Standardization of Workflow and Flow Cytometry Panels for Quantitative Expression Profiling of Surface Antigens on Blood Leukocyte Subsets: An HCDM CDMaps Initiative

Daniela Kužilková¹, Joan Puñet-Ortiz², Pei M. Aui³, Javier Fernández², Karel Fišer¹, Pablo Engel², Menno C. van Zelm³,⁴ and Tomáš Kalina*¹

¹ Childhood Leukaemia Investigation Prague (CLIP), Department of Paediatric Haematology and Oncology, Second Faculty of Medicine, Charles University, Prague, Czech Republic and University Hospital Motol, Prague, Czechia, ² Department of Biomedical Sciences, University of Barcelona, Barcelona, Spain, ³ Department of Immunology and Pathology, Central Clinical School, Monash University, Melbourne, VIC, Australia, ⁴ Department of Allergy, Immunology and Respiratory Medicine, Central Clinical School, Monash University and Alfred Hospital, Melbourne, VIC, Australia

Background: The Human Cell Differentiation Molecules (HCDM) organizes Human Leukocyte Differentiation Antigen (HLDA) workshops to test and name clusters of antibodies that react with a specific antigen. These cluster of differentiation (CD) markers have provided the scientific community with validated antibody clones, consistent naming of targets and reproducible identification of leukocyte subsets. Still, quantitative CD marker expression profiles and benchmarking of reagents at the single-cell level are currently lacking.

Objective: To develop a flow cytometric procedure for quantitative expression profiling of surface antigens on blood leukocyte subsets that is standardized across multiple research laboratories.

Methods: A high content framework to evaluate the titration and reactivity of Phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) was created. Two flow cytometry panels were designed: an innate cell tube for granulocytes, dendritic cells, monocytes, NK cells and innate lymphoid cells (12-color) and an adaptive lymphocyte tube for naive and memory B and T cells, including TCRγδ⁰, regulatory-T and follicular helper T cells (11-color). The potential of these 2 panels was demonstrated via expression profiling of selected CD markers detected by PE-conjugated antibodies and evaluated using 561 nm excitation.

Results: Using automated data annotation and dried backbone reagents, we reached a robust workflow amenable to processing hundreds of measurements in each experiment in a 96-well plate format. The immunophenotyping panels enabled discrimination of 27 leukocyte subsets and quantitative detection of the expression of PE-conjugated CD
markers of interest that could quantify protein expression above 400 units of antibody binding capacity. Expression profiling of 4 selected CD markers (CD11b, CD31, CD38, CD40) showed high reproducibility across centers, as well as the capacity to benchmark unique clones directed toward the same CD3 antigen.

**Conclusion:** We optimized a procedure for quantitative expression profiling of surface antigens on blood leukocyte subsets. The workflow, bioinformatics pipeline and optimized flow panels enable the following: 1) mapping the expression patterns of HLDA-approved mAb clones to CD markers; 2) benchmarking new antibody clones to established CD markers; 3) defining new clusters of differentiation in future HLDA workshops.

**Keywords:** flow cytometry, cluster of differentiation (CD), expression profiling, surfaceome, CD marker

### INTRODUCTION

Since the development of hybridoma technology in 1975 (1), monoclonal antibody (mAb) production has been instrumental in examining protein expression and delineate cell types. Following its wide adoption, the need for quality assessment of antibody clones and consistency in naming their reactivity was quickly recognized, leading to the initiative of the Human Leukocyte Differentiation Antigen (HLDA) workshops (2, 3). Currently organized by the Human Cell Differentiation Molecules (HCDM), these wet-lab workshops have been run since the 1980s for experimental validation of the reactivity and specificity of mAb clones (2). Two or more validated clones recognizing the same protein target were clustered and designated a cluster of differentiation (CD) number (3). To date, ~400 targets have been assigned CD nomenclature, which ranges from CD1 to CD372 (4).

Flow cytometry is undoubtedly one of the key methods in which mAbs have been applied to evaluate protein expression in single cells (5). Multiparametric applications have expanded our knowledge in immunology and related fields, where the combinatorial expression of surface proteins identifies a particular cell type (6). At the same time, immunophenotyping has become a key method to diagnose hematological malignancies, performing disease classification (7) and associating the expression of particular markers with underlying leukemogenic molecular changes (8, 9).

HLDA workshop reports provide basic information on the reactivity of mAbs. However, these reports have been completed sequentially over 3 decades, scattering the expression information over many publications with a generally low number of investigated subsets (4, 10–14). Thus, a catalog containing comprehensive, quantitative and searchable CD marker expression data was missing until the CD Maps pilot project was published by the HCDM organization (15). Although this pilot project demonstrated the feasibility of a standardized and reproducible collection of the expression patterns, aspects of the procedures still required further optimization and conceptually different approaches, enabling the large-scale deployment and continual updatability of the CD Maps resource.

The construction of a comprehensive resource of CD marker expression should ideally include appropriate and assay-specific titration of each mAb reagent to use the optimal concentration for accurate molecule quantification and limit undesired background staining. In addition to standardized experimental procedures that are reproducible in time and place, the resource should be updatable and handle challenges with data management and annotations. Ultimately, a comprehensive combination of backbone markers is required to define the many functionally defined immune cell subsets in blood.

Although mAbs recognizing the same protein and showing similar reactivity patterns were clustered in CD workshops and assigned CD nomenclature, mAb clone performance may differ, making particular clones better suited for particular applications (16). These mAb clone differences can be defined by direct comparison in a standardized workflow, providing critical information to select the appropriate reagent for clinical studies - e.g., multisite cohort studies that must combine data analyses (17, 18).

Here, we developed a standardized and semi-automated procedure for high-throughput expression profiling of surface protein expression. We evaluated the standardization and optimization of high-throughput reagent titration, the polychromatic panel design for innate and adaptive blood immune cells and a bioinformatics pipeline for data analysis. This approach was validated globally across multiple centers with HLDA-approved antibody clones to CD3, CD11b, CD31, CD38 and CD40 and demonstrates the feasibility of antibody reactivity benchmarking within this framework.

### MATERIAL AND EQUIPMENT

**Human Blood Samples and Cell Lines**
The use of blood samples from healthy adults was approved by the Human Ethics Committees of Monash University, the Motol

---

**Abbreviations:** ABC, Antibody binding capacity; CV, Coefficient of variation; EMT, Experiment Master Table; FMO, fluorescence minus one; HCDM, Human Cell Differentiation Molecules; HLDA, Human Leukocyte Differentiation Antigen; HTS, High Throughput Sampler; ILC, innate lymphoid cells; IQR, interquartile range; mAb, monoclonal antibody; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; PE, Phycoerythrin; RT, room temperature; SOP, Standard Operating Procedure; TCR, T cell receptor; Tfh, T follicular helper cells; Treg, T regulatory cells.
University Hospital, and the University of Barcelona and was contingent on informed consent in accordance with the Declaration of Helsinki. Blood buffy coats were obtained from the local blood banks. In addition, 4 human cell lines were selected as representatives of the cell types expressing the molecules targeted by the 11th HLDA workshop: Raji (B cell) (19), Jurkat (T cell) (20), THP-1 (monocyte) (21), U266 (plasma cell) (22) (American Tissue Culture Collection (ATCC), Rockville, MD, USA). The Mouse pre-B cell line 300.19 (23) (ATCC) served as a universal negative control.

Flow Cytometry Equipment

Data acquisition was performed at three different centers using LSRII and LSFortress (BD Biosciences, San Jose, CA, USA) and 8-peak Rainbow bead calibration particles (Spherotech, Lake Forest, IL, USA) for PMT voltages and light scatter setup to achieve interlaboratory standardization as developed by the EuroFlow consortium (24). The PE-conjugated target mAbs were excited by the 561 nm laser; for each staining (well), a minimum of 0.5 million events were acquired. The EuroFlow Standard Operating Procedure (SOP) for Instrument Setup and Compensation can be downloaded from www.europflow.org.

Titration Procedure of Target mAbs

To accurately quantify expression, the target antibodies were PE-conjugated. The experimental setup for titrating large amounts of PE-conjugated antibodies was designed to be feasible at a large scale. To this end, a cellular mixture containing representatives of positive and negative cell subsets was created by mixing defined quantities (1×10^5 cells) of human peripheral blood cells and selected human (Raji, THP-1, Jurkat, U266) and mouse (300.19) cell lines. The cell lines were barcoded with cell tracking dyes as follows: mouse 300.19 and human U266 cell lines were stained with 20 and 5 μM CellTracker Blue CMHC Dye (Thermo Fisher Scientific, Waltham, Massachusetts, USA), respectively; human THP-1 and Jurkat cell lines were stained with 0.5 and 0.05 μM CellTracker Deep Red Dye (Thermo Fisher Scientific), respectively; human peripheral blood cells were stained with both 20 μM CellTracker Blue CMHC Dye and 0.5 μM CellTracker Deep Red Dye trackers. The human Raji cell line was left unstained. Cell tracker staining was performed according to the manufacturer’s protocols before mixing cells at equal rates and incubating them with PE-conjugated mAbs. The antibodies used were kindly provided by Exbio Praha, Vestec, Czech Republic (CD31, MEM-05; CD38, HIT2; CD3, UCHT1; CD3, SK7; CD3, TB3; CD3, MEM-57) and BioLegend, San Diego, California, USA (CD40, 5C3; CD11b, and ICRF44). All the mAbs were evaluated in with dilutions ranging from 1/5 to 1/3200 to determine the optimal titer at the edge of saturation. The dilution recommended by the manufacturer was chosen as the starting point.

Computer-Assisted Experimental Protocol Setup

To enhance the ease of tracking and repeating the experimental procedure, we established an automated process for protocol preparation based on a manually completed “Experiment Master Table” (EMT) using R software (http://www.r-project.org/). Briefly, the EMT was prepared as an Excel table with information about the sample, backbone panel, antibodies, user and so on. Information in the EMT is used (via web front-end) to automatically generate an experimental protocol that is time-stamped and includes calculated amounts of master mixes and pipetting volumes for all wells in all plates. This setting minimizes user errors and allows the tracking and archiving of the complete procedure.

Single Cell Isolation and Preparation

The blood leukocyte isolation protocol was optimized to minimize platelet adhesion (satellitism) (25). Briefly, the buffy coat sample contained citrate phosphate dextrose as anticoagulant, and was diluted 6× in PBS containing 2 mM EDTA, followed by the addition of an equal volume of a 4% dextran solution (Sigma–Aldrich, Saint Louis, MO, USA) in 0.9% NaCl. The mixture was left for 30 min for erythrocytes to sediment before collecting the supernatant containing the leukocytes. Following spinning (670 g, 5 min, RT) and removal of the supernatant, the white blood cell count was adjusted to 5×10^7/ml in PBS supplemented with 0.09% NaN3, 0.5% BSA and 20% rabbit serum (Biowest, Nuaille, France).

Staining of Blood Leukocytes for Expression Profiling

Cells were stained in 96-well Polypropylene DeepWell plates in a total suspension volume of 50 μl. First, each of the PE-labeled mAbs was added to each well (the marker details are listed in Supplementary Table S1). The mAb amounts were derived from the titration experiment, and PBS supplemented with 0.09% NaN3, 0.5% BSA and 20% rabbit serum was added to a final volume of 10 μl. Subsequently, 40 μl of leukocyte cell suspension (2 × 10^6 cells) was added to each well. Following careful mixing, the suspensions were incubated for 30 min at room temperature (RT) in the dark. Next, 25 μl of backbone mAb reagent mix was added to each well. Following careful mixing, the plate was incubated for an additional 30 min (RT, in the dark). The compositions of the two backbone antibody panels (innate and adaptive) were optimized, and the reagents were titrated beforehand (the details are provided in Table 1). Most of the backbone reagents were custom provided in an mAb mix that was dried in 96-well plates as HLDA innate and HLDA adaptive panels within Dry Reagents (Exbio Praha, Vestec, Czech Republic), with polymer-conjugated mAbs (BioLegend, Inc., San Diego, CA, USA) added from a liquid stock. All the mAbs
conjugates were generously donated by Exbio and BioLegend. The residual erythrocytes were lysed by Excelyse Easy solution (Exbio) according to manufacturer’s instructions. Briefly, 1.5 ml of 10× diluted Excelyse Easy solution was added to the 75µl cell suspension incubated for 10 min at RT, in the dark. This procedure provided a mild fixation condition to preserve fragile subsets (e.g. T follicular helper cells - Tfh). The samples were centrifuged (670 g, 5 min, RT), supernatant was removed, and the pellet was dried with a wool pulp. The cell pellet was resuspended in 200 µl of PBS for acquisition and stored at 4°C overnight.

**Automated FCS File Check and Annotation**

All acquisitions were performed using default cytometer acquisition software settings for FCS file labeling (e.g., Specimen_001_A1_A01_001.fcs). The previously prepared EMT table was used for automated renaming of FCS files and their FCS header fields to include all relevant experimental information from the EMT table. This facilitated automated and standardized annotation of FCS files for further analysis.

**Conversion of PE Fluorescence Intensity to Antibody Binding Capacity (ABC)**

PE conjugation of mAbs is consistent with a 1:1 ratio of fluorochrome:antibody, facilitating the calculation of the antibody binding capacity (ABC) from PE fluorescence. To convert PE fluorescence to the amount of PE molecules bound to a target, we used the PE Fluorescence Quantitation Kit (BD Biosciences) with four known levels of PE. The pellet was resuspended in 500 µL of PBS supplemented with 0.09% NaN₃ plus 0.5% BSA and analyzed by flow cytometry in parallel with four known levels of PE. The measured PE signals for all stainings on all and standardized annotation of FCS files were exported as FCS 3.0. Next, the position of each PE reagent was assigned to a well plate format were printed out and the EMT tables and placed in a usable format.

**Analysis, Gating and Export of Values**

The leukocyte and lymphocyte subsets to be analyzed were predefined and gated uniformly by a single operator using FlowJo (v10, BD Biosciences). From each defined subset, the median intensity in the PE channel and median intensity of ABC were extracted. For each subset, the 90th percentile of the ABC value on empty PE channels (fluorescence minus one; FMO) was considered the cutoff for the subset-specific background. The interquartile range (IQR) was calculated as IQR = Q3 − Q1. The minimum cell count for statistical evaluation was set to 66, and subsets with lower cell counts were omitted from further analysis. The FCS data and the FlowJo workspaces were deposited on the HCDM website (https://www.hcdm.org/index.php/2016-12-06-21-38-08/cdmaps-data-repository).

**RESULTS**

**Automated Workflow for High-Throughput Expression Profiling**

The scale of the intended CD Maps project required the following: 1) the processing of hundreds of measurements a day; 2) interlaboratory collaboration; 3) reproducibility in time and place. Thus, we designed a structure for high-throughput experiment execution (Figure 1) sourcing the EMT of PE reagents to be tested. The EMT contained all identifiers of a reagent (clone name, origin, CD name, gene name) (Supplementary Table S1).

A SOP for leukocyte isolation and antibody labeling in a 96-well plate format was developed, and a website interface was made that allowed for the creation of customized experimental protocols using the EMT and SOP in a printable format (Data Sheet 1). Thus, the position of each PE reagent was assigned to a well of the 96-well plate, printed out and the EMT tables represented the history of the processed experiments.

Dried reagent cocktails in a 96-well plate format were designed, titrated and custom produced to ensure speed, precision and stability of the backbone reagents used for universal gating. After acquisition on the HTS, proper compensation of the PE channel was verified using FMO control wells, and the files were exported as FCS 3.0. Next, the

---

**TABLE 1 | Reagents used in the HLDA innate and HLDA adaptive panel.**

| Innate tube Fluorochrome | BV421 | Pac Orange | BV605 | BV711 | FITC | PE | PE-DyLight 594 | PerCP-Cy5.5 | PE-Cy5™7 | APC | Alexa Fl. 700 | APC-Cy7™7 |
|--------------------------|-------|------------|-------|-------|------|----|----------------|-------------|------------|----|-------------|----------|
| Target clone             | CD127 | CD45       | CD45  | BM16  | CD45 | CD66| CD117          | CD19        | CD14       | CD11c | CD123       | CD16     |
| Volume                   | 1.25 µl| 5 µl       | 5 µl  | 2 µl  | 1.25 µl| 2.5 µl|10402           | LT19        | MEM-15     | BU15 | 6H6         | L243     |
|                         |       |            |       |       |      |    |                |             |            |      |             | 2.5 µl   |

| Adaptive tube Fluorochrome | Pac Blue | Pac Orange | BV605 | FITC | PE | PE-Dazzle594 | PerCP-Cy5.5 | PE-Cy7™7 | APC | Alexa Fl. 700 | APC-Cy7™7 |
|----------------------------|----------|------------|-------|------|----|--------------|-------------|------------|----|-------------|----------|
| Target clone               | CD45RA   | CD45       | CXCR5 | CD27 | tested CD | CD127        | CD4         | IgD | TCRgd       | CD19     |
| Volume                     | 5 µl     | 5 µl       | 0.625 µl| 2.5 µl|    | 0.625 µl    | 2.5 µl       | 2.5 µl     | 5 µl|             | 2.5 µl   |

Dried reagents shaded.
batch of FCS files was processed using a website interface that annotated the FCS files with the set of reagent identifiers from the EMT. Finally, this workflow generated fully annotated FCS files with consistent reagent identifiers, allowing subsequent batch analysis (Data Sheet 2).

**Strategy for the Titration of PE Reagents**

For accurate quantification of protein expression in ABC, all the PE reagents must be optimally titrated (5). To optimize the procedure for high-throughput processing, a uniform titration protocol was developed using a mixture of defined cell lines corresponding to B and T lymphocytes, plasma cells and monocytes, and fresh human peripheral blood cells. Each cell line was uniquely barcoded with a combination of three barcodes and electronically gated based on the fluorescent parameters for the backbone from 7 to 10-11 (Table 1). The innate cell tube was extended with CD117, CD127 and CRTH2 to facilitate the identification of ILC-1, ILC-2 and ILC-3 subsets (27, 28), bringing the total number of innate cell subsets to 12 (Table 2). The lymphoid tube was expanded with CD25, CD127 and CXCR5, enabling the detection of T regulatory cells (Tregs) (29) and Tfh cells (30, 31) with a total of 15 defined cell subsets (Table 2). The detailed gating strategies for the innate and adaptive cell tubes are shown in Figures 3, 4.

In a previous study, autofluorescence and spreading error limited the sensitivity of the low PE signal because the 488 nm laser was used for measurement (15). Here, we sought to establish the lower limit of detection of the optimized panels using 561 nm laser excitation of PE. Advancing our study from the pilot using 8 fluorochromes (7 backbone + PE marker) to 11 or 12 with PE excitation with a 561 nm laser necessitated using 4 laser conventional flow cytometers. BD LSRII and BD Fortessa instruments were used with similar but not identical detection
where one instrument lacked the BV711 detection channel, and samples acquired on that analyzer were not stained with CD56-BV711 in the innate tube. In those samples, NK cells, ILC-2 and ILC-3 (but not ILC-1) were adequately resolved. The minimal ABC resolution was determined as the 90th percentile of the ABC value on the FMO control tube (Table 2). The median level of the minimal ABC resolution across subsets was 396 ABC units (229 to 786, minimum to maximum), and this threshold was used to gate positive events for each evaluated reagent.

**Feasibility and Reproducibility of CD Maps Resource Building**

The automated procedure and standardized experimental approach were evaluated by expression profiling of four HLDA-approved clones to CD markers. The four CD markers CD11b, CD31, CD38 and CD40 were selected based on their known distinct expression profiles across cell lineages: CD11b is expressed on myeloid cells (32), CD40 is expressed on B cells (33), and CD31 and CD38 show various degrees of expression across myeloid and lymphoid cell subsets (34, 35) but at different intensity levels.

CD11b expression was the highest (evaluated as median ABC) on neutrophils and classical monocytes but was moderately intensive on eosinophils, basophils and intermediate monocytes and lacking on nonclassical monocytes, dendritic cells, NK cells and adaptive lymphocytes (Figure 5A). CD31 and CD38 were expressed on naïve CD4 and CD8 T cells. However, although CD38 was absent at later differentiation stages in steady-state T cells, CD31 was also expressed on memory CD8 T cell subsets (Figures 5B). The highest expression of CD31 was found on subsets of monocytes, while neutrophils, eosinophils, basophils and dendritic cells showed threefold lower expression (Figures 5A). All the CD8 T cells were CD31 positive but presented lower levels than myeloid cells, while only naïve CD4 T cells showed partly positive expression (Figure 5B). B cells presented a heterogeneous staining pattern with lower intensity (Figures 5B). The highest level of CD38 expression was found on basophils and NK cells (heterogeneous), followed by myeloid dendritic cells (mDC), plasmacytoid dendritic (pDC) and B cell subsets (Figure 5A). CD38 expression was gradually decreased on monocyte subsets along with their maturation. Memory and effector stages of T cells lacked CD38 at steady state. However, among B cells, CD38 expression was heterogeneous, reaching high levels on naive B cells, spreading from negative to high on switched memory and mostly lacking on natural effector B cells. CD40 was expressed on all B cell subsets and absent from all other leukocyte subsets tested. The ABC units allowed for interpretable expression level evaluation, but the patterns did
not differ from those observed on PE fluorescence intensity (Data Sheet 5).

Similar patterns of expression were observed for the proportions of positive cells within a certain subset (Data Sheet 6) with several differences. Nearly 100% of ILC3 were CD11b positive, while the expression levels were rather low on the basis of the median ABC. Conversely, on the basis of the median ABC (649), Tfh cells were deemed CD31 negative, while 30% of events within the Tfh cell subset were positive for CD31.

Parallel evaluation of the procedure at three centers demonstrated highly similar results. For each PE reagent, we chose to closely examine one cell subset. The expression of CD40 on naive B cells (median ABC: 2963; IQR: 1420), CD38 on monocytes (median ABC: 14049; IQR: 1912) and CD31 on naive CD8 T cells (median ABC: 6813; IQR: 4242) was comparable across donors and sites (Figure 6). In contrast, CD11b (median 84100; ABC; IQR: 76212) showed site-dependent variation that was explained by the sample source. In the general CD Maps protocol, a buffy coat was used; however, the limited availability of a buffy coat forced one laboratory to use peripheral blood, which accounted for the difference (Data Sheet 7). The expression of CD11b on neutrophils isolated from freshly drawn peripheral blood (median ABC: 16614; IQR: 8320) is 5x lower compared to neutrophils isolated from buffy coat (median ABC: 94590; IQR: 33437).

Comparison of the three different donors analyzed in each laboratory showed highly similar results: the mean coefficient of variation (CV) reached 30% (Min: 1%; Max: 111%). The inter-laboratory mean CV was 55% (Min: 4%; Max: 137%). Full details are in the Supplementary Table S2.

Although aggregated expression data over all subsets and all donors provided a complete picture (Figure 7), the histogram distribution of measured single cells allowed us to explain greater heterogeneity of the median ABC values for subsets with heterogeneous (CD11b on B cells) or bimodal (CD31 on naive CD4 T cells) expression (Figure 5).

### TABLE 2 | Definitions of the leukocyte subsets defined in this study.

| Population name | Population code | Immunophenotype | Background cut-off (ABC units) |
|-----------------|-----------------|-----------------|-------------------------------|
| Granulocytes    | Granulocytes    | CD45+/CD3-/CD19-/ |                               |
| Neutrophils     | Neutrophils     | SSC high/CD16+   | 408                           |
| Eosinophils     | Eosinophils     | SSC very high/CD16- | 632                           |
| Basophils       | Basophils       | SSC low/CD123+-HLA-DR- | 357                           |
| Monocytes       | Monocytes       | CD45+/CD3-/CD19+/-SSC low/CD123-/HLA-DR+/CD11c+ |                               |
| classical monocytes | class Mono | CD14+CD16- | 384                           |
| intermediate monocytes | inter Mono | CD14+CD16+ | 353                           |
| nonclassical monocytes | nonc Mono | CD14-CD16+ | 350                           |
| Dendritic cells | Dendritic cells | CD45+/CD3-/CD19-/-SSC low |                               |
| myeloid dendritic cells | mDC | CD123-/CD11c+/CD16-CD14-/HLA-DR+ | 396                           |
| plasmacytoid dendritic cells | pDC | CD123+ HLA-DR+ | 309                           |
| Innate lymphoid cells | Innate lymphoid cells | CD45+/CD3-/CD19-/-SSC very low/CD123-/HLA-DR-/CD14-/CD127+/CD16- |                               |
| Innate lymphoid cells 1 | ILC-1 | ORTH2-/CD117- | 336                           |
| Innate lymphoid cells 2 | ILC-2 | ORTH2+/CD117- | 312                           |
| Innate lymphoid cells 3 | ILC-3 | ORTH2-/CD117+ | 459                           |
| NK cells | NK cells | CD45+/CD3-/CD19-/SSC very low/CD123-/HLA-DR-/CD14-/CD127-/CD56+ and/or CD16+ | 229                           |
| T cells | T cells | CD45+/SSC low/CD3+/CD19-/- |                               |
| TCRγδ+ T cells | TCRγδ+ T cells | CD45+/CD3+/CD19-/- |                               |
| CD4 helper T cells | CD4 | CD45RA+/CD27+ | 369                           |
| CD4 naive | CD4 naive | CD45RA+/CD27+ | 471                           |
| CD4 Central Memory | CD4 CM | CD45RA+/CD27+ | 406                           |
| CD4 Effector Memory | CD4 EM | CD45RA+/CD27+ | 360                           |
| CD4 CD45RA+ effector memory | CD4 TEMRA | CD45RA+/CD27+ |                               |
| Regulatory T cells | Treg | CD25+/CD127- | 305                           |
| Follicular helper T cells | Th | CD45RA+/CD56+ | 415                           |
| CD8 cytotoxic T cells | CD8 | CD45RA+/CD27+ | 384                           |
| CD8 naive | CD8 naive | CD45RA+/CD27+ | 425                           |
| CD8 Central Memory | CD8 CM | CD45RA+/CD27+ | 396                           |
| CD8 Effector Memory | CD8 EM | CD45RA+/CD27+ | 338                           |
| CD8 CD45RA+ effector memory | CD8 TEMRA | CD45RA+/CD27+ |                               |
| B cells | B cells | CD45+/SSC low/CD3-/CD19-/- |                               |
| B naive | B naive | CD45RA+/CD27- | 773                           |
| B double negative | B dn | CD45RA+/CD27- | 732                           |
| B nature Effector | B nat Eff | CD45RA+/CD27+ | 786                           |
| B switched Memory | B sw Mem | CD45RA+/CD27+ | 727                           |

The background cut-off value was determined as the median of the 90 percentile FMO values from all analyzed donors.
To further evaluate the reproducibility of the ABC data, we compared the currently measured data to the CD Maps pilot study for the four CDs (Supplementary Table S3). For the cell subsets depicted in Figure 6, the CD11b median ABC was 1.2-fold higher in the current study on neutrophils (the same Ab clone was used but different vendors), the CD31 median ABC was 1.12-fold higher (on CD8 naive T cells; MEM-05 vs. WM59 clone using different vendors), the CD38 median ABC was 1.5-fold lower (on monocytes; using the same clone and vendor, but a titer 4 times lower), and the CD40 median ABC was 1.8-fold lower in the current study (on naive B cells; using the same clone but different vendors).

Taken together, the differences in ABC expression levels between the current and CD Maps pilot study (15) were less than twofold.

**Reactivity Benchmarking of Multiple mAb Clones Targeting the Same CD Marker**

An important application of the CD Maps resource will be selecting appropriate mAb clones. Although all mAb clones that were validated in previous HLDA workshops will have similar expression patterns, their expression levels were not quantitatively defined, with the potential to show substantial differences. To address this issue, we applied our experimental setup with quantitative expression in ABC units to four CD3 mAb clones. These four clones were first titrated on lymphocytes, and highly different titration curves were observed, although all were reactive to the same target protein on T cells. The titration curve of the TB3 clone showed a prolonged plateau and was shorter for UCHT1 and SK7, and the expression levels of MEM-57 were decreased (Figure 8A).

Following optimal titer selection, all four clones were investigated in the context of the adaptive cell tube. As expected, all four clones were specifically reactive to all T cell subsets and did not stain any of the B cells (Figure 8B). However, within the T cell subsets, we observed quantitative differences depending on the mAb clone. T cell receptor (TCR) γδ T cells showed higher expression levels of CD3, a finding that was
FIGURE 4 | Gating strategy and identification of adaptive lymphocytes. Within total acquired events, first the debris (low FCS) was excluded (upper left plot) followed by doublets (non-linear events on FSC-A vs. FSC-H plot; 2nd plot upper row). Lymphoid blood cell types were defined as CD45SSClow (3rd plot upper row). Gamma delta T cells (Tgd) were gated as T cell receptor (TCR)gd+CD3+ (4th plot upper row). B cells were identified as CD3negCD19+ (4th plot upper row) and subsequently divided into naive (B naive), switched memory B cells (B sw Mem, CD27iggDneg), and double negative B cells (Bdn, CD27immunDneg, upper right plot). T cells were gated as CD3+CD19negTCRgd- (4th plot upper row) and on the basis of CD4 or CD8 expression divided into CD4 T helper cells (T CD4) and CD8 cytotoxic T cells (T CD8, 4th plot lower row). Within the T CD4 cells follicular helper CD4 T cells (Tfh) were gated as CXCR5+CD45RAneg (3rd plot lower panel), regulatory T cells (Treg) as CD25+CD127neg (2nd plot lower panel). Remaining CD4 T cells (left plot lower row) as well as CD8 T cells (right plot lower row) were subdivided based on CD27 and CD45RA expression to naive (CD45RA+CD27+), Central Memory (CD45RAnegCD27+), Effector Memory (CD45RAnegCD27neg), and Terminal Effector Memory RA+ T cells (CD45RA+CD27neg).

FIGURE 5 | Levels of Antibody Binding Capacity (ABC) on leukocyte subsets. (A) Histograms of expression levels of CD11b, CD31, CD38 and CD40 are shown on innate leukocyte subsets and (B) on adaptive lymphocyte subsets of a representative subject in comparison to fluorescence minus one (FMO) controls. ABC units show expression levels recalculated from PE-antibody conjugates providing a standardized quantification.
consistently observed for all four clones (from 71620 to 85479). Three clones (SK7, UCHT1 and TB3) showed similar levels of CD3 expression on naive CD4 T cells (71079, 71419, 80876 ABC units) and naive CD8 T cells (47735, 49610, 51909 ABC units, respectively). By contrast, the MEM-57 clone yielded lower CD3 expression levels for both CD4 (33540 ABC) and CD8 (20745 ABC) T cells (Figure 8C). These results suggest that the CD3 epitope recognized by MEM-57 is less accessible on TCRab+ T cells, resulting in lower measured ABC. Thus, the MEM-57 clone enables the distinction of TCRab+ from TCRgd+ T cells based on the CD3 expression intensity. However, the titration characteristics make MEM-57 suboptimal for CD3 expression quantification. The surface expression levels of CD3 on T cell subsets can be reproducibly quantified using three independently developed CD3 clones (SK7, UCHT1 and TB3) across measured donors.

**DISCUSSION**

Here, we demonstrate a high-throughput procedure for the expression profiling of surface antigens on 27 leukocyte subsets that is standardized across global laboratories for accurate quantification. This optimized procedure overcomes previous limitations observed in the CD Maps pilot project mapping the expression of CD1 to CD100 (dynamic web resource at hcdm.org) as follows (15): 1) Expansion of backbone markers enabled the distinction of additional T cell subsets (Treg and Tfh) and innate lymphoid cells (ILC-1, ILC-2 and ILC-3); 2) a universal titration procedure was adopted for each PE marker, improving the quantitation and 3) excitation of PE from the 561 nm laser led to increased sensitivity to markers with low expression levels. The procedure presented here will now facilitate re-evaluation of all approved CD markers (CD Maps) and validation of new mAbs for CD marker designation in HLDA workshops.

The same surface markers were used to define the leukocyte subsets that were previously defined in the CD Maps pilot project except for IgM. Thus, the definition for naive and natural effector B cells was slightly different. Because all naive B cells (CD27negIgD+) express IgD, these cells and natural effector B cells (CD27IgD+) were hardly affected (36, 37). The current approach will have left IgM-only memory B cells (CD27IgM Igdneg) in the CD27negIgdneg population, making this a mixture of unswitched and switched memory B cells (38).

The definition of DC subsets relies on excluding CD5+, CD19+, CD14+ and CD16+ cells, high HLA-DR expression and subdivision of CD123+ (pDCs) and CD11c+ (mDCs) (39, 40), while additional markers can be used to identify and separate myeloid DCs into two finer subsets (conventionalDC1: CD141, CD370 and conventionalDC2: CD1c, CD301) (39, 41).

For T cell subsets, we used CD45RA and CD27 to resolve the naive (CD45RA CD27+) and central memory (CD45RAnegCD27+) stages from the effector memory (CD45RAnegCD27neg) stage, in accordance with the definition strategy used by the EuroFlow group for human primary immunodeficiency (42–44). The advantage of this approach is that CD27 can be used for subsets of B cells and T cells, making the antibody backbone simpler. Furthermore, sample processing and antibody clone selection was reported as a source of false negative staining with CD62L and CD197 respectively (6). Although CD62L and CD197 define the same cells as naive cells (44), they delineate a small but consistent population of transitional effector (CD27CD62LnegCD197neg) (45), a population that is blended to central memory in our dataset. Effector memory (EM) and terminal effector memory (TEMRA) stages contain further subsets of early, intermediate and late stages defined by CD28 expression (42) and correlate with the chronic carrier status of Cytomegalovirus and Epstein-Barr virus (46, 47). Because expanding the knowledge of T cell subsets by polychromatic cytometry has led to identification of new functional subsets (48), subsets defined by checkpoint inhibitors (49) or tissue-specific subsets (28) at a fast pace, our simplified subset definitions may yield heterogeneous expression signals on some subsets containing finer subtypes, but the general description will be true and useful nonetheless.
Detection of the PE signal from excitation with the 561 nm laser in the current study improved signal sensitivity because of decreased autofluorescence of leukocytes and reduced the data spread from decreased spillover of the FITC reagent, as expected (50). These two effects combined led to a decreased background on myeloid subsets (384 ABC units) and allowed for more reliable detection of low-expressing markers than we had previously observed in the CD Maps pilot study (background of 1026 ABC units) (15).

We have addressed an important issue of proper titration (5) by building a uniform titration protocol, where we added cell lines representing leukocyte types and peripheral blood cells to allow for proper titer estimation. Our approach was specifically designed for mAbs submitted for 11th HLDA workshop and the CDMaps2 project. Although most antigens targeted in this study are expressed at sufficient levels on peripheral blood cells, other known markers such as activation markers (CD25, CD54, CD69 or CD80) are expressed at very low levels or small subsets but are expressed on the selected cell lines. Other cell lines may be appropriate for titration, when the surface markers investigated will include markers expressed solely on stem cells, platelets, or endothelial cells. Because nonspecific staining is a problematic aspect of antibody reagent binding at given concentrations, we used a mouse cell line to ensure the presence of a negative cell type that can be easily evaluated. We selected titers that balance the following two objectives: 1) mAb staining was near saturation to provide accurate ABC measurement and achieve reproducibility; 2) minimal background fluorescence on defined negative cells allow the specific quantification of target molecules with low levels of expression. The titration curves of the four CD3 clones illustrated that they depend highly on whether the mAb clone titer is an ideal balance of these two objectives, demonstrating the need to select the right mAb clone for the experimental objective.

Large-scale expression profiling studies involve an extensive experimental setup, with expansion of the backbone from 7 to 10-11 colors in our study, resulting in added experimental complexity. To optimize the sample preparation procedure, a dried antibody backbone mixture was custom produced in 96-well plates. This allowed fast and robust sample staining of
Another challenging aspect of CD Maps resource is the handling of large datasets. This issue was addressed using R-project scripts presented via the Shinny interface to annotate (clone names, titers, and manufacturers) the acquired measured FCS files.

To accurately quantify surface molecule expression and visualize intercell and interindividual variation, the technical variability must be minimized. Here, we build on previous expertise obtained from the CD Maps pilot study (15) with further refinement of titration and PE excitation. Compared with the CD Maps pilot project, we reached similar quantitative results for CD11b, CD31, CD38, and CD40. Comparable results were achieved despite using specimens from different donors, acquisition 5 years later using new instrumentation, different staffing and PE reagents obtained from different vendors (3 of four different) highlighting the robustness of the standardization procedure. This finding agrees with the long-term experience of the EuroFlow consortium, where reproducible signal intensity measurement is achievable using thorough standardization (51) and is exploited for quality assessment purposes applied worldwide (52). Thus, the EuroFlow consortium can use CD marker reagents from different vendors with comparable intensity measurements (18). Of the markers tested here, CD3 and CD38 are currently used in EuroFlow QA.

However, preanalytical sample handling procedures can alter the expression level of particular surface molecules on batches of 96-well plates and high interlaboratory data comparability due to limited pipetting errors.
granulocytes (53). Here, we observed a 4.5-fold increase in CD11b ABC after processing Buffy coat samples compared with freshly drawn peripheral blood cells; additionally, CD11b can increase with activation or with density gradient isolation (54). Lymphocyte subsets generally show higher stability of expression than myeloid subsets with prolonged storage; however, specimens measured within 24 h after the blood draw maintain stable expression (55).

Evaluating the surface expression and reagent performance at the level of defined subsets provides an opportunity to reach reproducible readouts for markers with complex expression profiles (e.g., uniform CD38 positivity on monocytes but heterogeneous expression on unselected leukocytes) (5). Furthermore, the comparison between four CD3 clones demonstrates that quantitative differences in the ABC exist among clones, in which three CD3 clones reach very similar ABC values, while one clone consistently differs on TCRαβ+ subsets. Thus, extension of the CD Maps project from one representative reagent against each CD to multiple (all available) reagents is warranted, providing reactivity benchmarking. Meaningful ABC evaluations must, however, be performed on correctly titrated antibody conjugates.

In conclusion, we have developed and optimized a method for reproducible, high throughput evaluation of CD marker expression on 27 human peripheral blood subsets. Its primary use is for the completion of the CD Maps project, aiming to quantitatively profile the expression of all surface molecules assigned with CD nomenclature within all 10 historical HLDA workshops. Furthermore, this method will be applied to evaluate reactivity of all newly submitted reagents within the current 11th HLDA workshop. The robust and standardized nature of our procedure will enable benchmarking the reactivity of PE-conjugated antibody reagents (new or established). These implementations will provide the CD Maps resource managed by HCDM.org with representative reagents to all CD markers, and it will catalog all submitted reagents against that CD target, thereby providing the community with an experimental benchmarking platform in a structured and searchable format.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the Supplementary Material. The original fcs files are deposited on the HCDM website (https://www.hcdm.org/index.php/2016-12-06-21-38-08/cdmaps-data-repository). Further inquiries can be directed to the corresponding author.

REFERENCES

1. Köhler G, Milstein C. Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. *Nature* (1975) 256:495–7. doi: 10.1038/256495a0
2. Bernard A, Dausset J, Milstein C, S S. Leucocyte Typing: Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies. *New York, NY: Springer-Verlag* (1984).
3. Engel P, Boumst I L, Balderas R, Bensussan A, Gattei V, Horejsi V, et al. CD Nomenclature 2015: Human Leucocyte Differentiation Antigen Workshops

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee of Monash University, Ethics Committee of Motol University Hospital, Ethics Committee of the University of Barcelona. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Contribution: TK, PE, and MZ conceptualized the study and designed the experiments. DK, JP-O, PA, and JF performed the experimental work. DK, TK, and JP-O performed data analysis. KF built the online web tools. DK, TK, and MZ wrote the paper. All authors commented on draft versions and approved the final manuscript.

FUNDING

The work was financially supported by the International Union of Immunological Societies (IUIS), projects NU20-05-00282 of the Czech Republic Ministry of Health to TK, Australian National Health and Medical Research Council (NHMRC) Fellowship GNT1117687 to MZ, SAF2015-69829 from Ministerio Ciencia e Innovación, Spain to PE.

ACKNOWLEDGMENTS

Kelly Lundsten (BioLegend) and Miloslav Suchanek (Exbio) for providing reagents, and to the Czech National Grid Infrastructure MetaCentrum under the programme “Projects of Large Research, Development, and Innovations Infrastructures” (CESNET LM2015042) for access to computing and storage facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.827898/full#supplementary-material

as a Driving Force in Immunology. *J Immunol* (2015) 195:4555–63. doi: 10.4049/jimmunol.1502033
4. Clark G, Stockinger H, Balderas R, van Zelm MC, Zola H, Hart D, et al. Nomenclature of CD Molecules From the Tenth Human Leucocyte Differentiation Antigen Workshop. *Clin Transl Immunol* (2016) 5:7–9. doi: 10.1038/cti.2015.38
5. Kalina T, Lundsten K, Engel P. Relevance of Antibody Validation for Flow Cytometry. *Cytom A* (2020) 97:126–36. doi: 10.1002/cyto.a.23895
11. Zola H, Swart B, Banham A, Barry S, Beare A, Bensussan A, et al. CD
12. Kishimoto T, Goyert S, Kikutani H, Mason D, Miyasaka M, Moretta L, et al. CD
13. Mason D, Andre
14. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K.
15. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-Genome
16. Pulvertaft JV. A Study of Malignant Tumours in Nigeria by Short-Term
17. Glier H, Holada K. Blood Storage Affects the Detection of Cellular Prion
18. Kalina T, Flores-Montero J, van der Velden VHJ, Martin-Ayuso M, Böttcher
19. 8. Hrusa
20. doi: 10.1016/j.jim.2012.04.002
21. Tissue Culture.
22. J Immunol Methods
23. Horváth-Hejtbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of Monocytes and Dendritic Cells in Blood. Blood (2010) 116: e74–80. doi: 10.1182/blood-2010-02-258558
24. van Dongen JMJ. Replication History of B Lymphocytes Reveals Homeostatic Proliferation and Extensive Antigen-Induced B Cell Expansion. J Exp Med (2007) 204:645–55. doi: 10.1084/jem.20060964
25. Agematsu K, Kagami H, Yang FC, Nakazawa T, Fukushima K, Ito S, et al. Human Memory B Cells Originate From Three Distinct Germinal Center-Dependent and -Independent Maturation Pathways. Blood (2011) 118(8):2150–8. doi: 10.1182/blood-2011-04-345579
26. van Zelm MC, Vlcek J, Stamatopoulos K, Cerutti A, et al. Human Memory B Cells Originate From Distinct Germinal Centers. Blood (2009) 114:2811–5.
27. Prager E, Sunder-Plassmann R, Hansmann C, Koch C, Knapp W, Napp et al. Interaction of CD31 With a Heterophilic Counterreceptor Involved in Downregulation of Human T Cell Responses. J Exp Med (1996) 184:41–50. doi: 10.1084/jem.184.1.41
28. van Zelm MC, Szczepanski T, van der Burg M, and Dongen JJM. Replication History of B Lymphocytes Reveals Homeostatic Proliferation and Extensive Antigen-Induced B Cell Expansion. J Exp Med (2007) 204:645–55. doi: 10.1084/jem.20060964
29. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of Interleukin (IL)-2 and IL-7 Receptors Discriminates Between Human Regulatory and Activated T Cells. J Exp Med (2006) 203:1693–700. doi: 10.1084/jem.20060646
30. Schafer P, Willmann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC Chemokine Receptor 5 Expression Defines Follicular Homing T Cells With B Cell Helper Function. J Exp Med (2000) 192:1553–62. doi: 10.1084/jem.192.11.1553
31. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B Helper T Cells Express CXC Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production. J Exp Med (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
32. Hickstein DD, Ozois J, Williams SA, Baenziger FU, Locksley RM, Roth GJ. Isolation and Characterization of the Receptor on Human Neutrophils That Mediates Cellular Adherence. J Biol Chem (1987) 262:5576–80. doi: 10.1016/S0021-9258(18)45611-6
33. Mason D, Andre P, Bensussan A, Buckley C, Civin C, Clark E, et al. CD Antigens 2002. Blood (2002) 99:3877–80. doi: 10.1182/blood.V99.10.3877
34. Kikutani H, Mason D, Miyasaka M, Moretta L, et al. CD Antigens 1996. Blood (1997) 89:3502. doi: 10.1182/blood.V89.10.3502
35. Kalina T, Fiser K, Perez-Andres D, Kuzikova D, Cuenca M, Bartol SJW, et al. CD Maps-Dynamic Profiling of CD1-CD100 Surface Expression on Human Leukocyte and Lymphocyte Subsets. Front Immunol (2019) 10:2434. doi: 10.3389/fimmu.2019.02434
36. Lanza F, Healy L, Sutherland DR. Structural and Functional Features of the CD34 Antigen: An Update. J Biol Regul Homeost Agents (2001) 15:1–13.
37. Kalina T. Reproducibility of Flow Cytometry Through Standardization: Opportunities and Challenges. Cytom A (2020) 97:137–47. doi: 10.1002/cyto.a.23901
38. van der Velden VHJ, Flores-Montero J, Perez-Andres M, Martin-Ayuso Y, Crespo O, Blanco E, et al. Optimization and Testing of Dried Antibody Tube: The EuroFlow LST and PIDOT Tubes as Examples. J Immunol Methods (2019) 475:112287. doi: 10.1016/j.jim.2017.03.011
39. Pulvertaft JV. A Study of Malignant Tumours in Nigeria by Short-Term Tissue Culture. J Clin Pathol (1965) 18:261–73. doi: 10.1136/jcp.18.3.261
40. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-Genome Negative ‘Null’ and ‘T Cell Lines Derived From Children With Acute Lymphoblastic Leukemia and Leukemic Transformed Non-Hodgkin Lymphoma. Int J Cancer (1977) 19:621–6. doi: 10.1002/ijc.2910190505
41. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and Characterization of a Human Acute Monocytic Leukemia Cell Line (THP-1). Int J Cancer (1980) 26:171–6. doi: 10.1002/ijc.2902602080
42. Nilsson K, Bennich H, Johansson SG, Pontén J. Established Immunoglobulin Producing Myeloma (IgE) and Lymphoblastoid (IgM) Cell Lines From an IgE Myeloma Patient. Clin Exp Immunol (1970) 7:477–89.
43. Alt F, Rosenberg N, Lewis S, Thomas E, Baltimore D. Organization and Reorganization of Immunoglobulin Genes in A-MULV-Transformed Cells: Rearrangement of Heavy But Not Light Chain Genes. Cell (1981) 27:381–90. doi: 10.1016/0092-8674(81)90421-9
44. Kalina T, Flores-Montero J, van der Velden VHJ, Martin-Ayuso M, Böttcher S, Ritgen M, et al. EuroFlow Standardization of Flow Cytometer Instrument Settings and Immunophenotyping Protocols. Leukemia (2012) 26:1986–2010. doi: 10.1038/leu.2012.122
45. Glier H, Holada K. Blood Storage Affects the Detection of Cellular Prion Protein on Peripheral Blood Leukocytes and Circulating Dendritic Cells in Part by Promoting Platelet Satellitism. J Immunol Methods (2012) 380:65–72. doi: 10.1016/j.jim.2012.04.002
46. Telford WG, Babin SA, Khorev SV, Rowe SH. Green Fiber Lasers: An Alternative to Traditional DPSS Green Lasers for Flow Cytometry. Cytom Part A (2009) 75A:1031–9. doi: 10.1002/cyto.a20790
47. Havenberg MD, Spits H. Human Innate Lymphoid Cells. Blood (2014) 124:700–9. doi: 10.1182/blood-2013-11-427781
48. Hertoghu N, Schvedelina KV, Stuart KD, McElrath MJ, De Rosa SC. OMIP- 064: A 27-Color Flow Cytometry Panel to Detect and Characterize Human NK Cells and Other Innate Lymphoid Cell Subsets, MAIT Cells, and γδ T Cells. Cytom A (2020) 97:1019–23. doi: 10.1002/cyto.a.24031
49. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson L, Landay A, et al. Expression of Interleukin (IL)-2 and IL-7 Receptors Discriminates Between Human Regulatory and Activated T Cells. J Exp Med (2006) 192:1553–62. doi: 10.1084/jem.192.11.1553
50. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K.
Immunophenotyping of Severe PID in Newborns and Young Children. *Front Immunol* (2020) 11:1–14. doi: 10.3389/fimmu.2020.00371

45. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The Who’s Who of T-Cell Differentiation: Human Memory T-Cell Subsets. *Eur J Immunol* (2013) 43:2797–809. doi: 10.1002/eji.201343751

46. Appay V, Dunbar PR, Callan M, Klegerman P, Gillespie GMA, Papagno L, et al. Memory CD8+ T Cells Vary in Differentiation Phenotype in Different Persistent Virus Infections. *Nat Med* (2002) 8:379–85. doi: 10.1038/nm0402-379

47. van den Heuvel D, Jansen MAEE, Dik WA, Bouallouch-Charif H, Zhao D, van Kester KAMM, et al. Cytomegalovirus- and Epstein-Barr Virus-Induced T-Cell Expansions in Young Children Do Not Impair Naive T-Cell Populations or Vaccination Responses: The Generation R Study. *J Infect Dis* (2016) 213:233–42. doi: 10.1093/infdis/jiv369

48. Swanson PA, Seder RA. OMIP-067: 28-Color Flow Cytometry Panel to Evaluate Human T-Cell Phenotype and Function. *Cytom Part A* (2020) 97:1032–6. doi: 10.1002/cyto.a.24189

49. Vanikova S, Koladiya A, Musil J. OMIP-080: 29-Color Flow Cytometry Panel for Comprehensive Evaluation of NK and T Cells Reconstitution After Hematopoietic Stem Cells Transplantation. *Cytom A* (2022) 101(1):21–6. doi: 10.1002/cyta.20182

50. Telford W, Murga M, Hawley T, Hawley R, Packard B, Komoriya A, et al. DPSS Yellow-Green 561-Nm Lasers for Improved Fluorochrome Detection by Flow Cytometry. *Cytom Part A* (2005) 68A:36–44. doi: 10.1002/cyta.20182

51. Kalina T, Flores-Montero J, Lecrevisse Q, Pedreira CE, van der Velden VHJ, Novakova M, et al. Quality Assessment Program for EuroFlow Protocols: Summary Results of Four-Year (2010-2013) Quality Assurance Rounds. *Cytom Part A* (2015) 87:145–56. doi: 10.1002/cyta.22581

52. Kalina T, Bridickova N, Giler H, Fernandez P, Bitter M, Flores-Montero J, et al. Frequent Issues and Lessons Learned From EuroFlow Qa. *J Immunol Methods* (2019) 475:112520. doi: 10.1016/j.jim.2018.09.008

53. Elghetany MT, Davis BH. Impact of Preanalytical Variables on Granulocytic Surface Antigen Expression: A Review. *Cytom B Clin Cytom* (2005) 65:1–5. doi: 10.1002/cyto.b.20051

54. Kuijpers T, Tool A, van der Schoot C, Ginsel L, Onderwater J, Roos D, et al. Membrane Surface Antigen Expression on Neutrophils: A Reappraisal of the Use of Surface Markers for Neutrophil Activation. *Blood* (1991) 78:1105–11. doi: 10.1182/blood.V78.4.1105.1105

55. Diks AM, Bonroy C, Teodosio C, Groenland RJ, de Mooij B, de Maertelaere E, et al. Impact of Blood Storage and Sample Handling on Quality of High Dimensional Flow Cytometric Data in Multicenter Clinical Research. *J Immunol Methods* (2019) 475:112616. doi: 10.1016/j.jim.2019.06.007

**Conflict of Interest:** BioLegend and Exbio kindly donated reagents used in this study.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher’s Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Kužlíková, Puhet-Ortiz, Aui, Fernández, Fier, Engel, van Zelm and Kalina. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.