric techniques have been used for more than two decades in clinical laboratories for the enumeration of $CD4^+$ and $CD8^+$ T cells, in the diagnosis of AIDS [1,2], and also in the characterization of lymphoma and leukemic immune cell expansions [3,4]. However, the implementation of standardized procedures within academic research laboratories has recently become a concern for the community, as the absence of such standardization has precluded comparison between studies and experimental settings. Indeed, greater attention is now placed on the requirement for optimized approaches and harmonization of methods [5,6]. Several international initiatives have supported increased standardization of flow cytometry protocols and applications across multiple laboratories that share common scientific or clinical interests. These include the EuroFlow Consortium, which focuses on laboratory procedures for the phenotyping of malignant leukocytes [7,8]; the Human Immunology Project Consortium (HIPC) and European Network for Translational Immunology Research and Education (ENTIRE), which are working together to develop panels for the phenotyping healthy donors [5,6,9]; the ONE study consortium, which is addressing cellular phenotyping in the setting of transplantation [10]; and the Association for Cancer Immunotherapy (CIMT), which have established proficiency panels for different cell populations [11].

The reproducibility of cytometric data depends on five principle criteria: sample type, sample handling, choice of reagents, instrument selection and qualification, and data analysis. In three coordinated reports, we detail the steps that have been taken by the *Milieu Intérieur* Consortium to control for the pre-analytic aspects of cellular phenotyping (reported here), to optimize the analysis of multi-dimensional data [Chen et al. co-submission], which applied together have allowed the characterization of immune phenotype variation in a population of healthy donors [Urrutia et al., in preparation]. Our approach to immune cell phenotyping supports our Consortium's long-term efforts in utilizing cytometric data as a quantitative intermediate phenotype for association studies. Only with accurate and reproducible methodologies can we begin to establish, integrate and share large data-warehouses of phenotypic and genetic data.

Several prior and ongoing efforts have contributed to the challenge of harmonizing methods in academic research laboratories. Particular attention has been given to sample type, with comparative assessments of fresh or frozen purified peripheral blood mononuclear cells (PBMCs) and whole blood [12–14]. Additional parameters that have been considered include panel design [6,8,10], the use of liquid, lyophilized or freeze-dried reagents [15] and the calibration and settings for the optical bench of multi-laser cytometers that permit longitudinal, multi-user or inter-laboratory standardization [7]. In academic studies, however, there is less attention given to the variability introduced by sample handling. In many instances, sample collection is not proximal to core facilities and despite the use of standard operating procedures (SOPs), studies have not evaluated the impact of manual sample handling on the measured cellular phenotypes such as size, granularity and activation state.

In this report, we detail the steps that were taken to establish a robust protocol for immunophenotyping from 100 μl of fresh whole blood, using four eight-color cytometry panels. We present the design of cytometry panels used for phenotyping and quantifying major cell populations present in human blood — T cells, B cells, NK cells, monocytes, dendritic cells, neutrophils, basophils and eosinophils. These data establish the foundation for the analysis of six hundred healthy donors, analyzed over a six-month time interval.

2. Materials and methods

2.1. Human subject materials, reagents and instrumentation used

For optimization studies and panel development, whole blood samples were collected from healthy volunteers enrolled at the Institut Pasteur Platform for Clinical Investigation and Access to Research Bioresources (ICAReB) within the Diagmicoll cohort. The biobank activity of ICAReB platform is NF S96-900 certified. The Diagmicoll protocol was approved by the French Ethical Committee (CPP) Ile-de-France I, and the related biospecimen collection was declared to the Research Ministry under the code N° DC 2008-68. Samples collected as part of the *Milieu Intérieur* population based study were procured by investigators working at BioTrial, Rennes [Thomas

et al., in preparation], and tracking procedures were established in order to ensure temperature controlled delivery to Institut Pasteur, Paris within 6 h of blood draw (Supplementary Protocol #1). In all cases, whole blood was collected using Li-heparin as an anti-coagulant and maintained at room temperature (18–25°) until processing. The cells were stained using commercially available monoclonal antibodies (Table S1), according to the operating procedure developed as described herein. The standardized protocol is provided (Supplementary Protocol #2). We evaluated three red blood cell lysis reagents to optimize the staining protocol: BD FACS lysing solution (BD Biosciences, ref. 349202), Red Blood Cell lysis (Miltenyi Biosciences, ref. 130-094-183) and RBC lysis buffer (eBioscience Inc., ref. 00-4333-57). Fc-receptor blocking antibodies were used (eBioscience ref. 14-9161). Dead cells were excluded using the Fixable Viability Dye (FVD) eFluor 506 (eBioscience, ref. 65-0866) in the PMN and DC panels. For establishing compensation matrices, Mouse (BD CompBead Set Anti-mouse Ig, k ref. 552843) and Rat (BD CompBead Set Anti-rat/hamster Ig, k ref. 552845) compensation beads were employed. The acquisition of cells was performed using two MACSQuant analyzers (Serial numbers 2420 & 2416), each fit with identical three lasers and ten detector (FSC, SSC and eight fluorochrome channels) optical racks. Calibration of instruments was performed using MacsQuant calibration beads (Miltenyi, ref. 130-093-607). The semi-automated staining was performed using the Evo-150 liquid handling system (Tecan). A detailed script for the semi-automated sample processing is provided in (Online Supplementary Data File #1, http://www.milieuinterieur.fr/en).

2.2. Staining protocol for cytometric analysis

Whole blood (2 mL) was washed by mixing fresh whole blood and PBS at a 1:1 ratio, followed by centrifugation at 500*g* for 5 min at 18–22 °C (room temperature). Washed blood and pre-mixed liquid reagents were loaded onto the Freedom Evo 150 liquid handling system. The supernatant was aspirated and discarded, followed by the addition of fresh PBS taking it to the same final volume as input whole blood. Antibody premixes were prepared, shortly spun (about 20 s) and 100 μl of the resuspended cells was aliquoted into tubes containing the pre-mixed antibody cocktail. The samples were shortly vortexed and incubated 20 min in the dark at room temperature (RT). In samples stained with the PMN and DC panels 1 mL of 1x viability dye solution was added, followed by incubation for 30 min in the dark at 4 °C. Thereafter, 1 ml of cold PBS (4 °C) was added to the tubes, which were centrifuged for 5 min at 500*g* and the supernatant was aspirated. All samples, irrespective of the panel used, were resuspended in 2000 μl of 1x RBC lysing solution, shortly vortexed and incubated 15 min at RT protected from light. After centrifugation for 5 min at 500*g*, the supernatant was aspirated, the samples were resuspended in 240 μl PBS and immediately acquired on the cytometer.

2.3. Data analysis and statistical methods

Flow cytometry data were generated using MACSQuantify™ software version 2.4.1229.1 and saved as .mqd files (Miltenyi). The files were converted to FCS compatible format and analyzed by FlowJo software version 9.5.3. Statistical graphs were prepared with the R Software version 3.0.1 (Ref.: R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/), using the ggplot2 graphical package version 0.9.3.1 [16].

3. Results

3.1. Panel design

To enable detection, enumeration and phenotyping of major leukocyte populations present in circulation — PMNs, T cell, B cells, NK cells, monocytes and DCs — we designed four 8-color cytometry panels. The "lineage" panel covered the major cell populations, providing a reference for comparison with other consortia and served as an internal control for other panels (Fig. 1A). The "PMN" panel enabled the classification of neutrophils ($CD16^+Fc\varepsilon RI\alpha^-$ cells), basophils ($Fc\varepsilon RI\alpha^+CD16^-$) and eosinophils ($CDw125^+$) (Fig. 1B). Activation status of neutrophils was assessed by CD62L expression, and used as a marker of healthy donor status. The "T cell" panel was designed to classify $CD4^+$ and $CD8^+$ naïve (T_{naive}), central memory (T_{CM}), effector memory (T_{EM}) and $EMRA^+$ T cell (T_{EMRA}) subsets, utilizing the relative expression levels of CD27, CD45RA and CCR7 (Fig. 1C) [17]. By combining anti-CD8α and anti-CD8β antibodies within the same panel, we were able to distinguish CD8αα, CD8αβ and CD4 CD8αα T cells [18] (Fig. 1C). Information on the activation status of T cells was obtained by surface expression of HLA-DR. The "DC" panel delineates three principle subsets of dendritic cells in peripheral blood: plasmacytoid dendritic cells (pDCs), $BDCA\text{-}1^+$ and $BDCA\text{-}3^+$ conventional dendritic cells (herein referred to as cDC1 and cDC3, respectively) (Fig. 1D).

3.2. Selection of reagents and optimization of semi-automated staining procedure

Careful selection of antibody clones and optimal combinations of compatible fluorochromes is key to establish robust flow cytometry panels. We worked with three antibody suppliers (BD Biosciences, eBioscience and Miltenyi), who provided us with several clones and fluorochrome combinations for each antigen of interest. As previously reported by others [6], significant differences were observed between the different reagents despite their targeting the same cell surface protein. Our selection criteria were (i) specificity of the signal, as based on the staining index that is defined as the difference between the positive and the negative populations and the spread of the negative population [19]; (ii) signal resolution; (iii) availability of desired fluorochrome; (iv) fluorochrome stability (tandem dyes); (v) price and availability of single lot of reagents for cohort study; and, when possible, (vi) availability of CE-IVD format. A complete list of tested antibodies and notable observations concerning their staining performance are reported in Table S1. Two examples (anti CD14 and anti CD8β) are shown to illustrate our testing and selection procedures (Figure S1).

A Lineage

fluorochrome	specificity	clone (vendor)	mg/100mL of blood
eF450	CD3	SK7 (eBio)	0.03
V500	CD14	M5E2 (BD)	0.5
FITC	CD56	NCAM16.2 (BD)	0.06
PE	CD45	HI30 (BD)	0.13
PerCP-Cy5.5	CD16	3G8 (BD)	0.06
PE-Cy7	CD8b	SIDI8BEE (eBio)	0.13
APC	CD19	SJ25C1 (BD)	0.13
APC-H7	CD4	SK3 (BD)	0.06

B PMN

fluorochrome	specificity	clone (vendor)	mg/100mL of blood
eF450	CD62L	Dreg 56 (BD)	0.125
eF 506	FVD	— (eBio)	*
FITC	FCeRIa	AER-37/ CRA1 (eBio)	0.06
PE	CDw125	A14 (BD)	0.12
PerCP-Cy5.5	CD16	3G8 (BD)	0.002
PE-Cy7	CD32	FLI8.26 (BD)	3.06
APC	CD203c	FR3-16A11 (Miltenyi)	0.8

C T cell

fluorochrome	specificity	clone (vendor)	mg/100mL of blood
eF450	CD3	SK7 (eBio)	0.25
V500	HLA-DR	L243/G46-6 (BD)	0.13
FITC	CD45RA	L48 (BD)	0.5
PE	CD8a	BW135/80 (Miltenyi)	0.8
PerCP-eF710	CD27	O323 (eBio)	0.03
PE-Cy7	CD8b	SIDI8BEE (eBio)	0.13
APC	CCR7	FR11-11E8 (Miltenyi)	1.5
APC-H7	CD4	SK3 (BD)	0.06

D DC

fluorochrome	specificity	clone (vendor)	µg/100µL of blood
VioBlue	CD14	TUK4 (Miltenyi)	1.5
eF 506	FVD	/ (eBio)	*
VioGreen	CD19	LT19 (Miltenyi)	0.8
VioGreen	CD3	BW264/56 (Miltenyi)	0.2
FITC	CD1c	AD5-8E7 (Miltenyi)	0.7
PE	CD301	AC144 (Miltenyi)	0.6
PerCP	HLA-DR	AC122 (Miltenyi)	1
PE-Vio770	CD86	FM95 (Miltenyi)	0.6
APC	CD304	AD5-17F6 (Miltenyi)	0.5
APC Vio770	CD141	AD5-14H12 (Miltenyi)	0.5

Figure 1 Organization of panels for whole blood immunophenotyping. Four eight-color panels were established in order to quantify and characterize the major leukocyte populations in circulation. (A) The lineage panel consisted of markers for T cell, B cell, NK cell and monocyte populations. (B) The polymorphonuclear cells (PMN) permitted classification of neutrophils, basophils and eosinophils. (C) The T cell panel assessed $CD4^+$ and $CD8^+$ naïve, central memory (T_{cm}), effector memory (T_{EM}) and effector memory RA^+ (T_{EMRA}) subsets. (D) The dendritic cell (DC) panel classified the three major DC subsets — pDCs, cDC1 and cDC3. Selection of fluorochrome, clone, vendor and optimal dilution for 100 µl of whole blood used in the study is indicated. FVD, fixed viability dye, was used at a 1:1000 dilution.

3.3. Gating strategies

3.3.1. The lineage panel

For the characterization of major leukocyte populations, we first identified $CD45^+$ hematopoietic cells, followed by exclusion of doublets (Fig. 2A). Subsequently, B cells were gated as $CD19^+CD16^-$, and T cells were identified as $CD19^-$ cells followed by $CD3^+$ staining, then analyzed for the expression of CD4 and CD8 (Fig. 2B). Within the $CD3^-$ cells, NK cells were identified as $CD56^+$ and analyzed for their expression of CD16 and CD56. In the population of $CD56^-$ cells, $CD16^{hi}SSC^{low}$ cells were selected in order to segregate monocytes from neutrophils. Further gating identified $CD14^+CD16^{int}$ monocytes and $CD14^{low}CD16^{hi}$ monocytes. Neutrophils were defined as $CD16^{hi}SSC^{hi}$ (Fig. 2B).

3.3.2. The PMN panel

To characterize granulocytes populations, doublets were first excluded (Fig. 3A) and neutrophils were identified as $CD16^{hi}CDw125^-$ live cells. We also assessed the expression of CD62L within this cell population as a marker of activation (Fig. 3B). Basophils and eosinophils were gated within the $CD16^{low/-}$ cells as $Fc\varepsilon RI\alpha^+CD203c^+$ and $CDw125^+$, respectively (Fig. 3C). Of note, we highlight a difference in the staining of different subpopulations of PMN for Fixable Viability Dye (FVD) (Fig. 3A–C), using saponin treated cells as a positive control for dead cells (Fig. 3D).

3.3.3. The T cell panel

T cells were identified as $CD3^+$ cells (Fig. 4A). Upon exclusion of doublets (Fig. 4A), $CD4^+$ and $CD8\beta^+$ were gated and analyzed. We characterized naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and effector memory expressing RA (T_{EMRA}) subpopulations of both T cell subsets, based on their expression of CD45RA and CD27 [17,20] (Fig. 4B). T_N and T_{CM} cells have also been defined by the expression of CCR7 [21]. We therefore assessed the expression of CCR7 by these cell populations. The activation status was determined

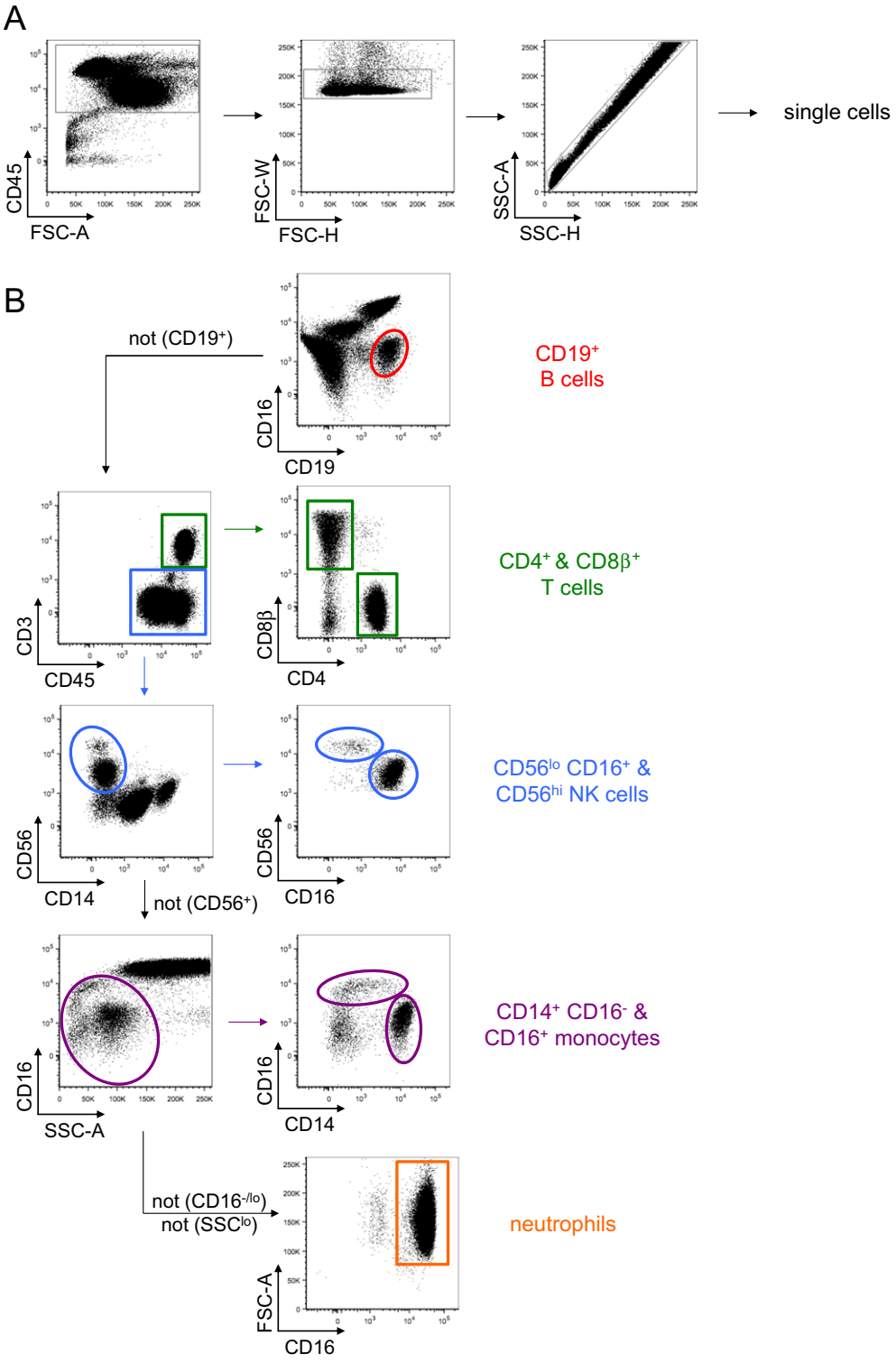
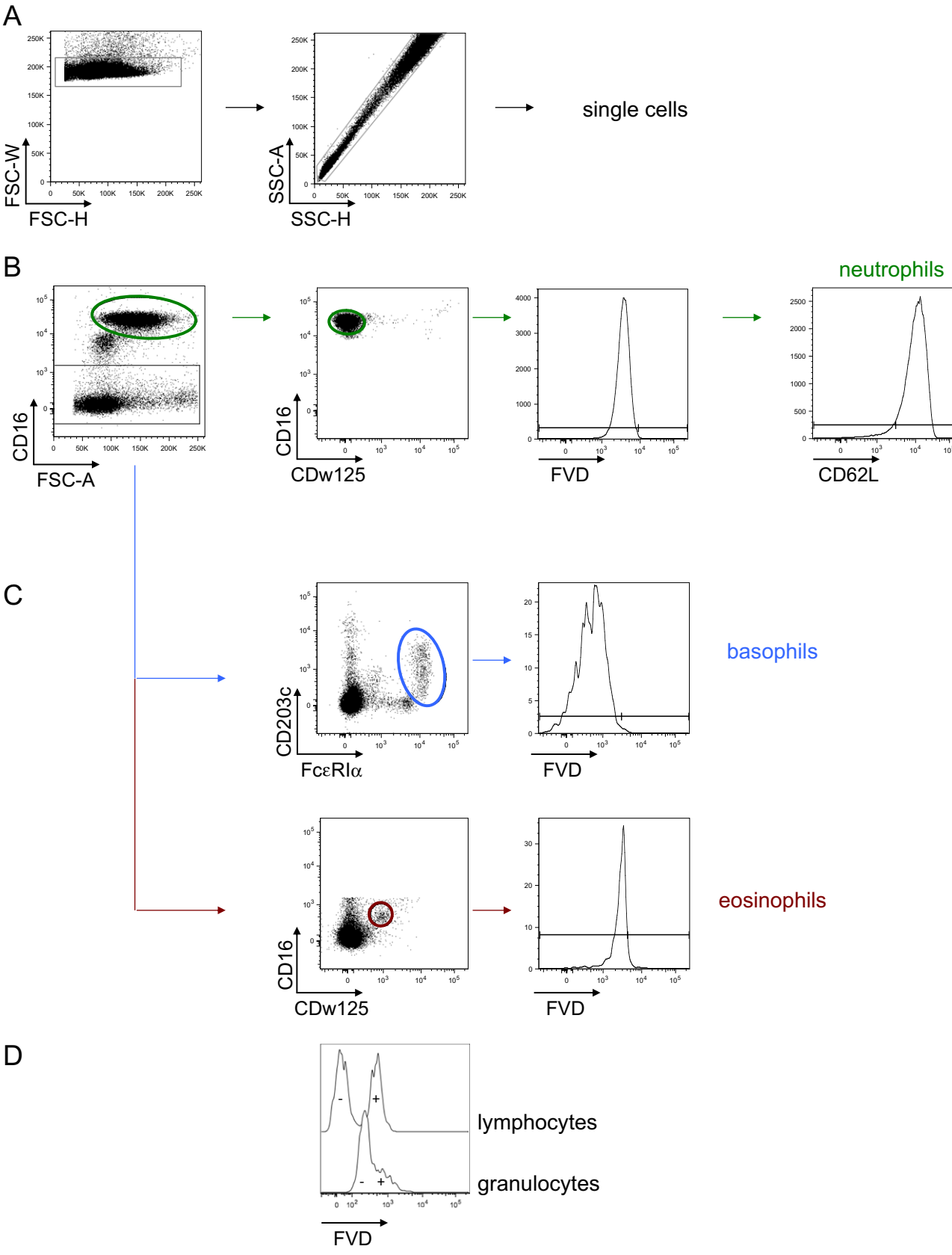


Figure 2 Gating strategy for the lineage panel. (A) $CD45^+$ cells were identified. Doublets were excluded using forward scatter height (FSC-H) and forward scatter width (FSC-W) followed by side scatter height (SSC-H) and side scatter area (SSC-A). (B) Expression of CD19 (red gate) was used to identify B cells. T cells were identified within the $CD19^-$ cells based on CD3 expression (green gate) and analyzed for the expression of CD4 and CD8β. Within the $CD3^-$ cells (blue gate), NK cells were identified as $CD56^+$ and analyzed for their expression of CD16 and CD56. In the population of $CD56^-$ cells, $CD14^+$ and $CD16^+$ monocytes were gated within the $CD16^{+/low}$ SSC^{low} cells (violet gates). Finally, neutrophils were identified as $CD16^+SSC^{hi}$ (orange gate).


A
FSC-W
FSC-H
SSC-A
SSC-H
single cells
B
neutrophils
CD16
FSC-A
CD16
CDw125
FVD
CD62L
C
CD203c
FcεRIα
FVD
basophils
CD16
CDw125
FVD
eosinophils
D
lymphocytes
granulocytes
FVD

by HLA-DR expression. In addition, the $CD4^+$ T cell population expressing CD8αα was identified (Fig. 4B).

3.3.4. The DC panel

To characterize DCs, we first gated on $HLA\text{-}DR^+CD14^-$ and excluded dead cell doublets, and $CD3^+$, $CD19^+$ or $CD14^+$ lineage positive cells using a cocktail of reagents (Fig. 5A). pDC, cDC1 and cDC3 populations were identified as $BDCA4^+BDCA2^+$ ($CD304^+CD303^+$), $BDCA1^+$ ($CD1c^+$) and $BDCA3^+$ ($CD141^+$), respectively (Fig. 5B). The activation status of the three DC subsets was assessed by their expression of HLA-DR and the costimulatory molecule CD86 (Fig. 5C). The position of gates to define cDC subsets was determined using $HLA\text{-}DR^-CD14^-$ cells as a negative control (Fig. 5D).

3.4. Standard operating procedures

To optimize the pre-analytical steps of immunophenotyping, we evaluated different conditions for each step of the protocol (Supplementary Protocol #2). We focused in particular on the temperature and duration of blood storage, as well as on red blood cell lysis and the staining protocol. Finally, to make the procedure amenable to a large cohort study, we implemented a semi-automated procedure using liquid handling robotics.

3.4.1. Sample

The treatment of blood samples has a large impact on cytometry data [15]. One of the biggest considerations was analysis of fresh blood as compared to freezing samples and batching analysis. To assess potential differences in results obtained by immunophenotyping of fresh whole blood versus thawed PBMC in our experimental conditions, we compared results from eighteen healthy donors. We did not observe a major difference in B cell frequencies (Figure S2A, upper panel) or of CD19 mean fluorescence intensity (MFI) values (Figure S2A, lower panel). Lower frequencies of $CD3^+$ lymphocytes and a significantly lower expression level of CD3 were observed in frozen/thawed PBMCs compared to whole blood (Figure S2A). While the frequency of $CD4^+$ and $CD8^+$ lymphocytes was similar in thawed PBMC and whole blood (Figure S2A, upper panels), we noted higher and more variable MFI values of CD4, and lower expression of CD8β in PBMCs as compared to fresh whole blood samples (Figure S2B, lower panels). Analysis of thawed PBMCs revealed lower CD56 expression, but no alteration in frequencies of NK cells (Figure S2B). Other differences were also noted, again with the observation that freeze/thaw introduced variance, especially in monocyte and DC populations (Figure S2C and S2D, lower panels). Based on these data, and the experience of other consortia [4,6], we utilized fresh whole blood samples for our study.

To assess the impact of time between blood draw and staining, we analyzed blood from three healthy donors at four different time-points: immediately after blood draw, and 2 h, 7 h and 24 h post-blood draw. The aliquots were kept at room temperature (18–25 °C) until the analysis. Staining patterns of the analyzed immune cell populations did not change within the first 7 h. However, further delay in time of sample staining and analysis (24 h) had a non-negligible impact on the size and granularity of cells, with an additional population of FCS^{high}/SSC^{low} cells appearing 24 h after collection (Fig. 6). Furthermore, there was a striking impact on the activation status of dendritic cells after 24 h, as observed by an increased expression of HLA-DR on the surface of cDC3 cells (Fig. 6).

We also evaluated the impact of the time between sample collection and processing on the cell numbers of selected immune populations. No differences were observed in the T cell, B cell, neutrophil or dendritic cell numbers (Fig. 7). The notable exception was the number of neutrophils in one of the three donors. Together, our data, based on cell phenotyping and enumeration studies, clearly showed that 6 h post-blood draw is the maximum permitted delay.

3.4.2. Staining protocol

Fresh whole blood samples were washed to eliminate soluble antibodies and other molecules that may interfere with staining. The duration of antibody incubation and staining temperature was evaluated (data not shown). Since blocking of FcR did not have a significant impact on the results (data not shown), it was not included in the staining protocol. The staining was followed by red blood cell lysis. We tested three red blood cell lysis solutions and identified the BD solution to be the most efficient, with reagents from other suppliers being either less efficient (Miltenyi) or slower in achieving RBC lysis (eBioscience) (data not shown). Additionally, we tested different criteria that impact lysis conditions and the reproducibility of the results: duration of incubation in lysis solution, use of mixing and the implementation of a wash step. We highlight that an additional wash step was introduced after red blood cell lysis to ensure complete elimination of the lysis solution, which also showed better preservation of size and granularity characteristics of leukocytes. The staining protocol, established for immunophenotyping of 100 μl of fresh whole blood, is detailed in Supplementary Protocol #2. All tested antibodies were titrated to fit the experimental conditions described in the protocol. To minimize variation of fluorescent signal intensity, only one lot of each antibody was used for staining throughout the whole study.

Figure 3 Gating strategy for the PMN panel. (A) Doublets were excluded from the analysis using FSC-W/FSC-H and SSC-A/SSC-H parameters. (B) Neutrophils were identified based on their high expression of CD16 (green gate), with hierarchical gating to select cells with low levels of CDw125 expression, low levels of FVD and high expression of CD62L. (C) $CD16^{low/-}$ cells were independently valuated for high FcεRIα expression and intermediate CD203c expression (blue gate), a phenotype characteristic of basophils; or intermediate expression of CDw125 and CD16 (red gate), hallmarks of eosinophils. (D) Whole blood was incubated with 1% saponin for 1 min, washed with PBS and stained with FVD. Granulocytes and lymphocytes were gated based on their size and granularity. The different levels of auto-fluorescence of lymphocytes and granulocytes used to set the gates in (B) and (C) are shown.

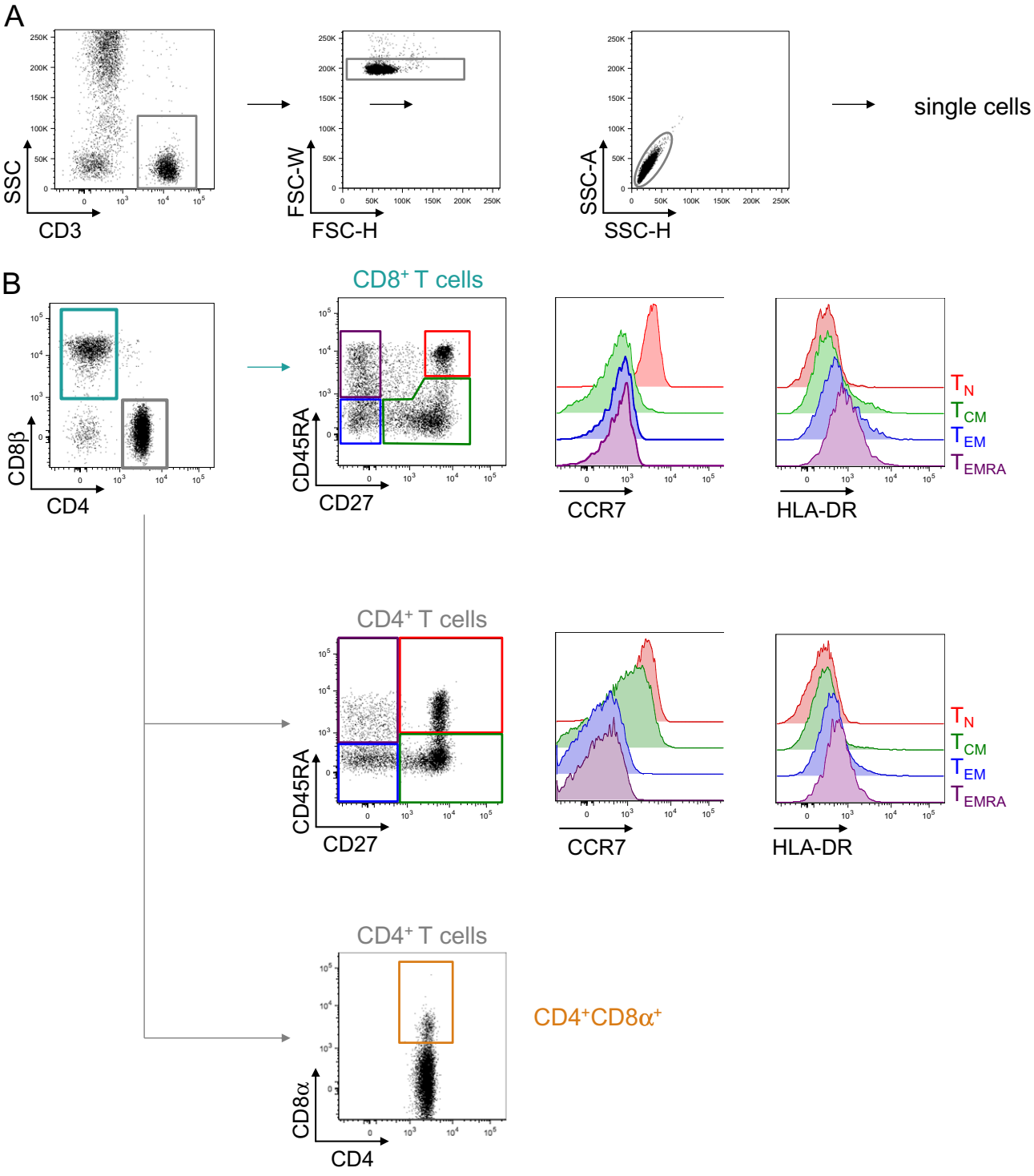


Figure 4 Gating strategy for the identification of T cell populations. (A) $CD3^+$ cells were identified. Doublets were excluded using FSC-W/FSC-H and SSC-A/SSC-H parameters. (B) Subsequent phenotypic analysis identified $CD4^+$ and $CD8^+$ T cell subsets based on CD4 and CD8β expression, respectively. $T_{Naïve}$ ($CD27^+CD45RA^+$ cells, and red shaded histograms), T_{CM} ($CD27^+CD45RA^-$ cells, and green shaded histograms), T_{EM} ($CD27^-CD45RA^-$ cells, and blue shaded histograms) and T_{EMRA} ($CD27^-CD45RA^+$ cells, and violet shaded histograms) were based on surface expression of CD27 and CD45RA. On each of the eight respective populations, CCR7 and HLA-DR expression was analyzed and plotted as a histogram. (C) The $CD4^+CD8\beta^-$ cells expressing CD8α were identified (orange gate).

While we were finalizing our study a comprehensive report by Kalina et al. described the efforts of the Euroflow Consortium to standardize cytometry protocols. We noted that our independently established procedures were very similar to the ones described by Kalina et al. and further emphasized the importance of reagent selection and staining conditions [7].

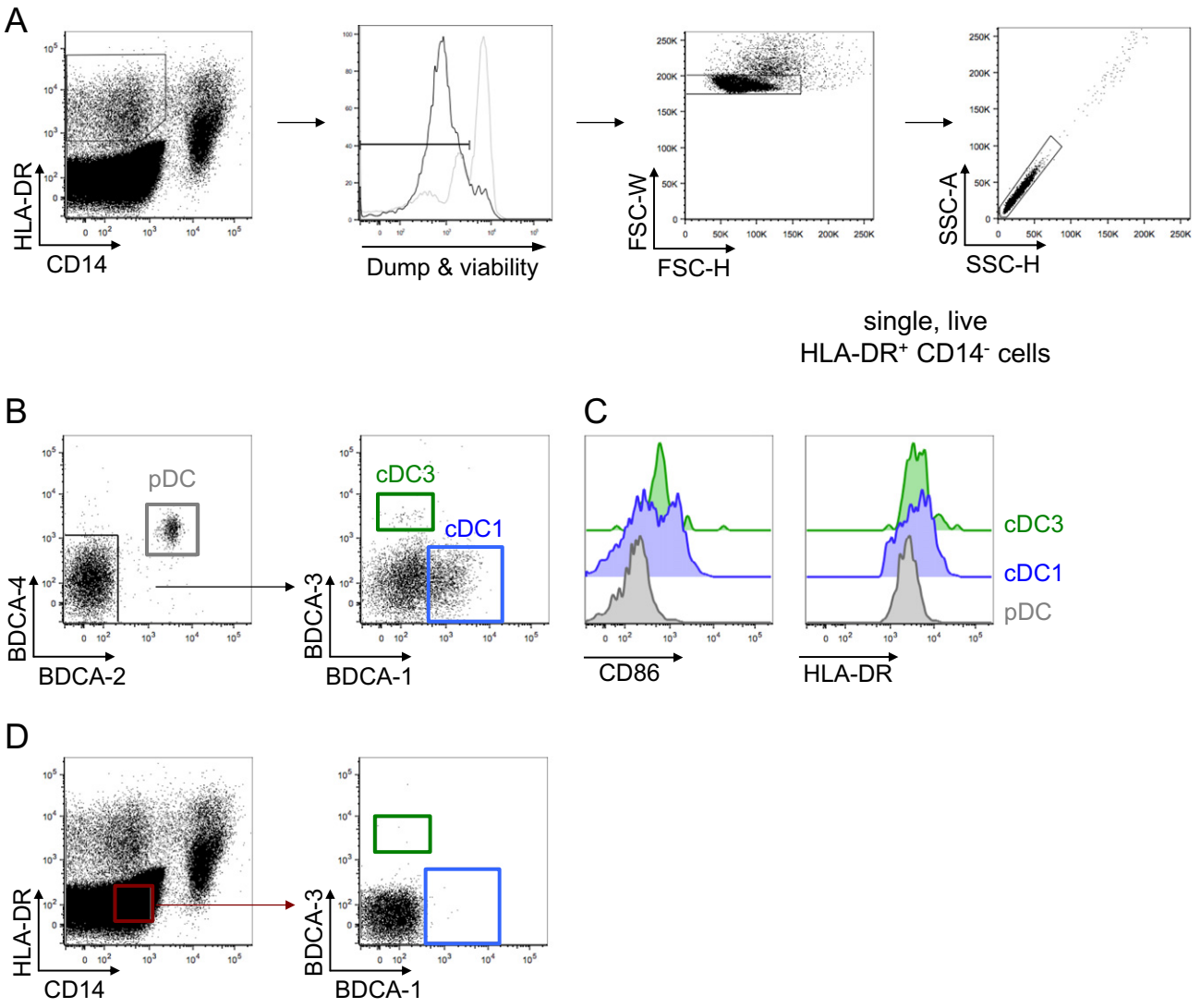


Figure 5 Gating strategy to identify dendritic cell populations. (A) HLA-DR^{+}CD14^{-} cells were identified and lineage-marker expressing cells and dead cells were excluded, followed by selection of single cells based upon FSC and SSC properties. (B) pDCs were identified by co-staining with BDCA-2 and BDCA-4 (gray gate). (C) BDCA2^{-}BDCA4^{-} cells were further characterized as cDC1 and cDC3 based on their expression of BDCA1 (blue gate) or BDCA3 (green gate), respectively. (C) All three DC subsets were evaluated for their expression of CD86 and HLA-DR with comparative histograms shown for the respective subsets. (D) HLA-DR^{-}CD14^{-} cells were used as an internal control for establishing the MFI cut-off values used for identification of the cDC1 subset.

3.4.3. Automation

All clinical laboratory tests use automation in sample processing and attempts have been made to implement automation in genomic assays (DNA/RNA extractions, genotyping, microarray assays, etc.). We decided to take advantage of automation in sample preparation for cellular immunophenotyping. To achieve this, we implemented our protocol using the EVO150 liquid handling platform (Tecan). The premix of antibodies was prepared manually on a daily basis, and all other steps for the staining protocol were performed using the liquid handling platform, with the exception of centrifugation. The pipetting scripts for the platform were created to enable staining of 4 to 12 samples, in parallel, in 96-deep well plates (Online Supplementary Data File #1, http://www.milieuinterieur.fr/en).

3.4.4. Setting of pre-acquisition parameters

Our study complies with the MIFlowCyt requirements [22]. Dead cells were excluded using FVD in the PMN and DC panels, in which either rare populations needed to be identified, or a high autofluorescence of target cells was expected. A dump channel was included in the DC panel to exclude CD19^{+}, CD3^{+}, NKp46^{+}, or CD14^{+} cells to further improve specificity, and in accordance to prior studies [23]. For antibodies that showed weak signals and did not enable clear separation of positive from negative cell populations (e.g., CCR7), we used FMO (fluorescence minus one) staining to set the positive/negative cell gates. During the first two months of the study, compensation controls were run every day, using automatic hardware compensation on MACSQuant. The compensation beads were used to calculate the

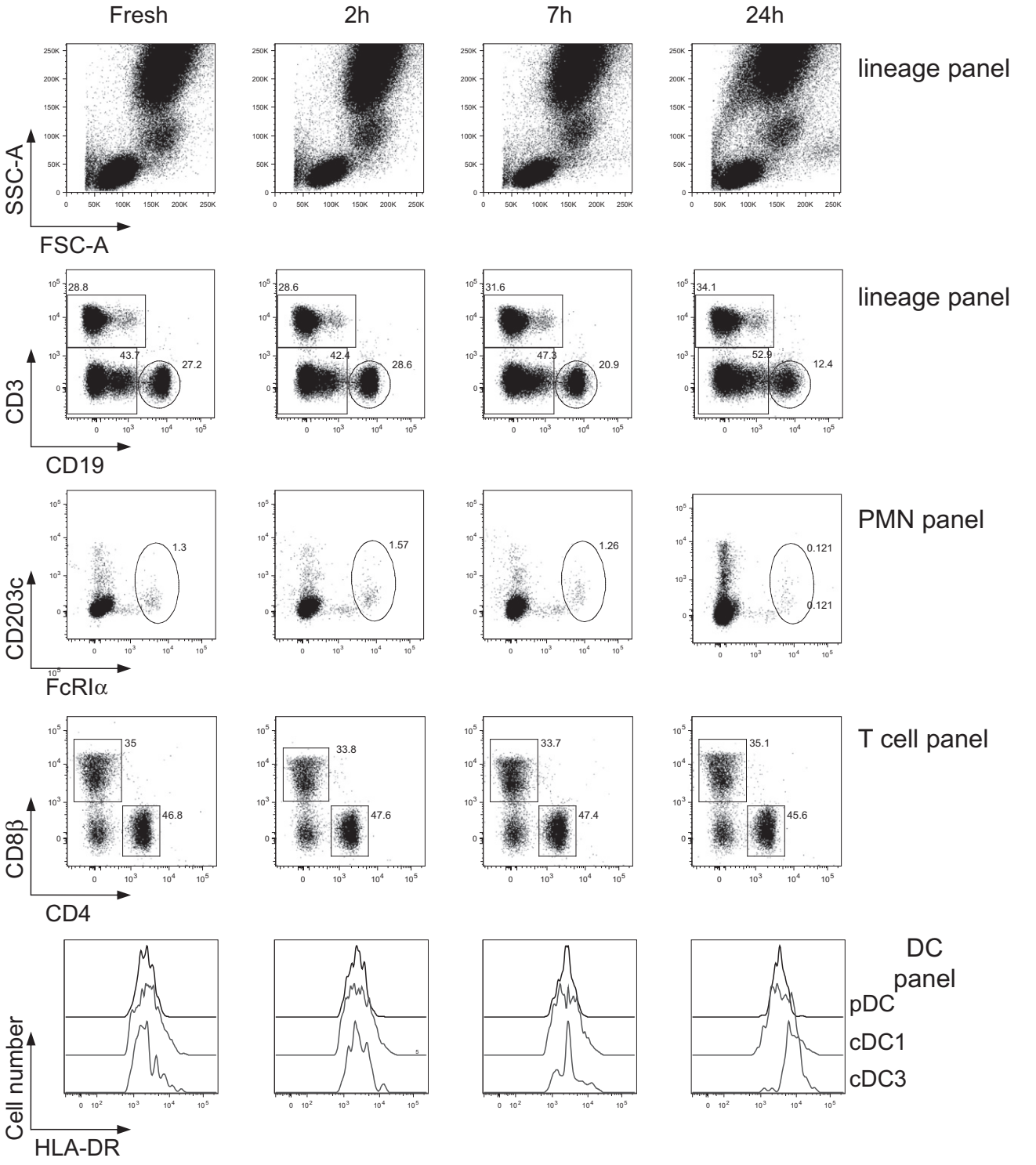


Figure 6 Optimization of sample processing and staining protocol. Whole blood was collected and stored at room temperature (18–25 °C) for 0 h, 2 h, 7 h or 24 h prior to the staining. Representative results of three independent experiments are shown for selected cell populations. The dot plots illustrate the impact of staining at different time-points after sampling of blood based on FSC/SSC characteristics and expression of selected cell surface markers, and on the MFI of the HLA-DR expressed on pDCs, cDC1 and cDC3.

compensation matrix for all antibodies, except for those labeled with Horizon V500 (BD) and dead cell marker (FVD eF506, eBio). For these reagents, cells were used as recommended by the suppliers. Consistent with EuroFlow consortium results [7], our compensation matrices did not change (PMT values varied <+/−5 V, data not shown). We thus decided to run compensation controls bi-weekly, unless the PMT voltage values reported by the cytometer after the daily set-up varied for >+/−5 V from the values obtained during the prior compensation run. In order to control for

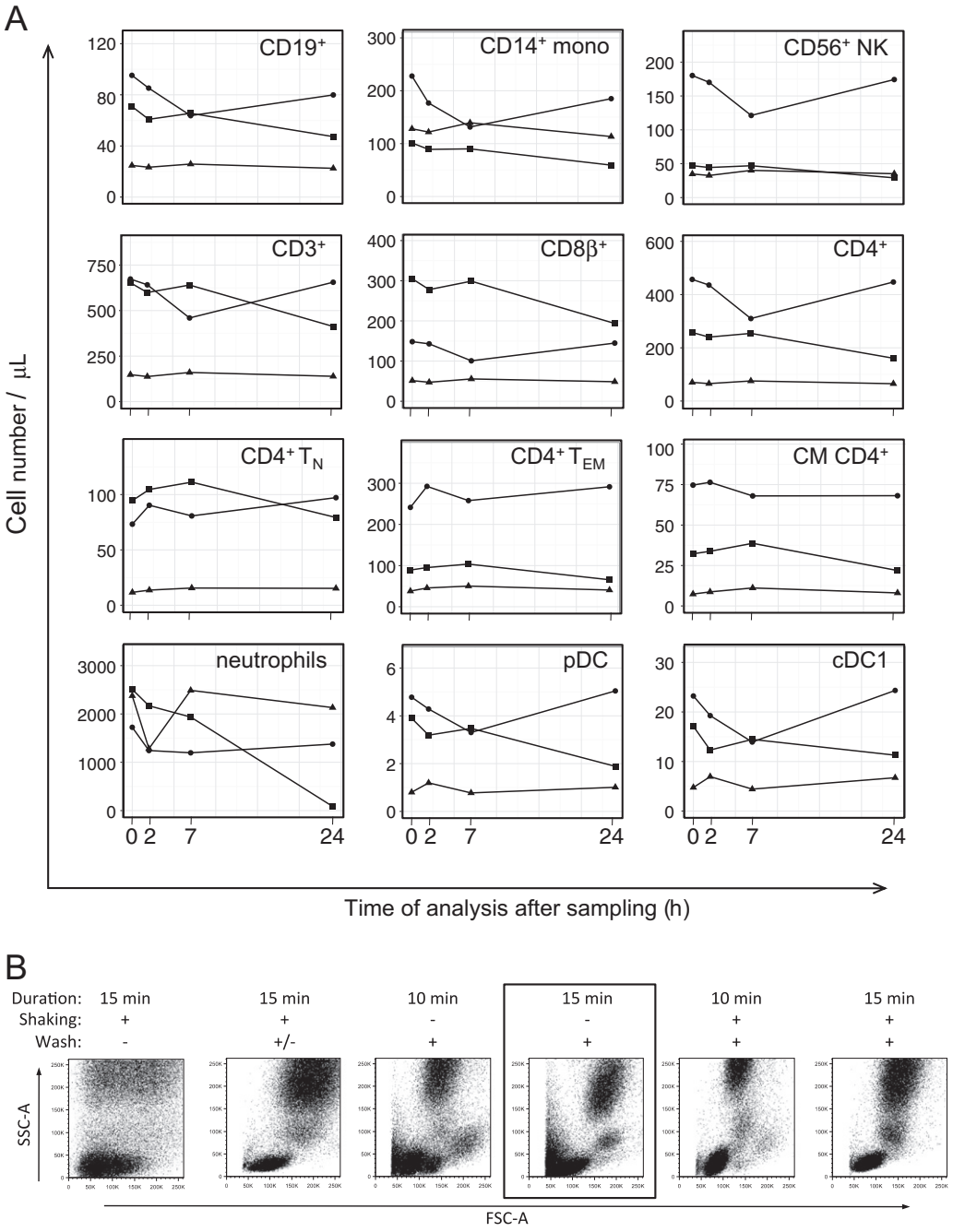


Figure 7 Optimization of sample processing and staining protocol. (A) Blood was stained as described in Fig. 6 and the impact of delayed staining is represented for samples obtained from three donors. Individual donors are indicated by symbols (filled triangle, square, circle). (B) Lysis procedures were evaluated using 100 µl of whole blood, mixed and incubated as indicated. The cells were acquired and their size and granularity characteristics were analyzed based on FCS and SSC parameters. The conditions selected for the study are highlighted by a black square.

cross-contamination, samples were plated with PBS filling every other well in the 96-well plates. None of the negative wells showed positive cells for any of the four panels (not shown).

3.4.5. Cytometer

Initial testing of panels was performed using an LSRII cytometer (BD) equipped with 4 lasers (488 nm, 405 nm and 630 nm and 658 nm). The design of our study (four 8-color cytometry panels) required an instrument that enables automatic acquisition from 96-well plates, the acquisition of absolute cell counts and at least 3-lasers. We compared two cytometers that corresponded to these criteria and that were commercially available at the beginning of the study (initiated in 2012): the BD FACSVerse and Miltenyi MACSQuant. The cytometers were evaluated based on their hardware, sample acquisition performance, software robustness, quality control and post-installation support provided by the suppliers

Table 1 Test of repeatability — technical replicates*.

Cell type	Parameter	Stat	Panel	Donor					
				1	2	3	4	5	6
T cells	CD3+	Median (CV)	T	1019 (9.2)	527 (14.4)	271 (9.2)	537 (5.8)	736 (13.0)	623 (4.1)
			Lin	1020 (9.8)	530 (09.0)	260 (4.0)	536 (4.2)	694 (09.7)	603 (3.9)
	CD8+	Median (CV)	T	165 (9.0)	127 (14.7)	66 (8.1)	194 (6.2)	149 (14.9)	151 (5.6)
			Lin	175 (9.8)	135 (10.1)	63 (3.8)	209 (3.9)	157 (09.8)	160 (4.0)
	CD4+	Median (CV)	T	768 (14.6)	359 (14.6)	170 (9.5)	768 (5.7)	516 (14.3)	347 (4.1)
			Lin	757 (09.6)	357 (08.3)	161 (4.1)	284 (4.5)	473 (09.6)	413 (3.9)
Monocytes	CD14+	Median (CV)	Lin	334 (10.0)	96 (16.1)	216 (02.7)	220 (3.5)	284 (11.3)	149 (7.8)
			DC	283 (06.4)	85 (16.3)	228 (10.5)	227 (7.6)	154 (22.9)	144 (7.4)
DC	pDC	Median (CV)	DC	3 (09.9)	2 (15.2)	2 (8.7)	5 (11.2)	4 (21.5)	2 (11.8)
NK cells	CD56+	Median (CV)	Lin	152 (11.1)	95 (10.9)	96 (5.1)	122 (04.2)	142 (10.4)	100 (03.4)
Granulocytes	Neutrophils	Median (CV)	Lin	2040 (4.4)	650 (11.1)	797 (6.2)	1060 (7.1)	1770 (06.0)	1590 (5.8)
			PMN	2652 (6.3)	1247 (08.4)	1135 (3.0)	1643 (5.5)	2052 (10.3)	1802 (7.4)

* Fresh blood samples from six healthy donors were divided in five aliquots each and immediately stained. Shown are absolute cell numbers (median value) of selected cell populations per 1 μl of blood. For each immune cell population identified, the intra-panel coefficient of variation (CV) was calculated. In instances where cell populations could be identified by two different panels, both data are reported. The cell numbers of each indicated cell population obtained by PMN, T and DC panels were calculated upon normalization of the total cell number obtained by each panel to the total cell number as quantified by the lineage (Lin) panel.

(Supplementary Table 2). Although the hardware characteristics, quality control and the concept of the software were comparable between the two systems, MACSQuant showed significant advantages concerning the software robustness and performance. In addition, its cell counting feature was reproducible and easy to assess. The MACSQuant's SOP requires a daily quality control check using control beads to monitor the performance of instrument. The cytometer enables identification of "bank settings" for up to 5 different panels. These settings store the target values of MFI for five combinations of up to eight antibodies/fluorochromes (5 eight-color panels). On each occasion that the control beads are run the system automatically sets the PMT values so that the intensity of signal matches predefined MFI values, and thus overcomes fluctuations or decrease of laser power ensuring data reproducibility. Two MACSQuant cytometers were installed with a distinct bank settings programmed on the instruments for the panels used in the study.

3.5. Data analysis

We selected the FlowJo software to analyze data. In order to improve standardization of analysis, we created analysis templates for each panel. A template consists of the gating strategy specific for the given panel, including a pre-defined table with parameters selected for statistical analysis. Magnetic gates were applied for the brightest and most clearly defined antigens to minimize bias introduced by manual repositioning of gates. Identical gate coordinates were selected to gate on the same cell populations across the four panels. The results obtained for each of the samples were verified by an operator prior to final validation. In order to minimize bias introduced by subjective analysis by different individuals, a given panel was analyzed by the same individual for all samples. The statistical parameters selected for the analysis included absolute cell number of each cell population of interest, its percentage in respect to relevant parent populations, gate coordinates to monitor fluorescence intensity and spread of fluorescent signal, and MFI values for cell populations in which activation markers were included (e.g., HLA-DR, CD86).

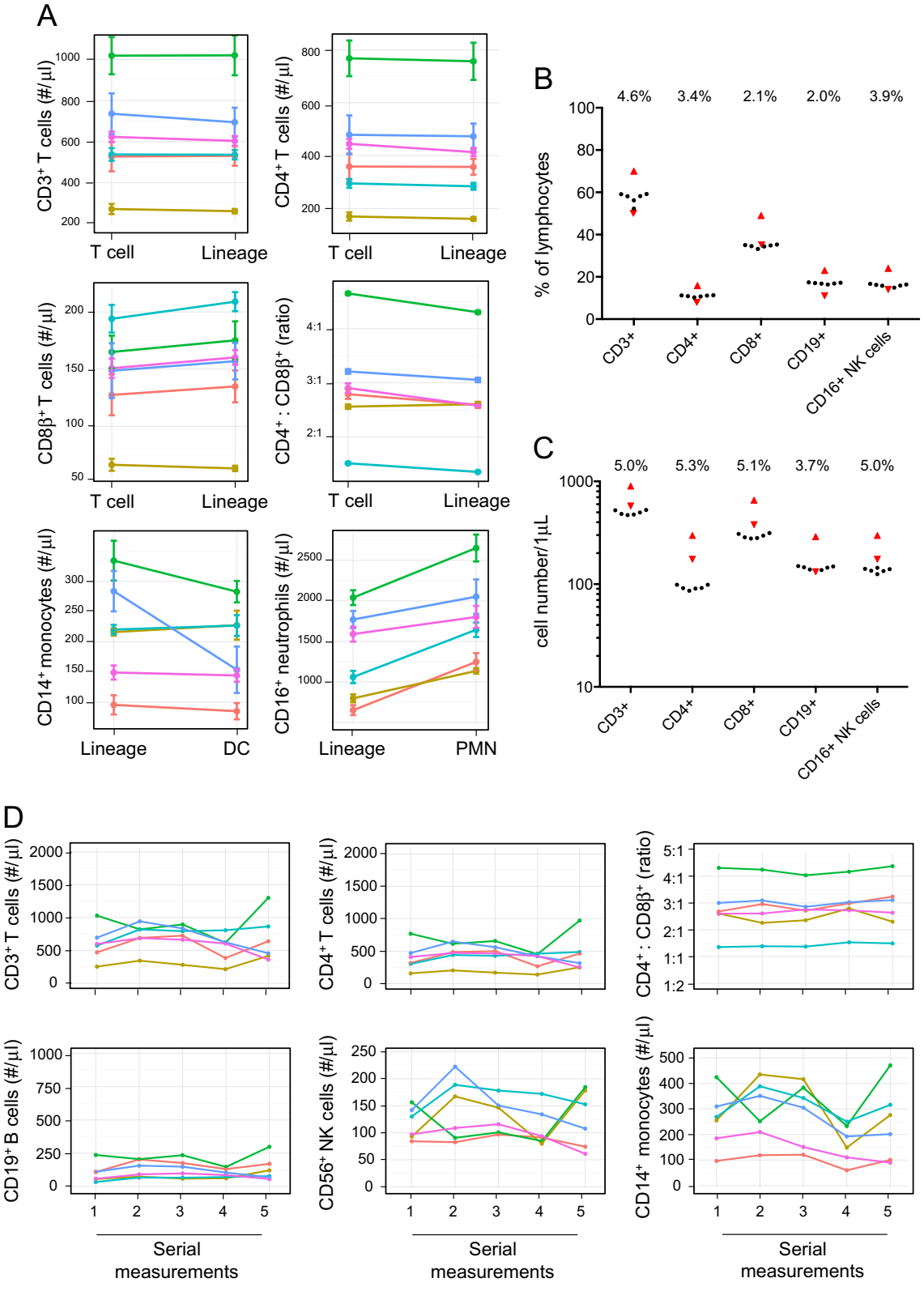
The bank settings programmed on the selected cytometers enable standardization of fluorescent signal, but do not enable standardization of cell size or granularity, features that vary based on sample handling (Supplementary Fig. 3). In order to take advantage of the standardization procedures of the newest cytometers, we omitted the

Figure 8 Repeatability studies, longitudinal studies and panel-to-panel correlations illustrate assay stability. (A) Fresh blood samples from six individual donors were divided in five aliquots for repeatability study each and immediately stained using defined semi-automated procedures. The immune cell populations were identified and absolute cell numbers obtained using above-indicated gating strategies. The graphs show inter-panel comparison of the indicated cell populations as obtained by two different panels (lineage vs. DC panel, lineage vs. T cell and lineage vs. PMN panel). Each dot represents the median value of the five replicate tests for a given donor. Data from individual donors are represented by distinct colors. Standard deviation (SD) between the median values obtained by two panels is indicated. (B, C) Stabilized human blood samples (Eurocell) were analyzed in six independent experiments. Cells were stained using the lineage panel cocktail and labeled by the semi-automated procedure. The percentages of lymphocytes (B) and absolute cell counts per μl of blood (C) are shown for the indicated cell subsets. The target value range determined by the manufacturer on BD and Beckman Coulter flow cytometers is indicated by the red triangles. The CVs for serial measurements are indicated for each analyzed immune cell population. (D) Whole blood samples from six individual donors were collected and stained by the lineage panel cocktail using the semi-automated procedure. Serial measurements were performed at five different time-points over five months. Each dot represents the absolute cell number of indicated cell types during serial measurements. Individual donors are represented by distinct colors.

typical starting gates of FSC/SSC, and directly queried fluorescent markers, using FSC/SSC when needed to exclude doublets or dead cells (Figs. 2–5). The FlowJo templates created for and used in the study are provided (Online Supplementary Data File #2, http://www.milieuinterieur.fr/en).

3.6. Assay validation: technical replicates and robustness of the staining procedures

To define the variance in our immunophenotyping procedures, we performed experiments to assess repeatability



and reproducibility. To assess repeatability, we analyzed the same sample, in independent runs, by a single operator and run on a single cytometer. In our experimental setting, fresh blood samples from six healthy donors were separated into five aliquots and stained using each of the four panels and run on the liquid handling platform, followed by acquisition on the corresponding MACSQuant cytometer. The results were highly reproducible, with intra-panel CVs below 15% for most of the analyzed cell subsets, irrespective of their absolute counts (Table 1). Higher CVs were observed for pDCs in one of the six donors (21.5%) and for $CD14^+$ monocytes in two panels in two donors (16% and 23%). The higher variance observed for monocytes may result from the fact that CD14 can be expressed on other cell types, such as neutrophils [24].

Most of the immune cell populations ($CD3^+$, $CD4^+$, $CD8^+$ T cells, $CD14^+$ monocytes) were assessed independently using two different panels, and as such cross-panel comparisons were possible (Table 1 and Fig. 8A). We normalized the total cell counts of PMN, DC and T cell panels to those obtained by the lineage panel because it identifies all "core" cell populations and does not include an additional washing step that was used during the staining of dead cells in PMN and DC panels.

To assess reproducibility, we evaluated the stability of staining over time, an important consideration for large cohort studies. To provide a stable reference, we utilized commercially available stabilized blood, analyzed over a period of one month. These data showed reproducible results with CVs in the range of 2.0–5.3% (Fig. 8B and C). The percentage of each analyzed cell population was within the range of values indicated by the manufacturer (Fig. 8B). The total cell numbers obtained, however, were under the expected value, and consequently so were the total numbers of each analyzed cell populations (Fig. 8C). This difference may be explained by additional washing steps included in our protocol, which are not used to set the reference values indicated by the supplier of the stabilized blood. Another factor contributing to the difference may be the utilization of different cytometric platforms or the use of beads for cell enumeration, as compared to a volume based calibrator built into the cytometer itself. Finally, we analyzed blood samples from six donors across five different time points. As shown in Fig. 8D, the counts of most cell populations were stable over time. The differences observed for NK and monocyte cell number may be due to biological variation and intra-individual variance can be factored into the interpretation of our future population-based results.

4. Discussion

Delivering on the promise of personalized medicine requires tools and techniques that allow both robust and reliable assessment of the immune status of individuals and comparisons between studies. Specifically, the adoption of universal, robust cytometric protocols will allow cross-population comparisons and the evaluation of the extent to which the proportion of different cell populations in patients presenting immunopathology deviate from "healthy" expectations. Flow cytometry is likely to play a key role due to recent technological advances in instrument design, and the availability of a large arsenal of reagents targeting specific molecules. Indeed, these two factors now permit low-cost, real-time and deep phenotyping of immune cell populations. A notable concern for comparative studies is the pre-analytic variation (sample processing, reagent selection, and instrument parameters). Although several international consortia have begun to tackle this issue, additional efforts need to be taken in order to establish flow cytometry as a tool applicable in routine clinical laboratories.

We report our advances in standardizing pre-analytic procedures for flow cytometry for the *Milieu Intérieur* Consortium, a single-center study aiming to defining reference values of immune parameters in healthy individuals. Our challenge was to establish a standardized procedure for flow cytometry allowing the analysis of 15,000 samples by one operator in a single center. In this context, we considered automation as a solution to facilitate the workflow and to standardize the pre-analytic procedures for flow cytometry. Since clinical studies with large sample numbers involve repetitive work, implementation of automated procedures also eliminates possible error or variation caused by fatigued technical personnel. Perhaps most importantly, an automated procedure allows full traceability at each step (e.g., distribution of antibodies, wash solutions). This is of particular importance if flow cytometry is to be used in a quality-controlled environment, such as a clinical laboratory.

Our work revealed that implementation of automated procedures for flow cytometry is time-consuming and requires extensive testing. Automation also inevitably drives up costs because of expensive consumables and increased reagent use (e.g., dead-volumes in the robotic system). Although the robotic system used in our study operated without technical problems, we acknowledge that a certain amount of daily and weekly maintenance was required. The investment, however, was considered worthwhile based on the quality of the data obtained and it is our hope that others can benefit from the standardization of pre-analytic approaches for sample handling. We have provided complete access to our scripts, and encourage other Consortia to make such procedures available to the community in order to facilitate future improvements in the standardization of flow cytometry procedures.

Our study was inspired by the work of H. Maecker and the FOCIS Human Immunophenotyping Consortium, who identified technical variables in flow cytometric procedures requiring standardization [5,6]. Here and in the accompanying article [Chen et al., co-submission], we present our efforts to optimize staining procedures, selection of reagents, instrument set-up and data analysis. Based on the results of extensive antibody testing (Table S1) we selected reagents from different suppliers and thus have opted not to use the preconfigured lyophilized reagents, as suggested by Maecker et al. [6,15]. To rank the performance of the antibodies of the same specificity, we assessed the staining index and the fluorochrome stability. We observed a significant difference in the performance of different clones from different suppliers, results that reinforce the need to follow MIATA guidelines [25].

Studies involving large-scale or longitudinal immunophenotyping projects analyze either fresh whole blood samples directly at the recruitment site, or PBMCs that are separated from whole blood, frozen and shipped to the analysis laboratory for centralized analysis. Both approaches have advantages and inconveniences. Several studies have

demonstrated that the Ficoll purification can alter the composition and frequency of leucocyte population and the expression of certain surface markers [6,15,26]. The advantage of analyzing frozen PBMCs is the possibility to perform the phenotyping of the entire cohort in the centralized laboratory, eliminating possible errors resulting from all preanalytical steps (reagent preparation, fluorochrome stability, staining protocol, instrument set-up and performance, etc.). We compared these two approaches by analyzing fresh whole blood and comparing it to a portion of the sample that was used for PBMC isolation, frozen and later thawed for comparative analysis. Although several markers showed no observable differences (cell number and MFI), a considerable number of cell surface molecules were affected by the isolation and freezing/thawing procedures. In line with other studies, we support the use of whole blood for immune phenotyping studies when possible.

Our gating strategy builds on the characteristics of the new generation of cytometers that allow precise standardization of the fluorescent signals (voltage-dependent settings). The FSC/SSC gates that are commonly used as a first step in gating strategy were omitted due to variance that could not be controlled. This approach facilitated the rational setting of gates and permitted batch-analysis using FlowJo. As a result of these efforts, only minimal gate positions adjustment was required for a small number of cell populations. Additional standardization of post-analytic gating procedures is addressed in an accompanying manuscript [Chen et al., co-submission].

In conclusion, our efforts are in line with several international consortia, with high coherence in the staining protocols reported by the EuroCell Consortium [7]. These independent approaches converge on the use of whole blood and not frozen PBMCs, procedures for sample handling, and criteria for reagent selection. The new generation of flow cytometers, if properly set up and calibrated, allow precise standardization of fluorescent signals, thus enabling reliable results in longitudinal studies. We believe that the approach and protocols described here provide a rational basis to establish internationally standard operating procedures for immunophenotying. This attention to standardized cytometric analysis is of paramount importance and will enable inter-institutional comparative studies in healthy and diseased populations.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

Consortia

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Additional information can be found at: http://www.pasteur.fr/labex/milieu-interieur.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2014.12.008.

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