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Flow cytometry reveals constant lymphocyte proportions after long-term cryopreservation of whole blood in TransFix® cell stabilization reagent

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ABSTRACT

Flow cytometry is an important technique for characterization of immune cells, with accurate lymphocyte profiling being essential for clinical diagnostics and research applications. While immediate processing of blood samples is ideal, long-term storage solutions are needed for large-scale studies or settings without immediate access to laboratory facilities. TransFix® is a chemical stabilization solution that allows delayed analysis by preserving cell morphology and surface markers. However, the impact of long-term cryopreservation in TransFix® on lymphocyte integrity remains underexplored. In this study, we evaluated the efficacy of cryopreservation in TransFix® for maintaining the proportions of key lymphocyte subsets, including $CD3^+$ T cells, $CD3^+CD4^+$ T helper cells, $CD3^+CD8^+$ cytotoxic T cells, $CD19^+$ B cells, and $CD16^+/CD56^+$ natural killer cells. Blood samples were cryopreserved in TransFix® for varying time periods, up to 48 months, and compared to fresh samples using flow cytometry. The results show that the proportions of lymphocyte subsets remain stable during cryopreservation for up to 48 months, with no significant differences observed between fresh and cryopreserved samples. This suggests that TransFix® can successfully preserve lymphocyte integrity for long-term storage, providing a reliable option for delayed analysis. These results highlight the usefulness of TransFix® for studies that require extended storage, making it easier to conduct immune monitoring in a wide range of settings.

1. Introduction

Flow cytometry is a cornerstone analytical technique in immunology, enabling detailed characterization of different immune cells, *e.g.* lymphocyte subsets (McKinnon, 2018). Lymphocytes are divided into several subpopulations depending on their origin, function and surface markers. The major subsets are $CD3^+$ T cells, $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T (Tc) cells, $CD19^+$ B cells and natural killer (NK) cells expressing CD16 and CD56 (Chaplin, 2010). Accurate and reliable lymphocyte profiling is essential for clinical diagnostics and research applications, including monitoring immune responses, disease progression, and the efficacy of therapeutic interventions. However, the integrity of lymphocyte subsets can be significantly influenced by the methods of blood collection, storage, and processing (Diks et al., 2019). Various preservation methods have been employed to maintain the viability and phenotypic stability of lymphocytes in blood samples, each with its own advantages and limitations. Traditionally, fresh blood samples are processed immediately after collection to ensure optimal cell integrity. However, immediate processing is not always feasible, especially in large-scale studies or in settings where laboratory facilities are not readily accessible. Several preservation methods have been developed to address this.

Short-term storage at room temperature or 4 °C is commonly used to preserve blood samples for up to 24–48 h. While this method slows down metabolic activity and reduces cellular degradation, it is not suitable for longer storage periods due to gradual loss of cell viability and phenotypic changes (Unalli and Ozarda, 2021; Ekong et al., 1993).

Chemical agents such as fixatives or preservatives added to blood

Abbreviations: PBMC, Peripheral blood mononuclear cell; CPT, Cell preparation tube; EDTA, Ethylenediaminetetraacetic acid; NK cell, Natural killer cell; Tc cell, Cytotoxic T cell; TF, TransFix®; Th cell, T helper cell; TVT, TransFix®/K_3-EDTA vacuum blood collection tube.

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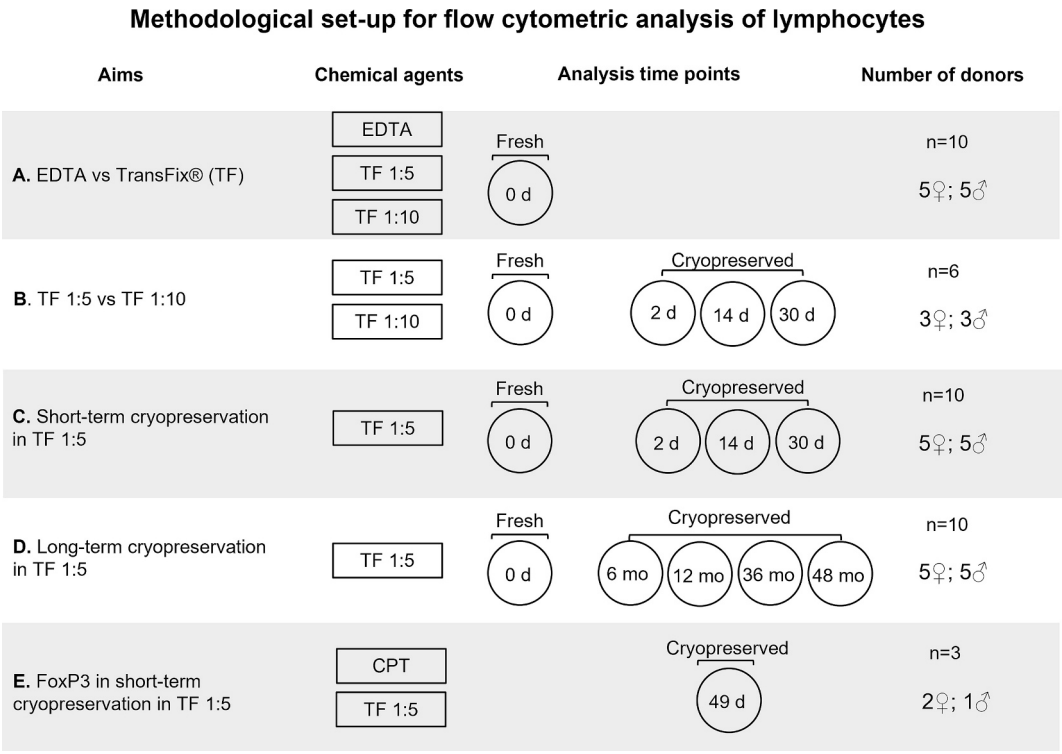

Fig. 1. Schematic overview of the methodological setup.
TF; TransFix®, CPT; Cell Preparation Tube.

samples stabilize cellular components and prevent degradation. These agents can extend the shelf-life of samples at room temperature or refrigeration. However, the choice of stabilizing agent is crucial as it must preserve the cells without adversely affecting their phenotypic properties (Sedek et al., 2020).

The use of cryoprotective agents and controlled freezing processes allows for long-term storage of blood samples at temperatures below −80 °C or in liquid nitrogen. Cryopreservation can effectively preserve cell viability and function over extended periods. However, it requires specialized equipment and careful handling to avoid freeze-thaw damage, which can impact cell recovery and marker expression (Capelle et al., 2021; Zhang et al., 2024; Whaley et al., 2021).

TransFix®/K_3-EDTA vacuum blood collection tubes (TVTs) from Cytomark offer an advanced solution for chemical cell stabilization, designed to preserve blood samples for delayed analysis by maintaining both cell morphology and surface markers. The stabilization solution within TVTs provide robust preservation, allowing for flexible sample processing without compromising the quality of flow cytometric analysis (Kaenzig et al., 2022). Previous studies have primarily investigated the use of TransFix® (TF) for short-term preservation of whole blood at room temperature or under refrigeration, with delayed analysis of up to 14 days (Sedek et al., 2020; Kaenzig et al., 2022; Nguyen et al., 2023). In contrast, the potential of TF for cryopreserving whole blood has not been extensively studied. A small study performed by Cytomark suggested that TVTs are suitable for cryopreservation of whole blood for up to 7 days (Coupar and Kaenzig, 2019), while a comprehensive study by Serra *et al* reported significant immunophenotypic differences between fresh blood and blood cryopreserved in TF for 18 months (Serra et al., 2022).

These findings underscore the need for additional research to evaluate the efficacy of TF in preserving lymphocyte subsets during cryopreservation of whole blood over varying timeframes. Understanding the effects of both short- and long-term cryopreservation in TF-stabilized blood is essential for expanding its application in clinical and research settings. In this study, we evaluate the efficacy of TF in maintaining lymphocyte subset integrity for flow cytometry analysis. We compare lymphocyte profiles from fresh blood samples with those from samples cryopreserved in TF for both short-term (several weeks) and long-term (several years) periods. Our analysis examines the proportional stability of lymphocyte subsets, providing insights into the feasibility of using TF in a wide range of clinical and research contexts. Overall, our findings demonstrate that cryopreservation in TF effectively preserves whole blood specimens for immunophenotyping by flow cytometry for up to 48 months.

2. Materials and methods

A schematic overview of the study aims, chemical agents used in the blood sample collection tubes, and timepoints for analysis of lymphocyte subsets in fresh or cryopreserved samples by flow cytometry are presented in Fig. 1.

2.1. Study population and sample collection

Blood samples were collected by venipuncture from a total of 13 anonymous donors. The subjects were informed on participation in the study by oral and written information. All collected samples were directly unidentified at the time of the collection. The study has no ethical approval since biological samples cannot be derived back to individuals, which is in concordance with the Swedish statue book (2003:460;3).

The sample cohort consisted of whole blood samples collected in TransFix®/K_3-EDTA Vacuum Blood Collection Tubes (TVTs; Cytomark, Buckingham, UK) and BD Vacutainer® K_2-EDTA tubes (EDTA; BD Biosciences, San Jose, CA, USA) (Fig. 1**, aims A-D**). To evaluate the effect of TF concentration on lymphocyte subsets, whole blood was collected in one 9 mL tube diluted 1 part TF to 5 parts whole blood (TF 1:5), and one 3 mL tube diluted 1 part TF to 10 parts whole blood (TF 1:10). Whole blood samples denoted as fresh were analyzed within 24 h from sampling. To evaluate the effect of cryopreservation on lymphocyte subsets, samples collected in TVTs were aliquoted into 0.5 mL cryovials

Table 1
Fluorochrome-conjugated monoclonal antibodies used for flow cytometric analysis.

Antigen	Fluorochrome	Clone	Aim
CD3	FITC	SK7	A-D
CD3	FITC	UCHT1	E
CD4	Pacific blue	RPA-T4	E
CD4	PE-Cy7	SK3	A-D
CD8	APC-Cy7	SK1	A-D
CD16/CD56	PE	B73.1/NCAM16.2	A-D
CD19	APC	SJ25C1	A-D
CD25	APC	M-A251	E
CD45	PerCP-Cy5.5	2D1	A-D
FoxP3	PE	259D/C7	E

FITC = fluorescein isothiocyanate, PE = phycoerythrin, Cy7 = cyanine 7, APC = allophycocyanin, PerCP = peridinin-chlorophyll-protein. All antibodies were from BD Biosciences (San Jose, CA, USA).

(Biosphere® plus; Sarstedt, Nümbrecht, Germany) and cryopreserved (−80 °C) within 4 h from blood sampling. The cryopreserved samples were thawed in a 37 °C water bath. Thereafter the cells were left to rest one hour prior to staining with fluorochrome labelled monoclonal antibodies.

For aim E of the study, where isolated peripheral blood mononuclear cells (PBMCs) were used as control samples, PBMCs were purified from BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes with sodium heparin (CPT; BD Biosciences) according to manufacturer's instructions (Fig. 1, **aim E**). Isolated PBMCs were resuspended in cryomedium consisting of 50 % RPMI (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 40 % heat-inactivated fetal bovine serum (Gibco) and 10 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, Merck, Darmstadt, Germany) before aliquotation into cryovials (TPP, Trasadingen, Switzerland) and subsequent transfer into a freezing container (Corning™ CoolCell™ LX; Corning Inc., Corning, NY, USA). The freezing container was placed at −80 °C for approximately one day, before cryopreserved samples were transferred from the freezing container to continued storage at −80 °C.

2.2. Flow cytometry

For aims A-D of the study (Fig. 1), lymphocytes in whole blood samples (fresh and thawed after cryopreservation) were stained and enumerated with fluorochrome-conjugated monoclonal antibodies in a 6-color antibody TBNK Reagent cocktail (BD Multitest™ 6-Color kit; BD Biosciences) in combination with Trucount™ tubes (BD Biosciences). The cocktail contains markers for T, Th, Tc, B and NK cells (CD45, CD3, CD4, CD8, CD19 and CD16/CD56) (Table 1). 5 μL of reagent cocktail were added to 50 μL samples to Trucount™ tubes and incubated for 15 min at room temperature (RT) in darkness. After incubation, the erythrocytes in the fresh whole blood samples were lysed with 450 mL of BD FACS™ Lysing Solution (BD Biosciences). Thereafter, the samples were incubated for 10 min at RT in darkness. Stained cryopreserved samples were diluted with 450 mL of phosphate-buffered saline (PBS; Gibco).

Flow cytometric analysis was performed on a BD FacsCanto flow cytometer (BD Biosciences) using BD FacsCanto clinical software v2.0 (BD Biosciences). The instrument performance was checked daily with the setup and tracking application BD FACS™ 7-Color Setup Beads (BD Biosciences). The acquisition gates were restricted to lymphocytes based on morphological characteristics and at least 5.000 lymphocytes were acquired and analyzed. Lymphocytes were gated based on CD45 expression and morphological characteristics on side scatter (SSC). For enumeration of lymphocytes a gate based on CD45 expression was used in combination with enumerated counting beads. The expression of CD3 on gated lymphocytes was used to determine T cells and further for the

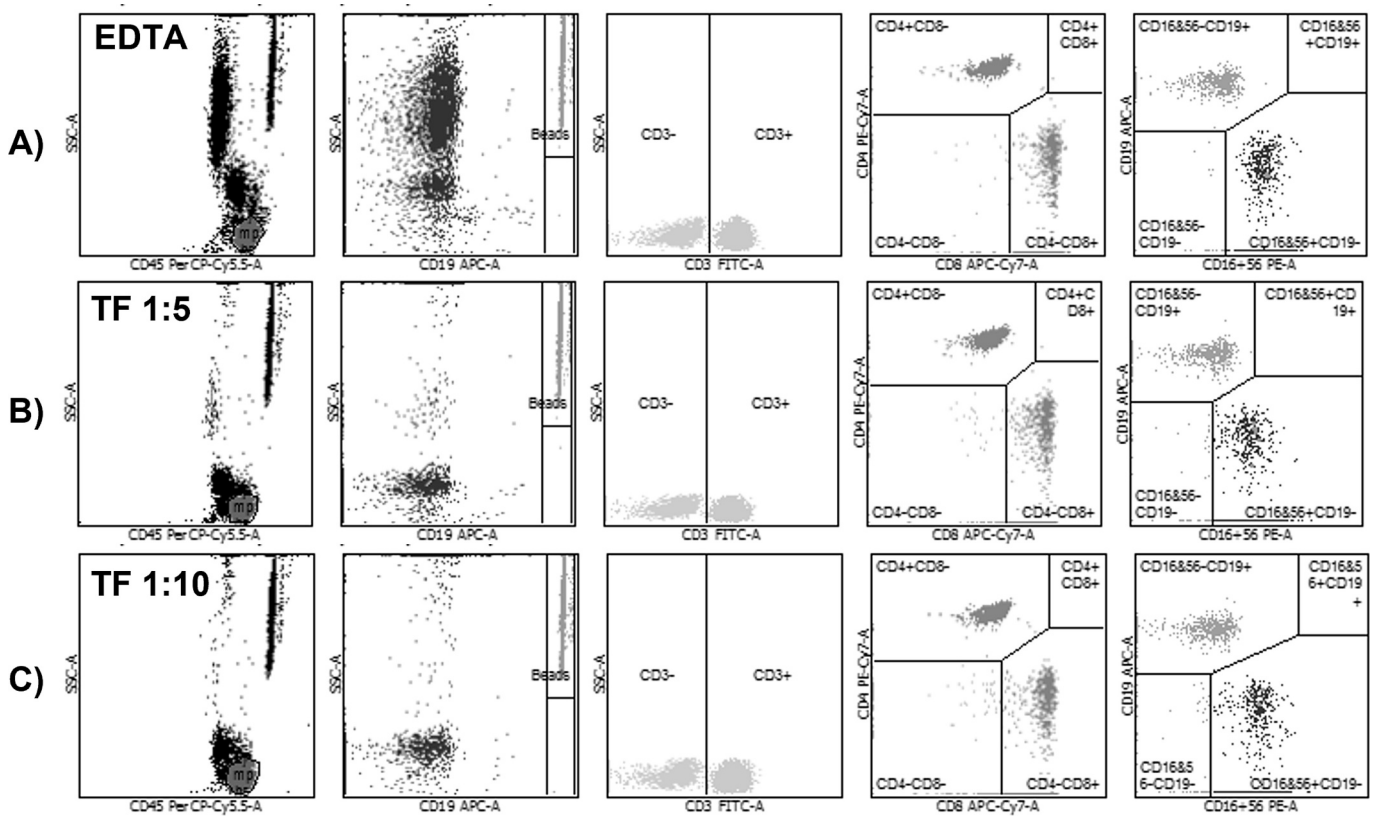

Fig. 2. Flow cytometric gating strategy.
Gating strategy for the flow cytometric analysis for aims A-C with one donor as a representative example. Whole blood sample in **A)** K_2-EDTA, **B)** TF at a 1:5 concentration and **C)** TF at a 1:10 concentration. TF; TransFix®.

Table 2
Corrected alpha levels using the Šídák multiple comparison test.

Statistical analysis of:	Variables	Possible pairs	Compared parameters	Corrected α-level
Aim A. EDTA *vs* TF for T, Th, Tc, B and NK cells (Fig. 3)	Fresh whole blood supplemented with EDTA, TF 1:5, or TF 1:10	3	5	**0.0034**
Aim C. Short-term cryopreservation in TF 1:5 for T, Th, Tc, B and NK cells (Fig. 5)	Fresh whole blood supplemented with TF 1:5 and whole blood cryopreserved in TF 1:5 for 2, 14, and 30 days	6	5	**0.0017**
Aim D. Long-term cryopreservation in TF 1:5 for T, Th, Tc, B and NK cells (Fig. 6)	Fresh whole blood supplemented with TF 1:5 and whole blood cryopreserved in TF 1:5 for 6, 12, 36, and 48 months	10	5	**0.0010**

T = T lymphocyte, Th = T helper cells, Tc = T cytotoxic cells, B = B lymphocyte, NK = Natural Killer cells, TF = TransFix.

determination of proportion Th ($CD3^+CD4^+$) and Tc ($CD3^+CD8^+$) cells. The proportion of B ($CD19^+$) and NK ($CD56^+/CD16^+$) cells were estimated from CD45-gated lymphocytes in combination with $CD3^-$/SSC. The gating strategies are presented in Fig. 2 and **Supplemental Fig. S1.**

For aim E of the study (Fig. 1), lymphocytes in either cryopreserved PBMCs or cryopreserved whole blood supplemented with TF 1:5 were thawed and stained with monoclonal antibodies for analysis of intracellular FoxP3 expression (Table 1). Staining of extracellular antigens (CD3, CD4, CD25) was first performed at 4 °C for 30 min in Dulbecco's PBS (DPBS; Cytiva, Marlborough, MA, USA) supplemented with 0.5 % (w/v) bovine serum albumin (BSA; Sigma-Aldrich) and 0.1 % (w/v) ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), followed by lysis of erythrocytes with BD FACS™ Lysing Solution. The cells were thereafter fixated and permeabilized with BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences) according to manufacturer's instructions before a final staining of intracellular FoxP3. The cells were resuspended in the DPBS buffer before analysis. Sample acquisition was performed on a CytoFLEX LX flow cytometer (Beckman Coulter, Indianapolis, IN, USA) using the software CytExpert 2.5 (Beckman Coulter). The instrument was calibrated before analysis utilising CytoFLEX Ready to Use Daily QC Fluorospheres (Beckman Coulter). At least 100.000 events were recorded in the lymphocyte gate during acquisition. Data were further analyzed and processed with the software Kaluza Analysis 2.2 (Beckman Coulter). Gatings were made in a similar way as for aims A-D, with initial gating on SSC and FSC followed by sequential determination of $CD3^+$, $CD4^+$, $CD25^+$ and $FoxP3^+$ populations. Gating strategy can be found in **Supplemental Fig. S2.**

2.3. Statistical analysis

All statistical analyses were conducted with GraphPad Prism 6.0 or 10 (GraphPad, San Diego, CA, USA). Since the sample size was limited, non-parametric tests were used throughout. Wilcoxon signed rank-test for paired observations was used to compare differences between the groups. To correct for the multiple statistical comparisons in aims A, C, and D of the study (Fig. 1), the probability level was adjusted using the Šídák multiple comparison test. Corrected alpha levels are presented in Table 2.

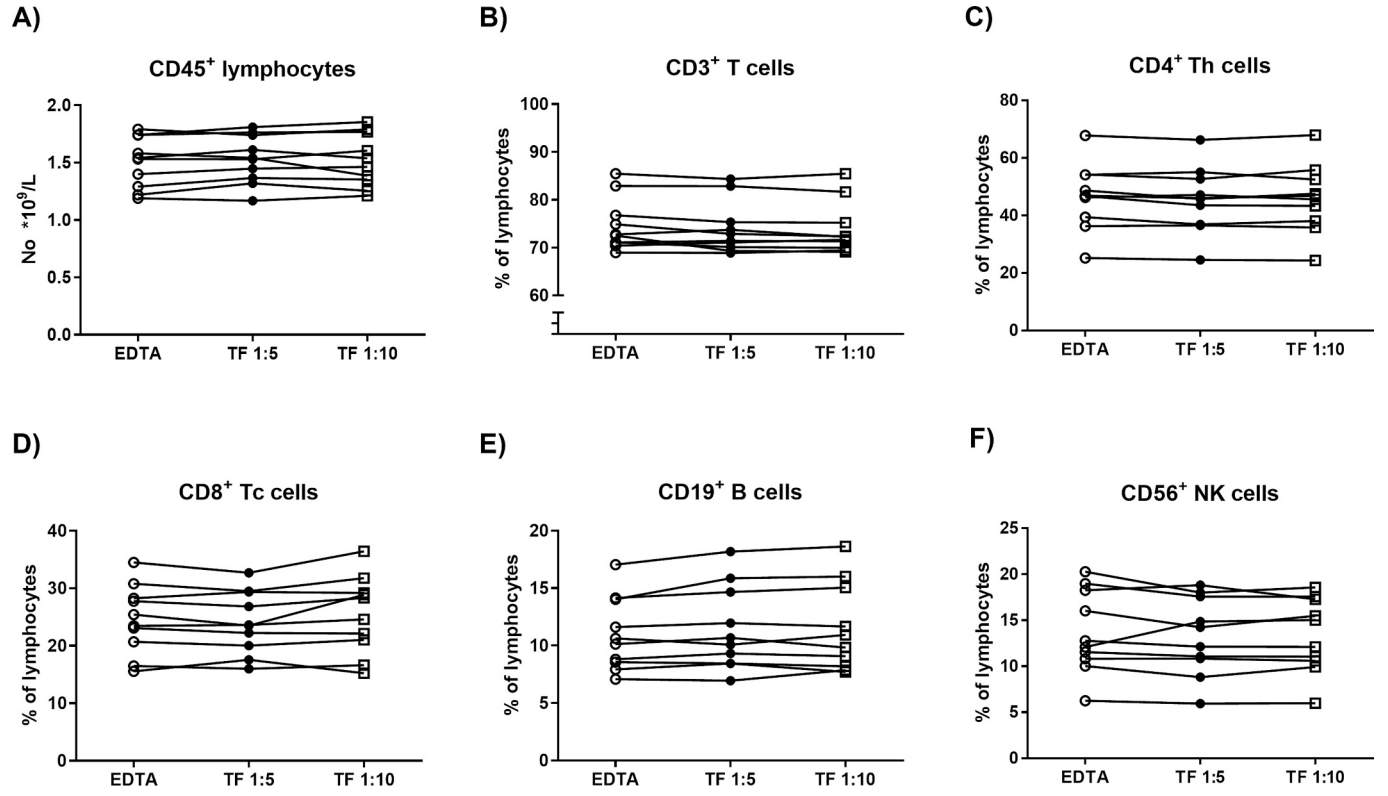

Fig. 3. No difference in proportions of lymphocyte subsets between fresh whole blood collected in EDTA, TF 1:5, or TF 1:10. Whole blood samples were collected in K_2-EDTA tubes (EDTA) or in TransFix®/K_3-EDTA vacuum blood collection tubes at 1:5 or 1:10 concentrations and directly analyzed by flow cytometry. **A)** Number of $CD45^+$ lymphocytes. Proportions of **B)** $CD3^+$(T) cells, **C)** $CD3^+CD4^+$ (Th) cells, **D)** $CD3^+CD8^+$ (Tc) cells, **E)** $CD19^+$ (B) cells, **F)** $CD56^+$ (NK) cells. TF; TransFix®. Wilcoxon signed rank-test followed by Sidáks multiple comparison test, $n = 10$.

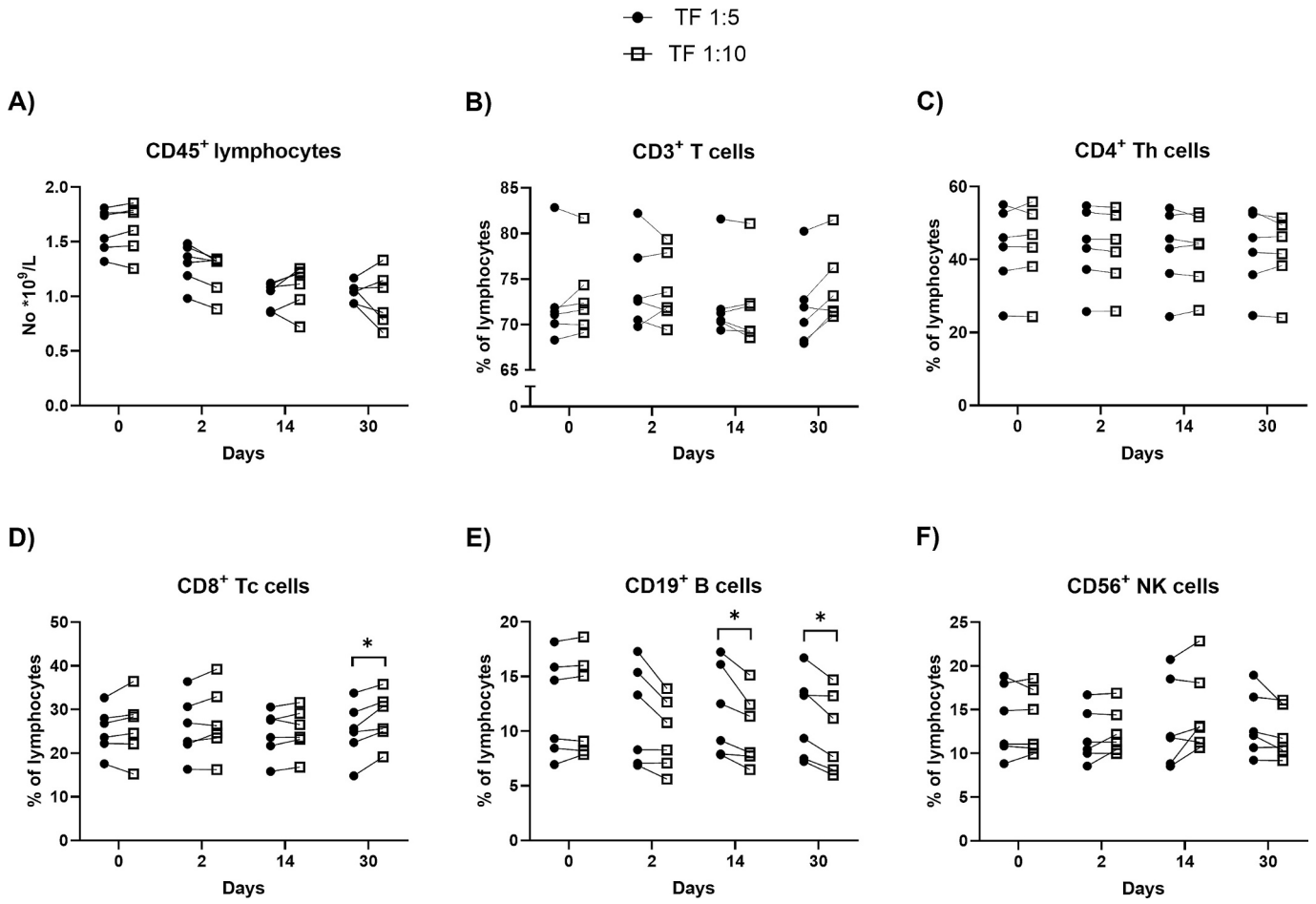

Fig. 4. Effect of short-term cryopreservation in different concentrations of TF. Whole blood samples were supplemented with TF at 1:5 or 1:10 concentrations and analyzed by flow cytometry either fresh, day 0, or after cryopreservation for 2, 14, or 30 days. **A)** Number of $CD45^+$ lymphocytes. Proportions of **B)** $CD3^+$(T) cells, **C)** $CD3^+CD4^+$ (Th) cells, **D)** $CD3^+CD8^+$ (Tc) cells, **E)** $CD19^+$ (B) cells, **F)** $CD56^+$ (NK) cells analyzed by flow cytometry. TF; TransFix® in 1:5 or 1:10 concentrations. Wilcoxon matched pairs signed rank test, $n = 6$, *indicates $P < 0.05$.

3. Results

3.1. Effects of anticoagulant and varying TF concentrations on lymphocyte subsets in fresh whole blood (aim A)

The total number of $CD45^+$ lymphocytes showed no differences between fresh whole blood samples treated with either EDTA or TF at 1:5 and 1:10 concentrations (Fig. 3A). Analysis of lymphocyte subsets revealed that the proportions of $CD3^+$ T cells, $CD3^+CD4^+$ Th cells, $CD3^+CD8^+$ Tc cells, $CD19^+$ B cells, and $CD56^+$ NK cells remained consistent across all groups (Fig. 3B-F).

3.2. Effects of different TF concentrations on lymphocyte subsets in fresh whole blood and after short-term cryopreservation (aim B)

The total number of $CD45^+$ lymphocytes showed no differences between fresh whole blood (day 0) collected in TF at 1:5 and 1:10 concentrations. Similarly, no differences in $CD45^+$ lymphocyte counts were observed between collection in TF 1:5 or TF 1:10 concentration after 2, 14, or 30 days of cryopreservation (Fig. 4A). The proportions of $CD3^+$ T cells, $CD3^+CD4^+$ Th cells, and $CD56^+$ NK cells remained consistent between TF 1:5 and TF 1:10, both in fresh samples and after 2, 14, or 30 days of cryopreservation (Fig. 4B-C, F). $CD3^+CD8^+$ Tc cell proportions were also unchanged between TF 1:5 and TF 1:10 in fresh blood and after 2 or 14 days of cryopreservation; however, after 30 days, the proportion of Tc cells was higher in TF 1:10 than in TF 1:5 (Fig. 4D). For $CD19^+$ B cells, there were no differences between TF 1:5 and TF 1:10 in fresh samples or after 2 days of cryopreservation, but the proportions were lower in TF 1:10 compared to TF 1:5 after 14 and 30 days (Fig. 4E).

3.3. Effects of freezing and short-term cryopreservation on lymphocyte subsets in fresh and cryopreserved whole blood supplemented with TF 1:5 (aim C)

No differences in total $CD45^+$ lymphocyte number were observed between fresh whole blood supplemented with TF 1:5 and whole blood supplemented with TF 1:5 and cryopreserved for two days, 14 days, or 30 days (Fig. 5A). Similarly, the proportions of $CD3^+$ T cells, $CD3^+CD4^+$ Th cells, $CD3^+CD8^+$ Tc cells, $CD19^+$ B cells, and $CD56^+$ NK cells were consistent between fresh whole blood and cryopreserved samples during the same time period (Fig. 5B-F).

3.4. Effects of freezing and long-term cryopreservation on lymphocyte subsets in fresh and cryopreserved whole blood supplemented with TF 1:5 (aim D)

The number of $CD45^+$ lymphocytes did not differ between fresh whole blood supplemented with TF 1:5 and whole blood supplemented with TF 1:5 and cryopreserved for 6 months, 12 months, 36 months and 48 months (Fig. 6A). Furthermore, no differences could be found between fresh whole blood and cryopreserved whole blood for the proportions of $CD3^+$ T cells, $CD3^+CD4^+$ Th cells, $CD3^+CD8^+$ Tc cells,

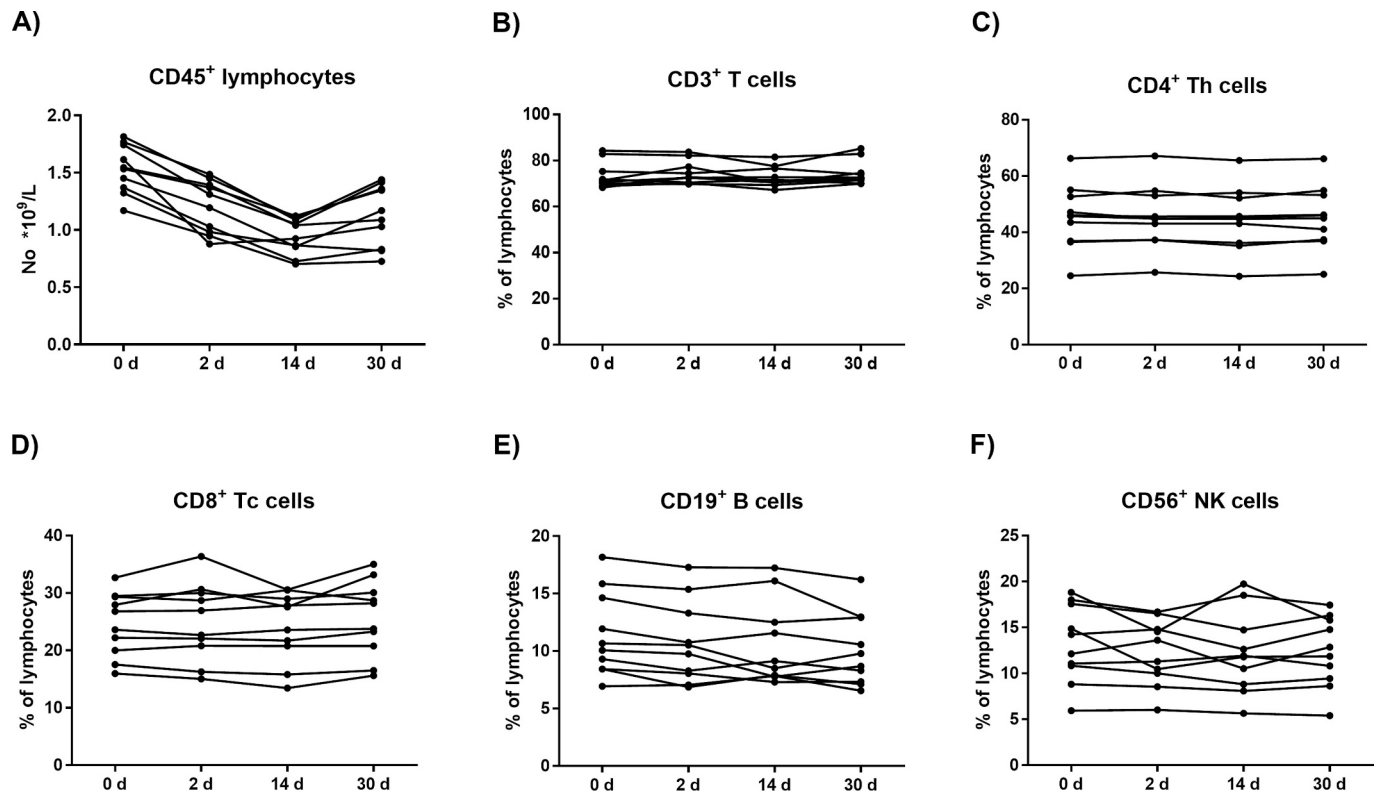

Fig. 5. No difference in proportions of lymphocyte subsets after short-term cryopreservation in TF. Whole blood samples were supplemented with TF at 1:5 concentration and analyzed by flow cytometry either fresh, day 0, or after cryopreservation for 2, 14, or 30 days. **A)** Number of $CD45^+$ lymphocytes. Proportions of **B)** $CD3^+$(T) cells, **C)** $CD3^+CD4^+$ (Th) cells, **D)** $CD3^+CD8^+$ (Tc) cells, **E)** $CD19^+$ (B) cells, **F)** $CD56^+$ (NK) cells. TF; TransFix®. Wilcoxon signed rank-test followed by Sidáks multiple comparison test, $n = 10$.

$CD19^+$ B cells, and $CD56^+$ NK cells (Fig. 6B-F).

3.5. *Effects of short-term cryopreservation of PBMCs and whole blood supplemented with TF 1:5 on FoxP3 expression in $CD4^+$ Th cells (aim E)*

$CD4^+$ lymphocytes from PBMCs collected in CPT tubes and whole blood supplemented with TF 1:5 were analyzed for intracellular FoxP3 expression after 49 days of cryopreservation. While the proportions of $CD4^+FoxP3^+$ cells and $CD4^+CD25^+FoxP3^+$ cells remained detectable in cryopreserved PBMCs, they were not detectable after cryopreservation in TF 1:5 (Fig. 7A-B).

4. Discussion

The results of this study demonstrate that long-term (up to 48 months) cryopreservation of whole blood samples using TransFix® (TF) cell stabilization reagent does not significantly affect the proportions of circulating lymphocyte subsets when assessed by flow cytometry. This finding is important, as it suggests that TF-stabilized blood samples can be used for delayed analysis, even after extended periods of cryopreservation, without compromising the integrity of key immune cell populations. Such findings hold potential for broadening the application of TF in both clinical and research contexts, where long-term storage of blood samples is required.

Our data demonstrate that the proportions of $CD3^+$ T cells, $CD3^+CD4^+$ Th cells, $CD3^+CD8^+$ Tc cells, $CD19^+$ B cells, and $CD56^+$ NK cells remain stable across multiple time points during cryopreservation. This indicates that TF effectively preserves lymphocyte subsets over extended storage periods. These findings align with previous research, which showed that TF maintains leukocyte subset distribution for up to 7 days of cryopreservation (Coupar and Kaenzig, 2019). Notably, our study expands on this knowledge by demonstrating that TF can preserve lymphocyte subset integrity even after cryopreservation for up to four years. However, it is important to highlight that our results differ from those of Serra *et al*, who reported significant immunophenotypic changes in blood samples cryopreserved in TF for 18 months (Serra et al., 2022). This discrepancy could be attributed to differences in cryopreservation conditions, sample handling, or analytical techniques between studies. Nevertheless, our results provide strong evidence that under the conditions used in our study, TF can maintain the stability of lymphocyte subsets over long-term cryopreservation, supporting its potential use in clinical and research settings requiring extended sample storage.

One notable finding is that we observed higher proportions of Tc cells in TF 1:10 compared to TF 1:5 after 30 days of cryopreservation, while B cells exhibited lower proportions in TF 1:10 compared to TF 1:5 after 14 and 30 days. These variations likely reflect minor methodological differences between TF concentrations rather than any fundamental instability of the lymphocyte populations. Thus, even though these differences were statistically significant they may not represent biologically meaningful deviations.

A trend towards a reduction in the total number of $CD45^+$ lymphocytes was observed when comparing fresh whole blood with cryopreserved samples. Although this decrease was not statistically significant with the tests applied, it aligns with the findings from the company manufacturing TF, which reported a slight decrease in $CD45^+$ cell counts following cryopreservation in their own study (Coupar and Kaenzig, 2019).

The results of our study align with and extend findings from previous research using chemical stabilizers for the cryopreservation of whole blood. Several studies have investigated the efficacy of various chemical stabilizers, such as Paraformaldehyde (PFA), Cyto-Chex BCT, and TF in preserving immune cell populations for flow cytometric analysis (Sedek et al., 2020; Canonico et al., 2004; Harrison et al., 2019; Ng et al., 2012).

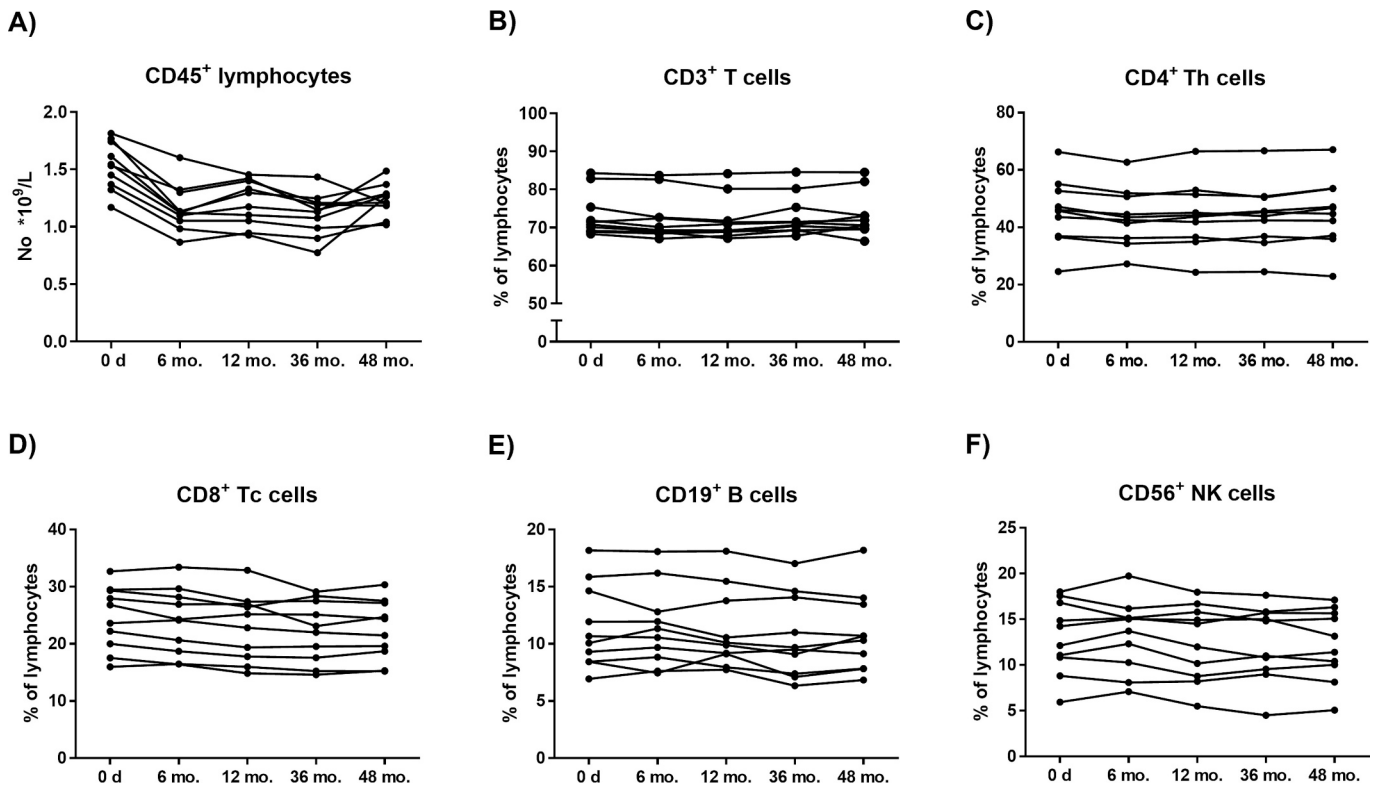

Fig. 6. No difference in proportions of lymphocyte subsets after long-term cryopreservation in TF 1:5. Blood samples were supplemented with TF at 1:5 concentration and analyzed by flow cytometry either fresh, day 0, or after cryopreservation for 6, 12, 36, or 48 months (mo). **A)** Number of $CD45^+$ lymphocytes. Proportions of **B)** $CD3^+$(T) cells, **C)** $CD3^+CD4^+$ (Th) cells, **D)** $CD3^+CD8^+$ (Tc) cells, **E)** $CD19^+$ (B) cells, **F)** $CD56^+$ (NK) cells. TF; TransFix®. Wilcoxon signed rank-test followed by Sidáks multiple comparison test, $n = 10$.

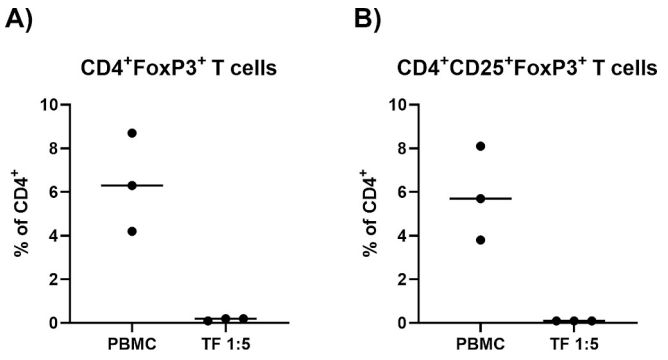

Fig. 7. FoxP3 is detectable in isolated PBMCs but not in whole blood supplemented with TF 1:15 after cryopreservation for 49 days. Whole blood samples were collected in Vacutainer® CPT™ tubes for isolation of PBMCs, or in TF at 1:5 concentration, and were analyzed by flow cytometry after 49 days of cryopreservation. Proportions of $FoxP3^+$ **A)** $CD3^+CD4^+$ T cells and **B)** $CD3^+CD4^+CD25^+$ T cells. PBMC; peripheral blood mononuclear cell, TF; TransFix®. $n = 3$. No statistical tests were performed.

However, these studies primarily focus on short-term preservation at room temperature or refrigerated conditions, with limited data available for long-term cryopreservation.

The ability to cryopreserve whole blood samples for long durations without affecting lymphocyte subset proportions has practical implications for various fields. In clinical diagnostics, flow cytometric analysis of immune cell populations is often required for the diagnosis and monitoring of diseases such as chronic infections, autoimmune disorders, and cancers. The option to cryopreserve samples without losing cell integrity would enable greater flexibility in sample handling, especially in settings where immediate analysis is not feasible or when samples need to be transported across long distances.

Dimethyl sulfoxide (DMSO) has long been widely used as a cryoprotective agent for the cryopreservation of cells and tissues, primarily due to its ability to prevent intracellular ice crystal formation (Whaley et al., 2021). Several studies have investigated the use of DMSO in the cryopreservation of whole blood for subsequent flow cytometric analysis, with most reporting positive outcomes (Alam et al., 2012; Verschoor and Kohli, 2018; Verschoor et al., 2018). One study specifically used CryoStor® CS10, a commercially available DMSO-based freezing medium, to cryopreserve whole blood for 3 months, successfully analyzing intracellular markers by flow cytometry and performing functional assays after thawing (Braudeau et al., 2021). However, DMSO is known for its toxicity at both the cellular and organismal levels (Awan et al., 2020), which limits its application, particularly in situations where samples cannot be immediately cryopreserved and must be stored at room temperature or under refrigerated conditions. Consequently, there is a growing need for alternative cryoprotective agents that are less toxic.

In research, particularly in large-scale studies or biobanking, long-term sample storage is crucial. Our findings show that TF-stabilized blood can be cryopreserved without compromising immune cell phenotyping, making it a valuable tool for longitudinal studies and retrospective analyses.

While the study demonstrates the effectiveness of TF in preserving lymphocyte subsets during long-term cryopreservation, there are some limitations to consider. *First*, while the major lymphocyte subsets (T cells, B cells, and NK cells) remained stable, we did not assess the effects of cryopreservation on myeloid cells, or more specialized lymphocyte populations, such as regulatory T cells, effector Th cell subpopulations (*e.g.* Th1, Th2, and Th17 cells) and memory T and B cells, which may respond differently to cryopreservation in TF. Additionally, the reduced

detectability of FoxP3 expression in cryopreserved whole blood supplemented with TF, compared to PBMCs, suggests that intracellular markers—especially those requiring specialized handling—may not be as well-preserved in TF-stabilized whole blood during long-term storage. This is an important consideration for studies targeting regulatory T cells or other intracellular markers, where a DMSO-based freezing medium like CryoStor® CS10 (Braudeau et al., 2021) may be more suitable than formaldehyde-based cell stabilization reagents like TF. *Second*, we did not investigate whether the expression levels of specific surface markers of the analyzed cell populations were influenced by cryopreservation in TF over varying timeframes, as observed in the study by Serra *et al* after 18 months of cryopreservation in TF (Serra et al., 2022). This is particularly relevant for studies focusing on cell activation or migration. *Third*, we utilized controlled cryopreservation conditions, which may not always be replicable in less-equipped laboratories or field settings, potentially affecting the consistency across different environments. Factors such as variability in freezing rates and storage temperatures could impact sample integrity. Therefore, it is important to standardize the freezing and thawing processes as much as possible to achieve consistent results.

In summary, our study provides strong evidence that long-term cryopreservation of whole blood using TF maintains the integrity of major lymphocyte subsets for up to 48 months, making it a viable option for delayed flow cytometric analysis. This has important implications for both clinical and research applications, particularly in large-scale studies and biobanking. However, further research is needed to assess the preservation of myeloid cells, more specialized lymphocyte populations, and intracellular- as well as extracellular markers to fully understand the potential and limitations of TF in cryopreservation.

CRediT authorship contribution statement

Maria Faresjö: Funding acquisition, Supervision, Writing – review & editing. **Junko Johansson:** Investigation, Visualization, Writing – review & editing. **Ulrika Islander:** Resources, Visualization, Writing – original draft, Writing – review & editing. **Andrea Tompa:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT to improve the language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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