Clinical Relevance

Cryopreservation Selectively Decreases Detection of Nine Clinically Relevant T Cell Markers

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Standard Peripheral Blood Mononuclear Cell Cryopreservation Selectively Decreases Detection of Nine Clinically Relevant T Cell Markers

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ABSTRACT

Biobanking is an operational component of various epidemiological studies and clinical trials. Although peripheral blood is routinely acquired and stored in biobanks, the effects of specimen processing on cell composition and clinically relevant functional markers of T cells still require a systematic evaluation. In this study, we assessed 25 relevant T cell markers in human PBMCs and showed that the detection of nine membrane markers (e.g., PD-1, CTLA4, KLRG1, CD25, CD122, CD127, CCR7, and others reflecting exhaustion, senescence, and other functions) was reduced among at least one T cell subset following standard processing, although the frequency of CD4, CD8, and regulatory T cells was unaffected. Nevertheless, a 6-mo-long cryopreservation did not impair the percentages of cells expressing many other membrane and all the eight tested intracellular lineage or functional T cell markers. Our findings uncover that several clinically relevant markers are particularly affected by processing and the interpretation of those results in clinical trials and translational research should be done with caution. ImmunoHorizons, 2021, 5: 711–720.
INTRODUCTION

Biobanks substantially contribute to the implementation of different types of clinical trials and translational research in all disease areas (1–4). Each year, millions of different types of samples are being collected, stored, and redistributed to perform biochemical, histopathological, or genomics, proteomics, and other types of “omics” analysis (5, 6), which are particularly important in the era of precision and personalized medicine (7). Among others, whole blood or isolated PBMCs are routinely collected and stored for either disease-focused cohorts or vaccine trial studies (8). The general cryopreservation effects have been long observed (9) and recently reviewed (10). Meanwhile, more precisely no effect has been observed in the percentages of major immune subsets, such as CD4 T cells, CD8 T cells, B cells, CD14+ monocytes, and CD56+ NK cells following cryopreservation (11, 12). The frequency of CD4 regulatory T cells (Treg) and effector CD8 T cells has also been found stable (13). Interestingly, the functional responses exhibited more contradictory results. For instance, proliferation responses and cytokine production in supernatants following cryopreservation of total PBMCs were found to be either maintained (14–17), enhanced (11), or decreased (18). After cryopreservation, a reduced cytokine production was observed in CD4 T cells (19) and more recently also in APCs (20). Additionally, a decrease in the expression of a selected marker of interest (e.g., PD-1 in CD8 T cells) was observed following cryopreservation, despite well-established cryopreservation practice in biobanking settings (21). Several modifications in NK cell activation markers, such as CD25, CD69, and NKP46 have been reported in frozen versus fresh samples (13). In short, progress has already been made in assessing the effects of cryopreservation on the expression of sporadic single markers of interests in T cells. However, no work has systematically investigated the effects of cryostorage on lineage and functional T cell markers to support evidence-based protocols for clinical trials or research studies. To this end, we analyzed up to 25 different T cell–related markers by comparing fresh and cryopreserved PBMCs from six different healthy donors aged between 20 and 40 y old, using multiple panels of state-of-the-art multicolor flow cytometry. We analyzed the markers defining T cell subsets, such as TBET, GATA3, RORgt, and FOXP3 for Th1, Th2, Th17, and Treg, respectively, as well as various functional markers in CD4 and CD8 T cell subsets, reflecting various functional attributes, including but not to limited to activation, proliferation, exhaustion, senescence, memory/naive status, metabolism, and other effector functions.

MATERIALS AND METHODS

Blood sampling, PBMC isolation, and cryopreservation

Blood collection was conducted according to Luxembourgish ethical guidelines. Six healthy volunteers donated blood after providing informed consent. Blood was collected in a single session for all participants via venipuncture into five 10-ml vacutainer K2EDTA blood collection tubes (367525; BD Biosciences) per donor. The blood collection tubes were transported at room temperature (RT) to the central processing laboratory.

The central processing laboratory (Integrated Biobank of Luxembourg, https://www.ibbl.lu/about-ibbl/) belongs to an accredited biobank as demonstrated by us (22). Within 4 h of collection, five blood collection tubes per donor were pooled and distributed over four 50-ml centrifugation tubes. Blood was diluted 1× with DPBS (14190-144; Life Technologies, Thermo Fisher Scientific). Diluted blood was transported in 50-ml SepMate tubes (86450; St cement Technologies) preloaded with Lymphoprep (07801; Stemcell Technologies) for PBMCs isolated by density gradient centrifugation. Tubes were centrifuged at 1200 × g for 15 min at RT. The interphase per tube was collected in a new 50-ml centrifuge tube and washed with DPBS using centrifugation at 500 × g for 10 min at 21°C. Cell pellets per donor were pooled in a single tube and washed with AutoMACS running buffer (130-091-221; Miltenyi Biotec, Bergisch Gladbach, Germany) using identical centrifugation conditions. Cell pellet per donor was resuspended in 20 ml of AutoMACS running buffer. Cell concentrations were determined by Cellometer (Nexcelom Bioscience, Manchester, U.K.). PBMCs per donor were diluted in cold (2–8°C) cryopreservation media (Cryostore CS10; Biolife Solutions) at 10 million per milliliter. One milliliter was transferred into a 2-ml cryovial (Greiner Bio-One, Vilvoorde, Belgium). The vials were frozen using a Mr. Frosty freezing container placed for up to 24 h in a −80°C freezer before being transported to liquid nitrogen tanks for a long-term storage. Meanwhile, 10 million of PBMCs were kept in AutoMACS running buffer at 2–8°C and used for multiple-panel analysis of baseline flow cytometry determination (i.e., fresh samples).

Thaw conditions, flow cytometry staining, and analysis

PBMCs were either stained freshly after isolation by density gradient centrifugation or following a period of cryopreservation (9 wk or 6 mo for this project) in the Luxembourg local biobank (Integrated Biobank of Luxembourg). The frozen PBMCs were first recovered by washing the cells in complete IMDM (supplemented with 10% FBS, penicillin-streptomycin, nonessential amino acids, and β-mercaptoethanol) and incubated overnight at 37°C in the same medium (23, 24). The detailed information about the components of the complete IMDM medium used for human T cells was already described in our previous work (25 and E. Danileviciute, N. Zeng, C. Capelle, N. Paczia, M.A. Gillespie, H. Kurniawan, D. Coowar, D.M. Vogt Weisenhorn, G.G. Giro, M. Grusdat, et al, manuscript posted on bioRxiv). The fresh and thawed PBMCs were stained following the same protocol as follows.

The PBMCs were first resuspended in flow cytometry buffer (PBS + 2% FBS) containing Fc-blocking Abs (564765; BD Biosciences) and incubated for 15 min at 4°C. After one
PBMC cryopreservation decreases the detection of clinically relevant T cell extracellular markers.

(A) Graphical representation of the experimental setup for analyzing a cohort of six healthy young donors. (B) Frequency of living lymphocytes among the gated singlets from either fresh or 9-wk–frozen or 6-mo–frozen samples (for the gating strategy, please refer to Supplemental Fig. 1). (C) Frequency of total CD4 cells, FOXP3^+^ Treg and total CD8 T cells among the parent gates (living cells for CD4 and CD8, and CD4 (Continued)
washing step (300 × g, 5 min, 4°C) in flow cytometry buffer, the cells were resuspended in Brilliant stain buffer (563794; BD Biosciences) containing the fluorochrome-coupled Abs (Supplemental Table I) and incubated for 30 min at 4°C in the dark. The surface staining was followed by three washing steps in flow cytometry buffer (300 × g, 5 min, 4°C). Next, the cells were fixed for 1 h at RT using the True-Nuclear transcription Factor Buffer Set (424401; BioLegend). Following the fixation, the cells were centrifuged down (400 × g, 5 min, RT), resuspended in 200 μl of flow cytometry buffer and left at 4°C overnight. The next morning, the PBMCs were washed once with permeabilization buffer (400 × g, 5 min, RT) and incubated in permeabilization buffer containing the Abs against the intracellular targets for 30 min at RT in the dark. Finally, the cells were washed three times in permeabilization buffer (400 × g, 5 min, RT) and resuspended in flow cytometry buffer for the acquisition on the BD Fortessa. The analysis of the data were performed using the FlowJo software (v10.5.3).

At each time point, single-stained compensation beads (01-2222-42; eBioscience, UltraComp eBeads,) were acquired to generate the spillover matrix and check the quality of the Abs. Cytometer setup was performed according to manufacture instruction using the CS&T beads (656505; BD Biosciences) to monitor optimal performance of the instrument over the time. CS&T beads were acquired manually and visually inspected. An irregular pattern only in the BUV395 channel among different time points was observed. The BUV395 has been used to monitor the expression of CD4, a marker known to be stable following cryopreservation and therefore our instrument setup allows the results comparable among different time points.

Ethic statement

We complied with all the relevant ethic regulations and informed consent was obtained from each healthy subject before the blood was drawn. The blood sampling was coordinated and performed through the Clinical and Epidemiological Investigation Centre of Luxembourg Institute of Health.

Statistics and reproducibility

We calculated all the p values using a nonparametric test (i.e., Wilcoxon-matched pairs signed rank test) implemented in Graphpad (Prism) as specified in the corresponding figure legends. When more than one test existed in one analysis, we applied the Graphpad-Prism–recommended multiple testing correction method (false discovery rate [FDR], two-stage step up [Benjamini, Krieger, and Yekutieli]) and used FDR-adjusted p values (also known as q values). With both criteria met (i.e., q values ≤ 0.05) and the average change fold between two groups ≥1.5, we considered the given marker among the given subset changed significantly. We also discussed the results showing a marginally significant q value (between 0.05 and 0.09) and an average change fold ≥1.5. The value of each individual sample was visualized at different time points.

RESULTS

PBMC cryopreservation selectively decreases detection of T cell markers

To assess whether the standard processing/cryopreservation conditions affect the detection of T cell–related markers, we used panels of multicolor flow cytometry to quantify and analyze up to 25 relevant T cell lineage or functional markers in fresh, 9-wk– or 6-mo–cryopreserved PBMCs (Fig. 1A, Supplemental Table I). The frequency of living cells among lymphocytes was unchanged following overnight recovery from 9-wk or 6-mo cryopreservation relative to the fresh samples (Fig. 1B). Similar to the results of others (11, 12), the frequency of the three major T cell subsets, namely, CD4+FOXP3+ cells (Tconv), CD4+FOXP3+ Tregs, and total CD8 T cells, among living lymphocytes was unchanged before and after 9-wk or 6-mo cryopreservation (Fig. 1C, Supplemental Fig. 1).

Unexpectedly, as early as 9 wk postcryopreservation, the frequency of cells expressing extracellular exhaustion, senescence, or effector/activation markers, such as PD-1, KLRG1, CD127, and CD25, started to significantly decrease among CD4 Tconv and already showed a decrease trend among CD8 T cells (q = 0.059, FDR-adjusted p value [also known as q value] by the Wilcoxon-matched pairs signed rank test; the test method will not be mentioned afterward unless a different test was used; Fig. 1D, 1E, 1J, 1K, Supplemental Fig. 2A). Following 9 wk of cryopreservation, PD-1 also started to show a decrease trend in the Tregs subpopulation (q = 0.059), which express relatively high levels of PD-1 in fresh samples (Fig. 1F). We also analyzed other cell surface and intracellular markers in 9-wk–frozen samples. Interestingly, TBET was the only one that already showed a decrease trend among CD8 T cells (q = 0.059) in 6-mo–frozen PBMCs. The enlarged number represents the percentage of the corresponding subset among the parent gate. (K) Representative flow cytometry plots showing the expression of KLRG1 and CD127 on CD8 T cells from fresh, 9-wk–frozen or 6-mo–frozen PBMCs. Of note, the Abs used for 6-mo–frozen and fresh samples might be different (refer to Supplemental Table I). Black circles denote staining on fresh PBMCs. Red circles denote staining on 9-wk–frozen or 6-mo–frozen PBMCs. The value of each sample from the cohort is displayed in the figures. The Wilcoxon-matched pairs signed rank test was used to determine p values and the two-stage step up (Benjamini, Krieger, and Yekutieli) was used to adjust p values (also known as q values). *p ≤ 0.05, **q ≤ 0.01. ***q ≤ 0.001. ns or unlabeled, not significant.
FIGURE 2. Six-month cryopreservation impairs the detection of specific markers only in certain subsets of T cells.

(A–C) Frequency of cells expressing CCR7, CD122, CTLA4, or ICOS among Tregs (A), Tconv (B), or CD8 T cells (C) from fresh or 6-mo–frozen PBMCs. (D–G) Representative flow cytometry plots showing the expression of CTLA4 and CD45RO on Tregs (D), the expression of CD122 and CD45RO on total CD8 T cells (E), the expression of CD28 and CCR7 on CD4 Tconv (F), the expression of ICOS and CD45RO on (Continued)
and a mean change fold of \( q = 0.10 \) between fresh and 9-wk–frozen samples (Supplemental Fig. 3A–D). To exclude the potential effects in binding affinities or fluorescence brightness/sensitivity due to the selected clone and/or fluorochrome, we chose another Ab for each of those affected markers to analyze 6-mo–frozen samples (for the detailed information of the selected Abs, refer to Supplemental Table I). Importantly, to guarantee having comparable results between the different Abs measuring the same markers, we mainly evaluated the percentage of the cells expressing the respective marker among the given T cell subset. Notably, despite having used distinct Ab clones, the frequency of cells expressing exhaustion, senescence, or effector markers, such as PD-1, KLRG1, CD127, and CD25, significantly decreased among all the three T cell subsets in 6-mo–cryopreserved PBMCs (Fig. 1G–K). These results indicate that the effect of cryopreservation is independent from Ab clones and storage periods at least for the tested markers. Our data were concordant with the previously reported observation about decreased PD-1 expression among CD8 T cells following cryopreservation (21). Although others have reported no effect of cryopreservation on CD127 expression (12), the authors had focused on Tregs, in which the expression of CD127 is known to be low or absent and therefore their report is not conflicting with our observations. For the Treg-functional marker CD39, no significant effect was observed in 9-wk–frozen versus fresh samples, regardless of the T cell subsets (Fig. 1D–F). However, the fraction of CD39\(^+\) cells was significantly decreased after 6-mo cryopreservation in all the three tested T cell subsets, despite the low-level baseline expression of CD39 in CD4 Tconv and total CD8 T cells (Fig. 1G–I). Because the same clone and fluorochrome for CD39 within the same panel was used to analyze both fresh and 6-mo–frozen samples, we also analyzed the mean fluorescence intensity (MFI) among the positive cells. Interestingly, the MFI of CD39\(^+\) cells was also significantly decreased among all the three T cell subsets (Supplemental Figs. 2B, 4A).

**PBMC cryopreservation selectively decreases detection of certain markers among specific T cell subsets**

We also observed a certain degree of cell type–specific effects for a few markers, such as CTLA4, CD122, CCR7, and ICOS. The frequency of CTLA4\(^+\) cells was significantly decreased among Tregs (Fig. 2A, 2D) and showed a decrease trend among CD4 Tconv (\( q = 0.066 \), Fig. 2B) following 6-mo cryopreservation. No significant effect on CTLA4 detection was observed among total CD8 T cells following either 9-wk (Fig. 1E) or 6-mo cryopreservation (Fig. 2C), possibly due to its low baseline expression even among fresh CD8 T cells. Unexpectedly, the frequency of CTLA4\(^+\) cells showed an increase trend among Tregs in 9-wk–frozen versus fresh samples (\( q = 0.059 \), Fig. 1F). The frequency of CD122\(^+\) cells among total CD8, but not among CD4 Tconv and Tregs (Fig. 2A–C, 2E), showed a decrease trend following 6-mo cryopreservation (\( q = 0.066 \)). Because the Abs used to analyze CD122 were identical between fresh and 6-mo–frozen samples, we also analyzed the MFI. Interestingly, the MFI of CD122\(^+\) cells showed a clear decrease already among CD4 Tconv and Tregs, but not among total CD8 T cells, further indicating existence of the cryopreservation effect (Supplemental Fig. 4B). Different from the observation on CD122, the frequency of CCR7\(^+\) cells was significantly reduced among Tregs (Fig. 2A, 2F) while also showing a clear decrease trend among CD4 Tconv and total CD8 T cells (Fig. 2B, 2C) following 6-mo cryopreservation. After 9-wk cryopreservation, the percentages of CCR7\(^+\) cells among CD4 Tconv and Tregs were unaffected (Fig. 1D, 1F). Nevertheless, the frequency of CCR7-expressing cells surprisingly showed an increase trend among total CD8 T cells in 9-wk-old samples (\( q = 0.059 \), Fig. 1E). For the 9-wk–cryopreserved samples, the effects on the percentages of CCR7- and CD122-positive cells swapped, (i.e., the expression of CD122 showed an increase trend among CD4 Tconv and Tregs [\( q = 0.059 \) and 0.074, respectively, Fig. 1D, 1F]), but remained unaffected among total CD8 T cells (Fig. 1E). The percentages of cells positive for the activation marker ICOS among Tregs (Fig. 2A, 2G), but not among CD4 Tconv and total CD8 T cells (Fig. 2B, 2C), were also significantly decreased following 6-mo cryopreservation. In fact, the frequency of ICOS\(^+\) cells among Tregs already showed a decrease trend even just after 9-wk cryopreservation (\( q = 0.059 \), Fig. 1F).

**PBMC cryopreservation does not impair the positive percentages of intracellular T cell markers**

The freeze/thaw effects were not consistent among all the tested cell surface markers. For instance, the frequency of other tested membrane functional markers, such as CD45RO, CD45RA, CCR7, CD28, and CD31, did not meet both criteria to be considered showing a robust change (i.e., a significant \( q \) value) and the average change folds \( \geq 1.5 \) among CD4 Tconv, Tregs, or total CD8 T cells following 6-mo cryopreservation (Fig. 3A–D, Supplemental Fig. 2C–E). The percentage of positive cells for the immunosenescence marker CD57 was also unaffected among both CD4 Tconv and Tregs, whereas it...
showed a modest increase trend among total CD8 T cells ($q = 0.055$, Fig. 3A–C). More encouragingly, all the examined intracellular lineage or functional markers, such as FOXP3, TBET, RORgt, GATA3, EOMES, HELIOS, Ki67, and pS6, were robustly detected among CD4 Tconv, Tregs, or total CD8 T cells (Figs. 1C, 3A–C, Supplemental Fig. 4C–G). Although the frequency of TBET-expressing cells was decreased consistently among total CD8 T cells of all the tested donors ($q = 0.077$, Fig. 3C, Supplemental Fig. 3B), the average change fold is still modest (~1.4). Both EOMES and Ki67 even showed an increase trend among total CD8 T cells ($q = 0.077$, Fig. 3C), possibly because of an increased accessibility of Abs to cellular nucleus proteins after cryopreservation and thawing procedures, although the average change magnitude was small. This shows that the protein markers within cells might be better protected from cryopreservation-related damage.
We also performed the analysis for the 14 unaffected markers in 9-wk-frozen samples. Interestingly, the MFI of the CD45RA\(^+\) or CD31\(^+\) cells among all the three T cell subsets (Fig. 4D–F) was significantly decreased. The MFI of CD45RA was already decreased among both CD4 Tconv and total CD8 T cells (Fig. 4D, 4F), but not that much among Tregs because of its low baseline expression of CD45RA (Fig. 4E). Moreover, the MFI of CD31\(^+\) cells also consistently decreased among total CD8 T cells of all the tested donors (Fig. 4F) and showed a modest but significant decrease among other subsets (Fig. 4D, 4E). Many of those 14 markers also showed a modest but significant
change in the MFI of the positive cells from either 9-wk- and/or 6-mo–frozen samples (Fig. 4A–F). These results indicate that a more universal effect of cryopreservation already occurred on many other markers, although the effect was not yet reflected in the percentage of positive cells.

DISCUSSION

In summary, we identified a so far unrecognized systematic cryopreservation effect on T cells: the decrease in expression and/or detection of nine clinically relevant markers among one of the three, or even all the three analyzed human T cell subsets (CD4 Tconv, Tregs, and CD8 total). Those markers correspond to the critical functions of immune exhaustion, immunosenescence, activation, or effector functions of T cells. As those markers are particularly important for diagnosis and treatment of cancer (28) and various aging related diseases (29) including infectious diseases (30), one might need to take specific caution on the readouts of the frozen samples related to those diseases. If the decrease of the measured marker was due to a lower expression, it might have functional consequences. However, if the decrease was only due to a lower detection of the markers by flow cytometry because of a change in the epitopes, there would not necessarily be a functional impact on the cells. Several groups have already particularly investigated the functional effects (mainly cytokine production or proliferation) of cryopreservation on PBMCs, although the results are still controversial (11, 14–18). With our data, we are unable to distinguish whether our observation was an issue of altered detection or actually altered expression. Nevertheless, regardless of the reason for the readout changes, the clinical impact remains: cryopreservation (even under the best biobanking practice) introduces a bias when the expression of certain clinical markers on immune cells is assessed.

Meanwhile, we did not observe a clear cryopreservation effect on the frequency of positive cells for at least six important cell surface markers and all eight examined intracellular markers among different subsets of T cells. Interestingly, when “zooming into” the expression levels of the positive cells for those 14 markers, we also noticed a quantitative change in the expression levels of many of those markers, indicating a more universal cryopreservation effect, which is otherwise ignored without digging into the MFI. However, we would like to point out that because MFI values are much more sensitive to the instrumental settings, the frequency of cells expressing a given marker should preferentially be used as more robust readouts when separate subpopulations (e.g., positive and negative) can be clearly distinguished. We also noticed a relatively smaller number of impaired markers in 9-wk-old– versus 6-mo-old–cryopreserved samples, indicating that the cryostorage duration might be another factor mediating the detection and/or expression levels of some specific markers. Furthermore, the dynamic effects on specific markers, for instance, the 9-wk cryopreservation-induced increase in the expression of CTLA4, CCR7, and CD122, followed by a 6-mo cryopreservation-induced decrease in their expression among specific T cell subsets, deserve even more attention and formal validation in future clinical research endeavors. These original data provide evidence about the potential preanalytical (cryopreservation) impact on the analysis of some of the most clinically relevant lineage and functional markers among major T cell subsets. Because cryopreservation is widely accepted and used among most of the completed and ongoing clinical trials worldwide, our work establishes the basis to critically assess conclusions based on immunophenotypic analyses of cryopreserved PBMCs. As described above, our results also reveal some robust intracellular and cell surface markers. These are preferred readouts in the context of future clinical trials because they can be more easily implemented in clinical analysis processes of various diseases involving immunological dysregulations.

DISCLOSURES

The authors have no financial conflicts of interest.

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PBMC CRYOPRESERVATION DECREASES T CELL MARKERS’ DETECTION


