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ORIGINAL ARTICLE

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A comprehensive assessment of four whole blood stabilizers for flow-cytometric analysis of leukocyte populations

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Abstract

Though cryopreservation of cell fractions is widely used in flow cytometry studies, whole blood cryopreservation is more challenging due to the presence of erythrocytes and effects of fixatives commonly used for preservation. Here, we evaluated and compared head-to-head the performance of four commercial whole blood cryopreservation kits; (1) Cytodelics, (2) Stable-Lyse V2 and Stable-Store V2 (SLSS-V2), (3) Proteomic stabilizer (PROT-1), and (4) Transfix. We found that PROT-1, Transfix, and Cytodelics maintained the distribution of major leukocyte subsets—granulocytes, T cells, natural killer cells, and B cells, on a comparable level to unpreserved samples, despite the attenuation of fluorescence intensities in flow cytometric assays. Moreover, these three stabilizers also maintained the activated phenotypes of neutrophils upon stimulation with *N*-formylmethionyl-leucyl-phenylalanine and lipopolysaccharides. The upregulation of adhesion molecules (CD11b), Fc receptors (CD16), and granule proteins (CD66b), as well as the shedding of surface L-selectin (CD62L), was conserved most efficiently in PROT-1 and Cytodelics when compared to samples only treated with erythrocyte lysing. However, none of the stabilizers provided a reliable detection of CCR7 for accurate quantification of T cell maturation stages. We also evaluated the performance of Cytodelics in longitudinal clinical samples obtained from acute COVID-19 patients, where it allowed reliable detection of lymphopenia and granulocyte expansion. These results support the feasibility of whole blood cryopreservation for immunophenotyping by flow cytometry, particularly in longitudinal studies. In conclusion, the performance of different stabilizers is variable and therefore the choice of stabilizers should depend on cell type of interest, as well as antibody clones and experimental design of each study.

KEYWORDS
COVID-19, cryopreservation, flow cytometry, immunophenotyping, method, whole blood, whole blood stabilizer, whole blood stabilizer

Xiaobo Huang and Luz E. Cabrera contributed equally to this study. Santtu Heinonen and Eliisa Kekäläinen contributed equally to this study.

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1 | INTRODUCTION

Technology advancements in cytometry and sequencing have made it possible to capture the vast complexity of human immune responses. Peripheral blood mononuclear cells (PBMC) are the most common type of leukocyte sample for immunological studies because PBMCs can be frozen alive, enabling a prospective sample collection. However, density gradient separation and the removal of plasma matrix in PBMC samples can alter cell composition and cause nonspecific activation of cells [1–3]. In addition to requiring a cell laboratory and trained staff for sample handling, PBMCs do not include granulocytes and thus only represent a part of all circulating leukocytes. In all, the process of PBMC isolation and freezing is time-consuming and laborious which causes challenges in many clinical studies.

Whole blood stabilization and cryopreservation immediately after blood collection offer a solution to solve many of the practical issues related to PBMC collection [4]. It provides a more accurate view of all the cells involved in the immune response [1–3]. Immediate whole blood cryopreservation can also reduce mechanical stress, sampling time, and cryoprotectant effects, which can cause activation and cell death among granulocytes [5–7]. In addition, whole blood offers technical and logistical benefits for clinical studies. It can be preserved in stabilizing media immediately after blood draw and stored for later analysis. Direct fixation using formaldehyde also ensures staff safety by inactivating infectious agents such as viruses [8, 9]. For cytometry analysis, as little as 100 μl of whole blood is sufficient, which is a significant advantage when studying populations with limitations to sample volumes collected (e.g., pediatric or severely ill patients) or when collecting sequential samples [1, 10]. However, most commercial stabilizing media for whole blood are used in mass cytometry and their performance in traditional flow cytometry has not been assessed.

In this study, we present a comprehensive evaluation of four commercial whole blood stabilizers. Three of the stabilizing reagents—Cytodelics from Cytodelics AB, Stable-Lyse V2 and Stable-Store V2 (SLSS-V2), and Proteomic stabilizer (PROT-1) from Smart Tube Inc, are primarily used in mass cytometry for long-term sample storage. Transfix from Cytomark, an Invitro diagnostic (IVD) product, is specifically designed for cryopreserving flow cytometric samples for up to 14 days [11, 12]. As Transfix is widely used in clinical laboratories, it is expected to be optimal for sample cryopreservation and could potentially act as a fixed control in addition to unpreserved controls in this study. Here, we hypothesized that these cryopreservation reagents would allow flow cytometric analysis of cellular characteristics comparable to fresh samples. Specifically, we aimed to measure their performance on common lineage markers and specific markers for T cells and granulocytes. To assess the feasibility of whole-blood stabilizers for clinical sample collection, we also analyzed real-life COVID-19 patient samples preserved in Cytodelics.

2 | MATERIALS AND METHODS

2.1 | Study design and ethical considerations

The study consists of two cohorts: (1) peripheral blood samples collected from healthy donors ($n = 10$, *Healthy donor cohort*) were used for head-to-head comparisons of different stabilizers; and (2) peripheral blood samples collected from patients hospitalized with COVID-19 at the Helsinki University Hospital, Helsinki, Finland ($n = 15$, *COVID-19 cohort*) were used to assess the feasibility and performance of Cytodelics with clinical samples. Characteristics of both cohorts are described in Table S1.

The study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (HUS/853/2020 and HUS/747/2019). All study participants provided written informed consent.

2.1.1 | Healthy donor cohort

The healthy *donor cohort* comprised adult (18 years or older) volunteers with no underlying immunodeficiencies diagnosed nor ongoing immunosuppressive medications.

2.1.2 | COVID-19 cohort

Inclusion criterion was hospitalization due to SARS-CoV-2 infection confirmed by polymerase chain reaction test. Patients with immunosuppressive treatments (e.g., dexamethasone) were excluded. The cohort comprised 15 hospitalized patients of which 7 were admitted to the intensive care unit (ICU). Samples taken within 15 days from symptom onset were categorized as acute, and those taken later than 21 days after symptom onset were categorized as convalescent. Among the 15 patients, 13 patients were sampled twice, that is, during both acute and convalescent disease phases (6 hospitalized and 7 ICU). The two patients with only single sample available were included only in the unsupervised clustering (one patient in acute disease phase and another patient in convalescent disease phase). For statistical analysis, the corresponding convalescent or acute samples of these two were treated as missing data.

2.1.3 | Sample collection

Blood samples were collected by venipuncture to BD Vacutainer EDTA blood collection tubes. For healthy donors, blood was added to cryo-tubes containing whole blood stabilizers within 3 h after the venesections and further processed according to manufacturers' recommendations. Four stabilizer protocols were processed simultaneously for each donor. For COVID-19 patients, 0.5 ml full blood

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samples were incubated with 0.5 ml Cytodelics stabilizer at room temperature (RT) for 10 min and then stored at −80°C.

2.2 | Sample processing and staining

2.2.1 | For comparison between stabilizers

On the day of blood collection, control samples were stained with fluorochrome-conjugated antibodies for 25 min at RT and subsequently subjected to 1× BD FACS lysing solution (BD Biosciences, United States) for 10 min at RT as recommended by the Euroflow standardization [11]. Cells were washed twice with a staining buffer (2% FCS + 2 mM EDTA in PBS) before being acquired on the flow cytometer.

Unstained whole blood samples were stabilized in (1) Cytodelics (Cytodelics AB, Sweden), (2) Proteomic stabilizers (PROT1) (Smart tubes, CA, United States), (3) Stable Lyse–Stable Store V2 reagent, and (4) Bulk Transfix (Cytomark, United Kingdom), according to manufacturers' instructions before cryopreservation (Table 1). Blood samples were incubated in Cytodelics and PROT-1 stabilizer for 10 min at RT before being stored at −80°C until analysis. To preserve in SLSS-V2, samples were treated with Stable Lyse solution (Smart tubes Inc) and Stable Store solution (Smart tubes Inc) subsequently, each with a 15-min incubation at RT before moving to −80°C. Samples treated with transfix solution had a 15-min incubation at RT before being transferred to +4°C. With all stabilizers, we employed a fix/freeze/stain procedure with the blood-to-stabilizer rations recommended by the manufacturers.

PBMCs were isolated using Ficoll Paque Plus (GE Healthcare, United States). Blood samples were layered on top of the Ficoll and centrifuged at 400*g* for 30 min at RT without brake. PBMCs were collected from the cloudy layer above Ficoll, followed by the cryopreservation in CTL-Cryo™ Media (ImmunoSpot, United States) and stored at −140°C according to the manufacturer's instruction.

After 3–7 days all samples were thawed according to manufacturers' instructions either in a +37°C water bath for 2 min (PBMC, Cytodelics), a +10°C water bath for ∼10 min (PROT-1, SLSS-V2), or RT for 2 min (Transfix). Red blood cell lysis was done for Cytodelics and PROT-1 treated samples using fix/lyse buffers (Cytodelics AB, Sweden) and 1x thaw-lyse buffer (Smart tubes Inc), respectively. Except for PBMCs washed twice with thawing media (90% RPMI, 10% CTL wash, 10 μg/ml DNase), all other samples were washed with a staining buffer (PBS with 2% FCS and 2 mM EDTA).

2.2.2 | For COVID-19 samples

Samples were thawed according to the instruction for Cytodelics as mentioned above. The samples were then stained with fluorochrome-conjugated antibodies in the flow panel COVID-19 (Table S2).

2.3 | Granulocyte activation in vitro

Whole blood was stimulated with 5 μg/ml LPS or 100 μmol/ml *N*-formylmethionyl-leucyl-phenylalanine (fMLP) for 1 h in a +37°C incubator with 5% CO_2. The reaction was stopped, and cells were washed with cold PBS by centrifugation at 330*g* for 10 min. The cell pellet was reconstituted in PBS and processed further with either BD FACS lysing solution for immediate analysis or stored in different cryopreservation reagents for later use.

2.4 | Flow cytometry

Flow cytometry panels (Table S2) were designed with the aid of BD Horizon Guided Panel Solution tool to maximize resolution and minimize spectral spillover [14]. Compensation matrices were established

TABLE 1 Overview of four commercial whole blood cryopreservation kits and BD FACS lysing solution used as a fresh control

	BD FACS lysing solution	Cytodelics	PROT-1	SLSS-V2	Transfix (bulk)
Erythrocyte lysis	Yes	Yes	Yes	Yes	Yes
Formaldehyde fixation	10%	<5%	3%–7%	unknown	<1%
Methanol permeabilization	3.50%	Unknown	Unknown	Unknown	Unknown
Cryoprotectant	Unknown	Unknown	3%–7% Diethylene glycol	Unknown	Unknown
Processing time (min)	∼20	10	10	30	15
Stabilizer–Blood ratio	-	1:1	7:5	3:1	1:5
Preserving temperature	-	−20 or −80	−80	RT/+4/−80°C	+4°C
Storage duration	-	1 year	13 months [13]	>2 weeks at +4°C Unknown at −80°C	14 days
Thawing/RBC lysing time (min)	∼20 (RBC lysing only)	30	∼50	∼10	∼15
Regulatory status[a]	IVD	RUO	RUO	RUO	IVD
Estimated price/100 μl blood[b]	0.2€	0.5€	1€	0.4€	0.3€

Abbreviations: PROT-1, proteomic stabilizer; SLSS-V2, Stable Lyse–Stable Store V2.
[a]Research use only (RUO) or in vitro medical device (IVD).
[b]Price of whole blood cryopreservation kit.

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from single staining files using CompBead Ig, κ/Negative Control Particles Set (BD Biosciences) [15].

All staining was done in dark at +4°C. One to five million cells were first suspended in Brilliant Stain Buffer (BD Bioscience, United States). In granulocyte activation experiments, samples were first treated with 2.5 μg Human BD Fc Block™ (BD Biosciences) or 100 U/ml Heparin (Sigma-Aldrich, United States) for 20 min. Subsequently, an antibody cocktail for surface markers was added, followed by a 30-min incubation. If needed, the samples were then washed with 1X permeabilization buffer (eBioscience, United States), followed by staining with antibodies for intracellular markers for 30 min in the dark at +4°C. After washing twice with FACS staining buffer (PBS with 2% FCS and 2 mM EDTA), they were ready for acquisition by BD FACSDiva version 8.0.1 software in LSRII Fortessa (BD Biosciences). About 120,000–250,000 cells were recorded for studying the whole blood stabilizers, and about 1–1.5 million cells were recorded for the COVID-19 experiments.

2.5 | Data processing and statistical analysis

The obtained flow cytometry data were analyzed with Flowjo software (v10.7.1, BD Bioscience). The statistical analysis and graph design were performed using Prism 9 (GraphPad Software Inc, United States) and R software v3.6.3 (R core team).

Uniform Manifold Approximation and Projection (UMAP) and Self-Organizing Maps (FlowSOM) clustering was performed on the Flowjo software. All FCS files were preprocessed to correct for spectral spillover and remove debris, singlets, eosinophils, and B cells. An equal sampling of 10,000 events from each FCS file was done using DownSample plugin in Flowjo, which were then concatenated into a single flow cytometry file of 280,000 cells. The following markers were used for FlowSOM and UMAP analysis: CD3, CD15, CD16, CD14, HLA-DR, CD11b, CD33, CD274 (PD-L1), ARG1, LOX-1, Ki-67. All cells were projected on UMAP's two-dimensional space. UMAP plots were generated with Euclidean distance, 10 for nearest neighbor, 0.5 for minimum distance, and 2 for total components. Resulting UMAP plots were fed into the FlowSOM clustering algorithm (cluster number = 13) [16].

3 | RESULTS

3.1 | Robust preservation of major immune subsets after whole blood fixation and cryopreservation

All studied whole blood stabilizers changed cell morphology, particularly that of eosinophils, as shown in forward scatter/side scatter (FSC/SSC) (Figure S1A). This finding was expected due to the formaldehyde in the fixatives and the low preservation temperature, both of which are known to impact the cell morphology [17]. SLSS-V2 severely altered FSC/SSC plots, making it impossible to distinguish monocytes by size (Figure S1A). Fixation and cryopreservation also altered median fluorescence intensity (MFI) of cellular markers, making it inappropriate to use this parameter for direct comparison between the fresh controls and stabilized samples (Figure S2A).

The proportions of main leukocyte and lymphocyte populations from total CD45-positive leukocytes were mostly comparable between the control samples treated with BD FACS lysing solution and stabilized samples (Figure 1). In general, PROT-1 was the most robust in detecting common immune cell lineages, particularly lymphocytes. All other stabilizers also preserved well major lymphocytes, namely CD4 and CD8 T cell populations; CD19 B cells; CD56dim and bright NK cells. However, Cytodelics-treated samples had a slightly larger proportion of CD15+ granulocytes and a slightly lower CD4+ lymphocytes fraction, reflected in the total CD3+ lymphocyte population as well, when compared to the control samples (Figure 1A–C). In Transfix-treated samples, there was a smaller fraction of NK cells, mostly due to the reduced signal of CD16 (Figure 1G–I).

The results were more variable for innate immune cell subsets. PROT-1, SLSS-V2 and Transfix retained the frequency of CD15+ granulocytes and CD14+/CD16-classical monocytes reliably. However, a partial loss of CD14 signal was observed in all cryopreserved samples, rendering the identification of intermediate monocytes challenging (Figures 1K and S2B).

Compared to the stabilizers, PBMC did not represent whole blood populations due to the loss of granulocytes. The distribution of all studied populations in PBMC were significantly different from the fresh samples (Figure 1).

3.2 | Poor separation of T cell subpopulations in the whole blood stabilized samples

The reliable detection of CD4 and CD8 cells prompted us to look further into different functional subsets of T lymphocytes. We analyzed gamma delta (TCRγδ^{+}); naïve (CCR7+CD45+), effector memory (EM) (CCR7−CD45−), central memory (CM) (CCR7+CD45−), CD45RA+ effector memory (EMRA) (CCR7−CD45+) and recent thymic emigrant (RTE) (CCR7+CD45+CD31+CD95−) T cells. Though differences were present, PBMC and fresh controls had comparable frequencies for the majority of TCRγδ^{+}, naïve, and memory T subpopulations of the total CD3-positive T cells. Compared to PBMCs, the data was more variable in whole blood preserved samples, such as a two-fold reduction of γδ T cells in these samples (Figures 1L and S3).

Loss of CCR7 positive signals in all stabilized samples resulted in poor identification of naïve and memory T cells, particularly within the CD4 subset. Even IVD-certified Transfix performed poorly in detecting CCR7 signals despite having the antibody clone recommended by the manufacturer. On the other hand, CCR7 signal resolution was worse in CD4+ T cells than in CD8+ cells (Figure S4). There was a

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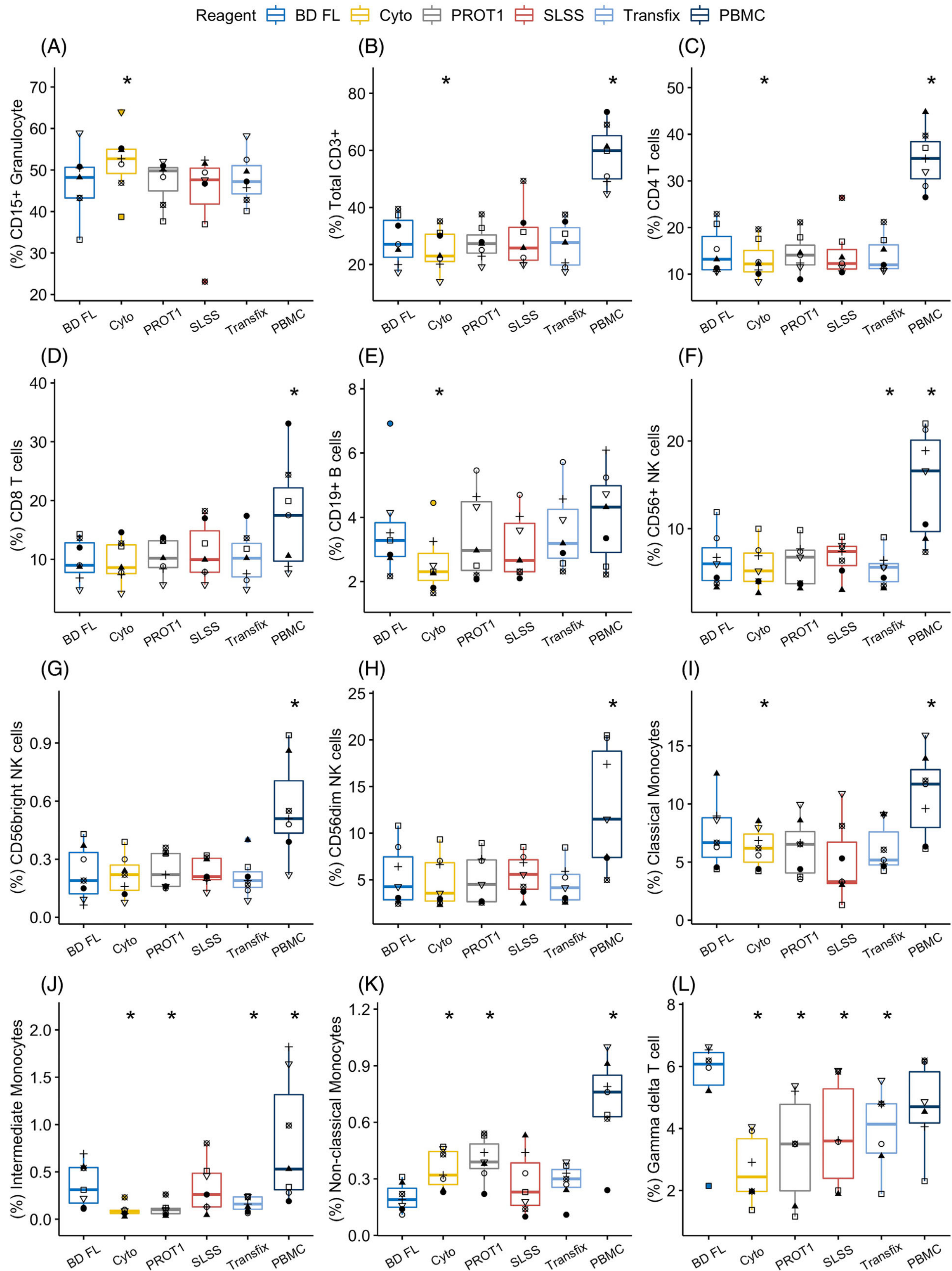

FIGURE 1 Main lineage populations in samples treated with different whole-blood stabilizers. (A–K) Proportions within total CD45-positive single cells—(A) Granulocytes; (B) total T cells; (C) CD4 T cells; (D) CD8 T cells; (E) B cells; (F) total natural killer (NK) cells; (G) CD56 bright NK cells; (H) CD56 dim NK cells; (I) classical monocytes; (J) intermediate monocyte; (K) non-classical monocytes. (L) Frequency of $\gamma\delta$ T-cells out of total CD3+ cells. Datapoint shapes correspond to different donors. Asterix indicates statistical significance in analysis with Wilcoxon matched-pairs signed-rank test when compared to the control-treated samples (BD FACS lysing, blue box plot). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. BD FL, BD FACS lysing solution; Cyto, Cytodelics; SLSS, Stable Lyse, Stabled Store V2 [Color figure can be viewed at wileyonlinelibrary.com]

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20%–40% loss of CD4+ naïve and CM cells, which subsequently had an impact also on the frequency of CD4+ EM and RTE populations (Figure 2A–D).

The higher CCR7 signal resolution with CD8 positive cells in stabilized samples allowed better identification of CD8+ T cell subsets. Notably, the quantification of CD8+ EMRA, CM, and EM subsets in

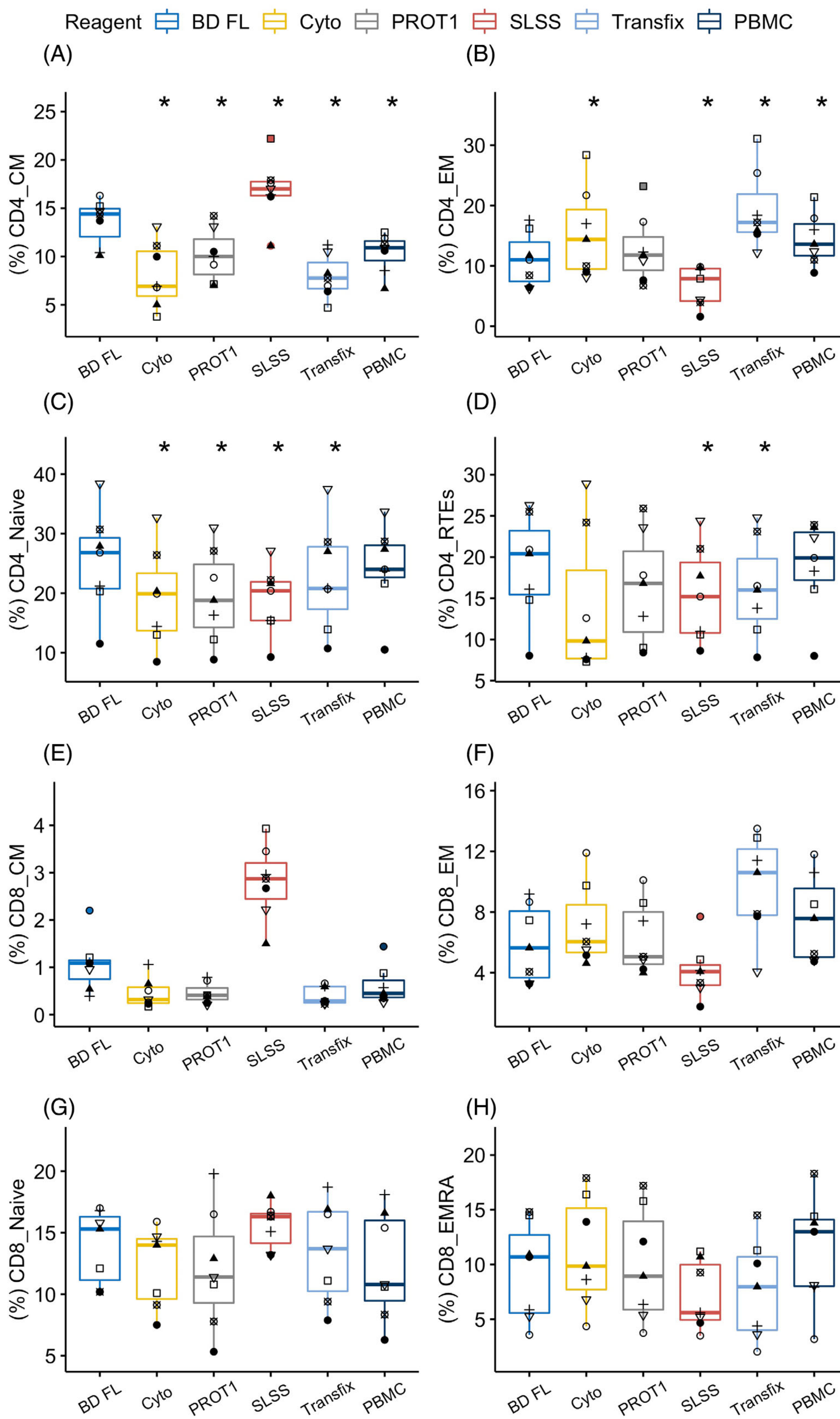

FIGURE 2 Detection of proportions of naïve and memory T cell subsets of total CD3-positive T lymphocytes in samples treated with different whole blood stabilizers ($n = 7$). (A–D) CD4; (E–H) CD8. Datapoint shapes correspond to different donors. Asterix indicates analysis with Wilcoxon matched-pairs signed-rank test when compared to the control-treated samples (BD FACS lysing, blue box plot). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. BD FL, BD FACS lysing solution; CM, central memory; Cyto, Cytodelics; EM, effector memory; EMRA, terminally differentiated effector memory; RTE, recent thymic emigrants; SLSS, Stable Lyse, Stabled Store V2 [Color figure can be viewed at wileyonlinelibrary.com]

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PROT-1 were comparable to the controls. Transfix also performed well in detecting naïve T cells but was unreliable for EM and EMRA subsets. SLSS-V2 and Cytodelics preserved the majority of the CD8 sub-populations poorly (Figure 2E–H).

3.3 | Granulocyte activation status was conserved by the whole blood stabilizers

We stimulated granulocytes with fMLP and lipopolysaccharides (LPS) for 1 h at +37°C and evaluated the expression of activation markers both pre- and post-cryopreservation. LPS stimulation significantly reduced the expression of CD62L and increased the expression of CD11b and CD66b while CD16 was only slightly increased in both neutrophils and eosinophils in the controls treated with BD FACS lysing solution (Figures 3 and S5B–D). A similar trend for the other markers was observed in fMLP stimulated granulocytes (Figures 3 and S5). We can conclude that our stimulation assay caused robust activation of granulocytes.

All whole blood stabilizers preserved the expressional trend of the neutrophil activation markers after the fMLP and LPS stimulation, despite the attenuated MFI signal. The data is more variable and less conclusive for eosinophils (Figure S6). In neutrophils, CD16, CD11b and CD66b markers were more suitable to study granulocyte activation in whole blood stabilizers, whereas a major signal reduction in CD62L in all stabilizers caused no distinction between activated and non-activated samples (Figure S7). PROT-1 had the best performance for neutrophils, preserving the activation profiles of all four markers in LPS-treated samples (Figure S7). However, PROT-1 did not reflect the full scale of change in CD66b expression since we could detect ~a 50% increase in CD66b in PROT-1-treated cells compared to more than 150% in the fresh controls. (Figure 3D).

The second-best performance was by Cytodelics, which preserved the expressional trend of CD66b and CD16, as well as CD62L to some extent, in neutrophils upon LPS stimulation. Again, the MFI and percentage of MFI changes in CD62L and CD66b after both stimulations were much lower than in the controls (Figure S7). Strikingly, CD11b signal was almost absent when using the ICRF44 clone that the manufacturer had validated (Figure 3B). However, the problem was mitigated by using D12 or M1-70 clones (Figure S5E).

Contrary to our expectations, Transfix performed poorly for both CD62L and CD16: half of the markers commonly used to assess neutrophil activation. SLSS-V2 produced the worst result, where CD11b, CD66b, and CD16, three out of four activation markers, showed either no response to stimulation at all or even a reverse trend compared to the control.

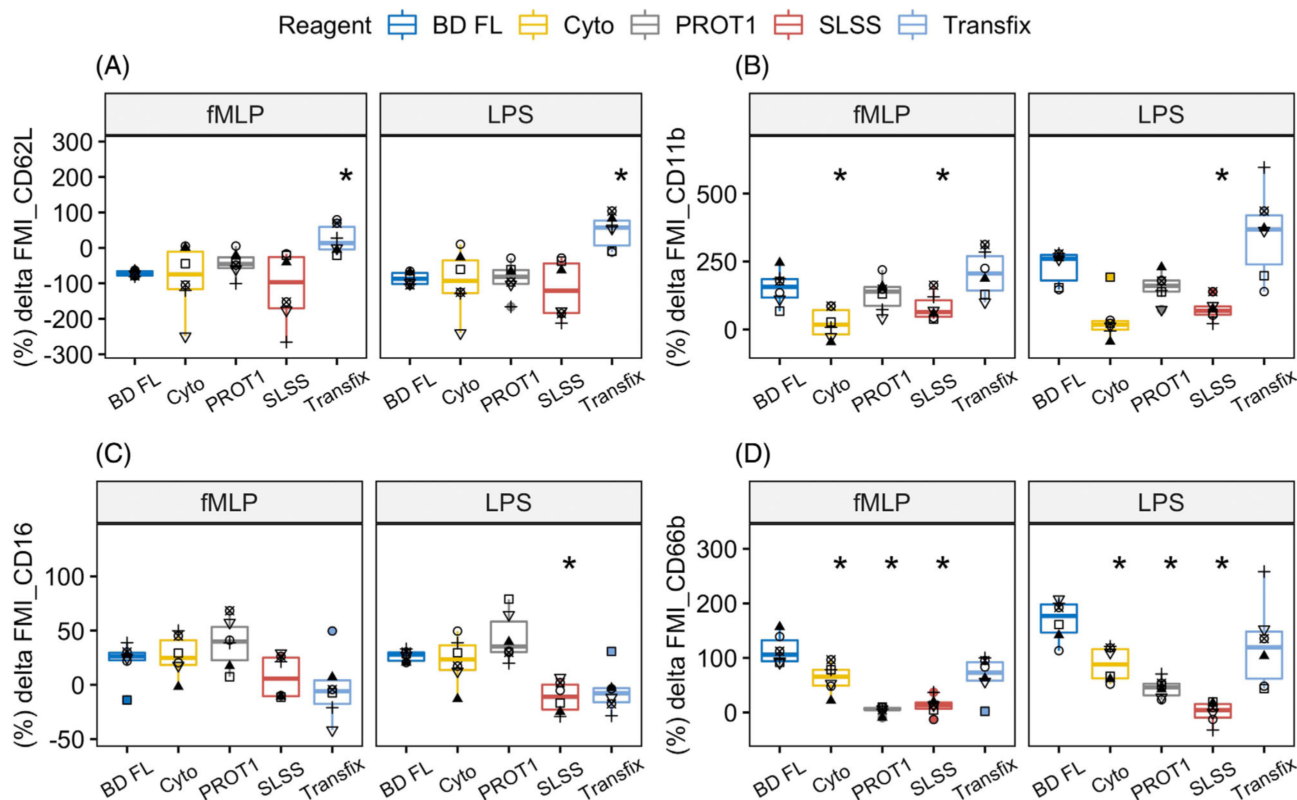

FIGURE 3 Retention of neutrophil activation marker expression on stimulated neutrophils by different whole blood stabilizers. (A) CD62L, (B) CD11b, (C) CD66b, (D) CD16. The frequency indicates the percentage change in expression relative to the unstimulated blood sample. Different shapes of datapoints correspond to different donors. Asterix indicates statistical significance in analysis with Wilcoxon matched-pairs signed-rank test when compared to the control-treated samples (BD FACS lysing, blue box plot). *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$. BD FL, BD FACS lysing solution; Cyto, Cytodelics; fMLP, *N*-formylmethionine-leucyl-phenylalanine; LPS, lipopolysaccharides; SLSS, Stable Lyse, Stabled Store V2 [Color figure can be viewed at wileyonlinelibrary.com]

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3.4 | Reliable longitudinal analysis of major immune subsets in Cytodelics-treated COVID-19 clinical samples

Since Cytodelics was robust and reliable in preserving common immune lineages and neutrophil activation markers, we further validated its performance using COVID-19 patient samples that were longitudinally collected and preserved in Cytodelics during acute disease and convalescence, with flow cytometry data being acquired later. Samples were stained with a 12-marker flow cytometry panel. We used the unsupervised analysis based on our previous publication to identify different subpopulations of granulocytes [18]. FlowSOM analysis of the flow cytometric data identified 13 meta clusters, of which lymphocytes and granulocyte subsets were segregated, as shown on UMAP (Figure S8B).

T cell frequency increased in COVID-19 patients by almost two-fold after 90 days from symptom onset, reflecting the T cell lymphopenia often found during acute COVID-19 (Figure 4A,B), which correlates with disease severity and poor outcome [19–21]. In addition, acute patients had a lower frequency of CD14+ monocytes, whereas the frequency of CD15+ neutrophils was significantly elevated (Figure 4C,D). In all, these data indicate that Cytodelics conserved known differences in major immune subsets between acute and convalescent COVID-19 samples [19, 20].

3.5 | Inconclusive detection of temporal changes of neutrophil subpopulations in Cytodelics-treated COVID-19 clinical samples

FlowSOM clustering also identified different subsets of neutrophils: immature, mature, and activated cells, based on CD16 expression (Figure 5A). FlowSOM Cluster 1 was entirely negative for CD16, indicating the presence of immature cells. These CD16− immature neutrophils increased significantly in acute samples from hospitalized and ICU treated patients (Figure 5B).

On the other hand, no statistical difference was detected between the acute and convalescent samples regarding immature (CD33+ CD16+) and mature neutrophils (CD33−/+ CD16+/++) (FlowSOM cluster 3, 2, 5, and 7, Figure 5C,D).

Similarly, the hospitalized and ICU cohort showed no difference in the frequency of suppressive-like neutrophils, as indicated by their expression of $CD16^{high}$, ARG1 and LOX-1 in cluster 3. ARG1 and LOX-1 expression accounted for about 20% of total cells analyzed.

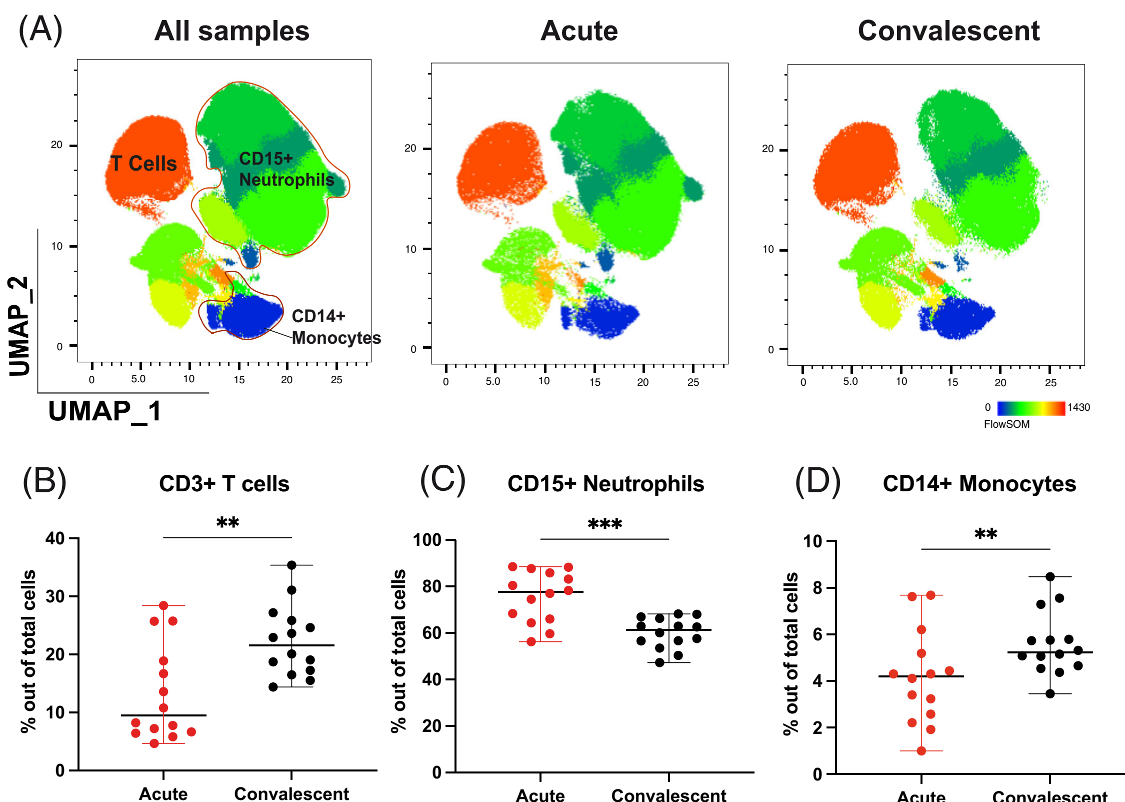

FIGURE 4 Detection of highly expressed immune markers in COVID-19. (A) UMAP projection of aggregated flow cytometry data for all participants with FlowSOM meta cluster overlaid. (B–D) Percentages of CD3+ T cells, CD15+ neutrophils and CD14+ monocytes respectively, among total cells from the full COVID-19 patient cohort used in UMAP analysis. Statistical analysis was performed with the Wilcoxon matched-pairs signed-rank test for nonparametric data. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

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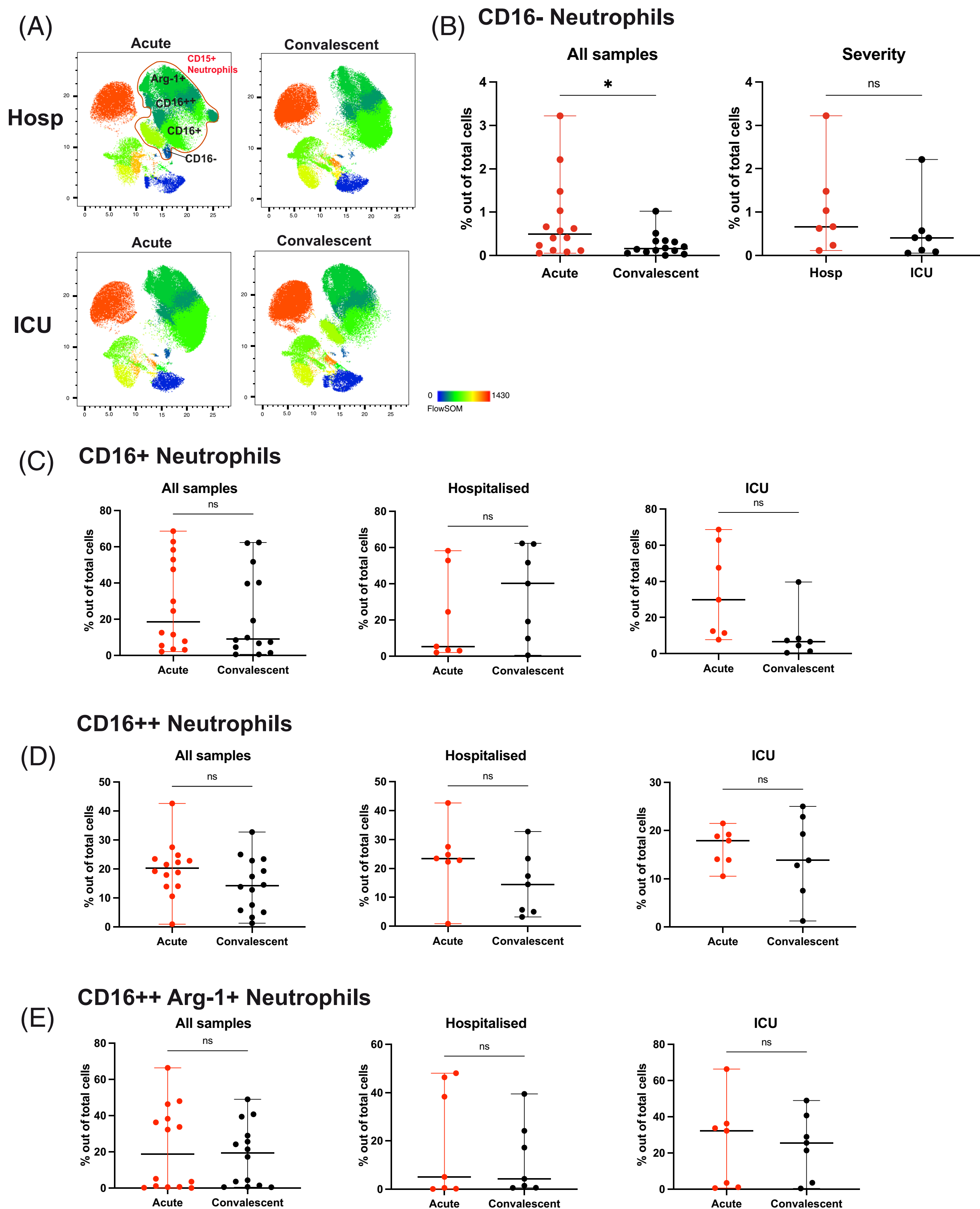

FIGURE 5 Detection of neutrophil sub-populations in COVID-19. (A) UMAP projection of different subgroups among hospitalized and ICU samples with FlowSOM metaclusters overlaid. (B–E) Percentage of neutrophil subpopulations of total cells according to FlowSOM metaclusters, CD16– (B); CD16+ (C); CD16++ (D); CD16++ ARG1+ (E). Statistical analysis was done with Wilcoxon matched-pair signed rank test for nonparametric data. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ [Color figure can be viewed at wileyonlinelibrary.com]

However, the frequency of suppressive-like neutrophils varied dramatically between patients. Out of the total cells, some subjects had up to 40% suppressive-like neutrophils, whereas others had less than 5%. In addition, these suppressive-like cells seemed to be highly proliferative, as suggested by a high expression of Ki-67 compared to other subsets (Figure S8B). In conclusion, we were unable to confirm if cluster 3 represented solely suppressive neutrophils or if it also contained other mature and activated neutrophils (Figure 5 E).

4 | DISCUSSION

We demonstrate here that despite the overall reduction in the fluorescence intensity, Cytodelics, PROT-1, SLSS-V2, and Transfix preserved the distribution of well-defined and highly expressed immune cell lineages. A fix/freeze/stain procedure was employed by all these stabilizers, and the sample processing time was much shorter than that required for PBMC isolation. In particular, the stabilizers properly conserved the lymphocyte subtypes, a finding in line with a previous study by Pinto et al. [22]. The best performance was found for PROT-1 and Cytodelics, followed by Transfix and SLSS-V2. Although the use of Cytodelics resulted in a small deviation in the frequency of CD3 cells between the controls and stabilized samples in the healthy cohort, it clearly reflected lymphopenia often found in between acute COVID-19 samples [19–21, 23]. Moreover, Cytodelics allowed the detection of the expansion of CD15+ neutrophils, particularly in immature CD16− subsets, consistent with findings in the previous report [13]. Together, these data provide evidence that these stabilizers can be used in clinical studies.

PROT-1 and Cytodelics also conserved the activation status of granulocytes regardless of differences in MFI. The finding is in accordance with a previous study by Ruiter et al., exploring neutrophil activation markers following a fix/freeze/stain procedure [17]. However, our data showed that whole blood cryopreservation did not fully capture the scale of activation and cellular changes in neutrophils, as indicated by the differences in the magnitude of MFI changes between test samples and the controls.

Importantly, we found that stabilization and cryopreservation decreased the median intensity of some myeloid markers. Partial loss of CD14, CD66b, CD15, and CD62L were observed, making it challenging to quantify monocyte subsets and the granulocyte activation status. Similar findings of the effects of cryopreservation on these markers have been documented [17, 24–27]. This also explained why we were unable to reliably identify granulocyte subpopulations, particularly suppressive neutrophils in Cytodelics-treated COVID-19 samples. The result agrees with a previous study describing the loss of suppressive granulocytes in frozen samples [27]. However, Serra et al., found no substantial differences in CD66b and CD14 expression in PROT-1 and Cytodelic compared to the fresh samples [28]. Similarly, we were unable to accurately quantify T cell subpopulations due to signal reduction in TCR-γδ and CCR7 in all stabilizers. We speculated that formaldehyde fixation might have destroyed antibody binding sites in TCR-γδ, yet this could not be confirmed due to the lack of fluorochrome-conjugated antibodies with gamma3.20 or H-41 clones, which is standard for detecting TCR-γδ in formalin-fixed samples [29]. Moreover, there are currently no reports on the performance of these clones in flow cytometry analysis. CCR7 signal was also attenuated in samples fixed with Cytodelics, PROT-1, SLSS-V2, and Transfix, making it impossible to accurately distinguish between naïve and memory T cell subsets. Comparable results were reported previously in mass cytometry by Sakkestad et al. [30] and Nassar et al. [31] and in flow cytometry by Serra et al. [28], suggesting that chemokine receptors, such as CCR7, CXCR3, and CCR6, are not compatible with whole blood preservation methods involving a fix/freeze/stain procedure [30, 31].

Cytodelics, PROT-1 and SLSS-V2 showed less than optimal results on classical fluorescence-based flow cytometry than in cytometry by time-of-flight (CyTOF). This is because fluorescence spillover and cell-associated autofluorescence make flow cytometry less sensitive and prone to error [32–34]. More in detail, correction for spectral overlap requires compensation, which could introduce variation to data interpretation. High-dimension flow cytometry panels increase the complexity of compensation matrices, making flow panels more prone to the loss of signal resolution [35–37]. Thus, fluorescence spillover and autofluorescence background are detrimental to detecting low abundance antigens, particularly when their sensitivity to antibody staining has been reduced by fixation and cryopreservation. By contrast, CyTOF avoids the spectral overlapping or autofluorescence issues by using element isotopes, making it excellent for low abundance markers [38, 39]. The technical differences between flow cytometry and CyTOF methods explain the discrepancy in the detection of certain antigens. For example, we were unable to reliably identify CD62L and CD14 signals in Cytodelics-fixed samples, contrary to our previous study where detection was done using CyTOF [40].

Whole blood stabilization and cryopreservation offer several advantages over the standard procedures such as PBMC isolation and cryopreservation with cryo-protectants [1]. They not only provide more accurate data on whole blood compositions, but the rapid process also offers the benefit of minimizing changes in cellular structures after the removal of physiological environments [1, 3]. Additionally, the procedure is simple and inexpensive while requiring a smaller sample volume than PBMCs, allowing for the collection of samples simultaneously, without the need for a highly trained staff and complex equipment. Small sample volume is also highly beneficial for sample collections done in pediatric patients. Moreover, cell fixation makes the method safer for infectious agents, such as SARS-CoV-2 [41].

To our knowledge, our study is one of the most comprehensive analyses of multiple whole blood cryopreserving stabilizer reagents for flow cytometry, and the most extensive in terms of covering different lineages. This is particularly relevant in the case of granulocytes, as their isolation can be challenging, with sampling time and mechanical stress playing a major role in their inadvertent activation and cell death. Additionally, unlike PBMCs, granulocytes are poorly cryopreserved by standard freezing procedures, causing clumping and damage to other cells by releasing genetic material and lysosomal enzymes upon thawing. Our results showed that, overall, the detectability of well-defined and highly expressed immune cell lineages was reasonably accurate in preserved samples. However, further optimization is needed to increase

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the efficacy of these whole blood stabilizers for low abundance markers. Our data suggests that PBMC isolation outperforms the whole blood stabilizers when focusing on lymphocyte and monocyte lineages. A stain/fix/freeze procedure would reduce the detrimental effects of formaldehyde fixation on epitope structures.

One of the limitations of our study is that the assessment on the effects of the different whole blood stabilizers concentrates on widely used extracellular markers. Two intracellular markers, namely Arg1 and Ki-67, were assessed indirectly, as these were only included in the COVID-19 panel. Previous studies by Paredes et al. and Braudeau et al. showed that leukocyte expression of intracellular markers, such as FOXP3, Helios and inflammatory cytokines, were detectable by flow cytometry after undergoing cryopreservation [42, 43]. However, it is important to investigate in the future how cryopreservation methods involving whole blood stabilization and fixation affect intracellular markers for flow cytometry detection, since it remains unclear whether the stabilizing buffers can increase cell permeability and have an impact on the staining of intracellular markers. Therefore, selection of compatible antibodies and experimental workflow are essential for achieving the most accurate result.

In conclusion, Cytodelics, PROT-1, SLSS-V2, and Transfix allow robust identification of cell lineages, but their usage for further phenotyping of smaller subpopulations need to be carefully considered. Selection of the whole-blood stabilizer needs to be carefully evaluated for each application regarding storage, since Cytodelics, PROT-1, and SLSS-V2 can store samples for up to 1 year, unlike the 14-day storage time for Transfix. Considering their variable performance in epitope preservation, there was no single best stabilizer that worked for all cell types. The cell types, markers, antibody clones, processing time, and techniques need all to be determined before selecting the most suitable stabilizers.

AUTHOR CONTRIBUTIONS

Conceptualization: Ngoc Anh Nguyen, Santtu Heinonen, Eliisa Kekäläinen. *Methodology*: Ngoc Anh Nguyen, Xiaobo Huang, Luz E. Cabrera, Kirsten Nowlan. *Data curation*: Ngoc Anh Nguyen, Xiaobo Huang, Pirkka T. Pekkarinen. *Investigation*: Ngoc Anh Nguyen, Luz E. Cabrera, Tomas Strandin. *Formal analysis*: Ngoc Anh Nguyen. *Supervision*: Santtu Heinonen, Eliisa Kekäläinen. *Funding acquisition*: Santtu Heinonen, Eliisa Kekäläinen. *Project administration*: Santtu Heinonen, Eliisa Kekäläinen. *Resources*: Pirkka T. Pekkarinen, Anu Kantele, Olli Vapalahti. *Writing – original draft*: Ngoc Anh Nguyen. *Writing – review and editing*: Ngoc Anh Nguyen, Xiaobo Huang, Luz E. Cabrera, Pirkka T. Pekkarinen, Kirsten Nowlan, Tomas Strandin, Anu Kantele, Olli Vapalahti, Santtu Heinonen, Eliisa Kekäläinen. [Correction added on 29 November 2022, after first online publication: The contributions of the author, Ngoc Anh Nguyen has been updated in this version.]

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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PATIENT CONSENT STATEMENT

All study participants provided written informed consent.

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