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EFFECT OF DELAYED CELL PROCESSING AND CRYOPRESERVATION ON IMMUNOPHENOTYPING IN MULTICENTER POPULATION STUDIES

Bharat Thyagarajan^{1,4}, Helene Barcelo¹, Eileen Crimmins², David Weir³, Sharon Minnerath¹, Sithara Vivek¹, and Jessica Faul³

¹Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis MN

²Davis School of Gerontology, University of Southern California Davis, Los Angeles, CA

³Institute for Social Research, Survey Research Center, University of Michigan, Ann Arbor, MI

Abstract

Variability induced by delayed cell processing and cell cryopreservation presents unique challenges for immunophenotyping in large population studies. We conducted a pilot study to evaluate the effect of delayed cell processing and cryopreservation on cell percentages obtained by immunophenotyping. We collected blood from 20 volunteers and compared the effect of (a) delayed cell processing up to 72 hours (b) cryopreservation and (c) the combined effect of delayed cell processing and cryopreservation on immunophenotyping of 31 cell subsets that included several subsets of T, B, Natural Killer (NK) cells, monocytes and dendritic cells using both whole blood collected in EDTA tubes and peripheral blood mononuclear cells collected in CPT tubes. We found the delayed cell processing up to 72 hours or cryopreservation alone did not significantly affect the percentages T cells, dendritic cells or monocytes but significantly increased the percentage of B cells and NK cells (p for trend = 0.01) but. However combination of delayed cell processing up to 72 hours and cryopreservation significantly increased the percentage of T cells as compared to cells processed immediately (p for trend <0.0001) while a delayed cell processing followed by cryopreservation decreased the percentage of NK cells (p for trend <0.0001). Total B-cells increased significantly with a 24-48 hour delay in cell processing and cryopreservation but not at 72 hours. The percentages of monocytes and dendritic cells remained unaffected by the combination of delayed cell processing and cryopreservation. These findings suggest that immunophenotyping of several immune cell subsets can be successfully implemented in large population studies as long as blood is processed within 48 hours of biospecimen collection though some cell subsets may be more susceptible to a combination of delayed cell processing and cryopreservation.

⁴**Corresponding Author** Dr. Bharat Thyagarajan, Department of Laboratory Medicine and Pathology University of Minnesota, MMC 609, 420 Delaware street, Minneapolis MN 55455, Phone: 612-624-1257, thya0003@umn.edu.

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Keywords

Immunophenotyping; cryopreservation; cohort study; cell processing

INTRODUCTION

Large population based cohort studies have successfully collected a variety of biospecimens using standardized protocols to measure biomarkers that are of interest in understanding etiology, early detection and prognosis of various diseases. However, since appropriate cryopreservation techniques to store viable cells are both costly and labor intensive, viable peripheral blood mononuclear cells (PBMCs) are not commonly stored in large population studies. Since cryopreserved PBMCs have several research applications including immunophenotyping and functional assays that can be performed even after completion of participant recruitment and collection of study endpoints, several population studies are now storing cryopreserved whole blood or PBMCs for use in future biomarker development. However, large cohort studies present unique problems for implementation of cryopreservation protocols as there is typically a delay of at least 24–48 hours between blood collection and processing the samples in a central laboratory, which may affect the viability of cryopreserved cells. Previous studies have reported that differences in cryopreservation and thawing protocols influence distribution of cryopreserved cells¹⁻¹⁴. Since there are no harmonized protocols for cryopreservation in large cohorts the optimal cryopreservation protocol to be used in large cohorts remains unclear.

We conducted a pilot study to evaluate the effect of delayed cell processing and cryopreservation procedures on immunophenotyping prior to implementing these procedures in the Health and Retirement Study (HRS). The HRS is a nationally representative longitudinal survey of more than 37,000 individuals over age 50 in 23,000 households in the USA, where we collected venous blood samples and cryopreserved PBMCs from 9,938 HRS participants in ~7300 households in 2016-2017. We identified 31 immune cell subsets which included T-cells, B-cells, Natural Killer (NK) cells, dendritic cells (DCs) and monocytes along with 17 T-cell subsets, 3 B-cell subsets, 2 NK-cell subsets, 2 DC subsets and 2 monocytes subsets. We describe the effect of (a) time delay between blood collection and blood processing (b) cryopreservation and (c) the combined effect of both delayed cell processing and cryopreservation on immunophenotyping of 31 immune cell subsets.

MATERIAL AND METHODS**Study Design:**

The study design is summarized in Figure 1.

Twenty healthy volunteers aged 40-80 years donated 8 tubes of blood (four CPT™ tubes (BD Biosciences, San Jose, CA) and four 4 ml EDTA tubes, (BD Biosciences, San Jose, CA)). Each of the four EDTA tubes was immunophenotyped either immediately (WB-D0), after a delay of 24 hours (WB-D24), 48 hours (WB-D48) or 72 hours (WB-D72). The EDTA tubes that were processed after delay of up to 72 hours were left standing at room

temperature before immunophenotyping. Using the WB-D0 sample as the reference, these EDTA tubes were used to evaluate the effect of time delay on immunophenotyping. Each of the four CPT™ tubes was cryopreserved either immediately (PBMC-D0), after a delay of 24 hours (PBMC-D24), 48 hours (PBMC-D48) or 72 hours (PBMC-D72). The CPT™ tubes that were processed after a delay of up to 72 hours were left standing at room temperature before cryopreservation. Using the WB-D0 sample as the reference, the PBMC-D0 sample was used to estimate the effect of cryopreservation on immunophenotyping while the remaining CPT™ tubes were used to estimate the combined effect of both time delay and cryopreservation on immunophenotyping. In addition to these primary comparisons, we also compared the effect of collecting blood in EDTA tubes (WB-D0) vs. CPT tubes (without cryopreservation) among 5 individuals. Four million cells were cryopreserved using ice-cold RPMI with 10% DMSO and stored at -80°C in a standard freezer box that was placed within a styrofoam container to ensure gradual cooling of samples. Samples were transferred to a liquid nitrogen freezer after 8-24 hours and stored at -135°C in the vapor phase till analysis. All cryopreserved samples were stored for 6-10 weeks prior to immunophenotyping. The cells were thawed for 60 seconds in a 37°C water bath. Prewarmed (37°C) 1X RPMI supplemented with 10% FBS was slowly added dropwise to the cells.

Immunophenotyping protocol:

The two panels of antibodies used to determine the percentages of various immune cell subsets are listed in Table 1.

The choice of markers to identify the cell subsets followed the guidelines of the Human Immunology Project¹⁵ with the addition of CD28 and CD95 to better differentiate subsets of effector and naïve T cells. The concentration of each antibody was determined by titration experiments. The various cell subsets identified in this study are listed in Table 2.

After a wash with 1X PBS, thawed PBMC samples were resuspended in 1X RPMI supplemented with 10% FBS and 50U of DNase (Life Technology, Carlsbad, CA) for a rest period of an hour at 37°C , 5% CO_2 and 95% humidity. The PBMCs were washed, resuspended in 1X PBS. One volume of whole blood samples was lysed using 9 volumes of 1X Red Blood Cell lysis buffer (BioLegend, San Diego, CA). Both lysed whole blood and thawed PBMCs were stained with the antibodies listed in Table 1 for 20 minutes at room temperature in the dark. The samples were washed once with 1X PBS and resuspended in 1X PBS. The samples were kept on ice for a maximum of 4 hours before being run on a Fortessa X20 flow cytometer (BD Biosciences, San Jose, CA).

Gating Strategy:

The gating strategies used to identify T and B cell subsets in panel 1 and the NK cells, monocytes and DC subsets in panel 2 are described below

Panel 1 gating strategy: Lymphocytes were initially gated on an FSC-A/SSC-A dot plot (Supplementary Figure 1A). Among the lymphocytes identified by the FSC-A/SSC-A dot plot, single cells were selected on an FSC-W/FSC-H dot plot (Supplementary Figure 1B).

Live single lymphocytes were then gated on viability dye/SSC-A dot plot (Supplementary Figure 1C). T cells (CD3⁺ CD19⁻) and B cells (CD3⁻ CD19⁺) were gated on a CD3/CD19 dot plot (Supplementary Figure 1D). B cells were further gated on an IgD/ CD27 dot plot (Supplementary Figure 1E) into three subsets: IgD⁺ memory B cells (CD3⁻ CD19⁺ CD27⁺ IgD⁺), IgD⁻ memory B cells (CD3⁻ CD19⁺ CD27⁺ IgD⁻) and naïve B cells (CD3⁻ CD19⁺ CD27⁻) (Supplementary Figure 1E). T cells were divided into cytotoxic T cells (CD4⁻ CD8⁺) and helper T cells (CD4⁺ CD8⁻) using a CD4/CD8 dot plot (Supplementary Figure 1F). Both cytotoxic and helper T cells were gated into four subsets using a CCR7/CD45RA dot plot (Supplementary Figure 1G&H): Effector (EFF, CD45RA⁺, CCR7⁻), Effector memory (EM, CD45RA⁻, CCR7⁻), Central memory (CM, CD45RA⁻, CCR7⁺) and Naïve (N, CD45RA⁺, CCR7⁺) cytotoxic or helper T cell subsets. Effector cytotoxic T cells were divided into pE (CD27⁻ CD28⁻), pE1 (CD27⁺ CD28⁺) and pE2 (CD27⁺ CD28⁻) subsets using a CD27/CD28 dot plot (Supplementary Figure 1I). Similarly Effector memory were divided into EM1 (CD27⁺ CD28⁺), EM2 (CD27⁺ CD28⁻), EM3 (CD27⁻ CD28⁻) and EM4 (CD27⁻ CD28⁺) subsets using a CD27/CD28 dot plot (Supplementary Figure 1J).

Panel 2 gating strategy: PBMCs were gated first on the SSC-A/FSC-A dot plot (Supplementary Figure 2A), single cells were then selected on FSC-W/FSC-H dot plot (Supplementary Figure 2B) and live hematopoietic cells were further selected on viability dye/CD45 dot plot (Supplementary Figure 2C). Using a CD3/CD19 dot plot (Supplementary Figure 2D), DC, NK and monocytes were selected as CD3⁻ CD19⁻ live single PBMCs. Monocytes were characterized as CD14⁺ cells using a CD14/CD20 dot plot (Supplementary Figure 2E). A subsequent HLA-DR/SSC-A dot plot (Supplementary Figure 2F) further identified the monocytes (removing potential NK cells contamination) as CD14⁺ HLA-DR⁺ monocytes. Lastly total monocytes were divided into CD16⁺ and CD16⁻ monocytes on a CD16/CD14 dot plot (Supplementary Figure 2G). NK cells were identified as CD14⁻ CD20⁻ cells on a CD20/CD14 dot plot (Supplementary Figure 2H). NK cells were further characterized as CD16⁺ using a CD16/SSC-A dot plot (Supplementary Figure 2I) and NK cells subsets were defined as CD56HI (CD56⁺⁺) and CD56LO (CD56⁺) on a CD56/CD16 dot plot (Supplementary Figure 2J). DCs were identified as CD14⁻ CD20⁻ cells on a CD20/CD14 dot plot (Supplementary Figure 2H). Subsequently, a HLA-DR/SSC-A dot plot was used to differentiate HLA-DR⁺ DCs from NK cells (Supplementary Figure 2K). DCs were further divided into two subsets, plasmacytoid DC (CD123⁺CD11c⁻) and myeloid DCs (CD123⁻ CD11c⁺) on a CD123/CD11c dot plot (Supplementary Figure 2L).

In both panels 1 and 2, we collected a minimum of 200,000 events after excluding dead cells and debris. All samples were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Total T-cells and B-cells were expressed as a percentage of total lymphocytes. CD4⁺ and CD8⁺ subsets of T-cells were expressed as percentage of T-cells. Subsets of CD4⁺ and CD8⁺ cells (effector, effector memory, central memory and naïve cells) were expressed as percentage of CD4⁺ and CD8⁺ cells respectively. The cytotoxic pE, pE1 and pE2 subsets were expressed as a percentage of cytotoxic effector cells. Cytotoxic EM1, EM2, EM3 and EM4 subsets were expressed as a percentage of cytotoxic effector memory cells. Subsets of B-cells were expressed as percentage of B-cells. Monocytes, DCs and NK cells were

expressed as a percentage of PBMCs. Subsets of monocytes, DCs and NK cells were expressed as a percentage of monocytes, DCs and NK cells respectively.

STATISTICAL ANALYSIS

All statistical analyses were conducted in R software package. All cell frequencies were evaluated for normality and there were no substantial deviations from normality. Mean percentage and 95% confidence interval for each cell subset in all experimental conditions are reported. We used paired t-tests to analyze the differences between experimental conditions. Since we compared seven experimental conditions with the reference, which was EDTA blood processed immediately (WB-D0) we used Bonferroni correction to adjust for multiple comparisons and used $p=0.007$ ($0.05/7$) as the level of significance in this study. In addition, we also used linear repeated measures regression to evaluate a linear trend across samples processed at different time intervals. We evaluated linear trend among samples used to evaluate effect of delayed processing on immunophenotyping using WB-D0, WB-D24, WB-D48 and WB-D72 and we evaluated the linear trend among samples used to evaluate effect of delayed processing and cryopreservation on immunophenotyping using WB-D0, PBMC-D24, PBMC-D48 and PBMC-D72. We used $p=0.05$ as the level of significance for the trend analyses. To evaluate the contribution of delayed cell processing on immunophenotyping, we compared the percentage of T, B, NK cells, monocytes and DCs in the EDTA blood tube processed immediately (WB-D0) to the percentage of these subsets in EDTA tubes processed after a delay of 24, 48 and 72 hours. To evaluate the contribution of cryopreservation on immunophenotyping, we compared the distribution of T, B, NK cells, monocytes and DCs in the EDTA blood tube processed immediately (WB-D0) to the blood that was collected in CPT tubes and cryopreserved without any delay in processing (PBMC-D0). To evaluate the combined effect of delayed cell processing and cryopreservation on immunophenotyping, we compared the distribution of T, B, NK cells, monocytes and DCs in the EDTA blood tube processed immediately (WB-D0) to the blood that was collected in CPT tubes and cryopreserved after a delay of 24 to 72 hours (PBMC-D24, PBMC-D48 and PBMC-D72). Finally, we used paired t-tests to compare the differences in cell subsets between blood collected in EDTA tubes and processed immediately vs. blood collected in CPT™ tubes and processed immediately.

RESULTS

Delayed cell processing up to 72 hours has minimal effect on T cells immunophenotyping

Cell viability was significantly lower when cell processing was delayed for 24 to 72 hours (WB-D24-D72) as compared to blood that was processed immediately after collection (WB-D0) (93.0% viability for WB-D0 vs. 81.1% for WB-D24, 80.4% for WB-D48 and 88.3% for WB-D72; $p < 0.001$ for all pairwise comparisons) (Table 3A). However, there was no significant linear trend of decreasing cell viability with increased delay in cell processing ($p=0.07$) (Table 3A). For the most part, percentages of T cells and the helper CD4+ and cytotoxic CD8+ were unaffected even when sample processing was delayed up to 72 hours (Figure 2A, Table 3A). Though none of the pairwise comparisons were significantly different for any of the T cell subsets, there was a significant trend towards an increase in the

cytotoxic (CD8+) effector cells (p for trend = 0.01) and a corresponding significant decreasing trend in the cytotoxic effector memory cells (p for trend = 0.002) (Table 3A). The percentage of B cells increased from 6.8% to 9.1% over 72 hours. Though none of the pairwise differences were statistically significant, there was a significant trend for increase in percentage of B cells with delayed cell processing (p for trend = 0.01)(Figure 2A, Table 4A). IgD– memory B cells showed a significant increase with delayed cell processing at 24 and 48 hours (12.5% at 24 hours and 17.2% at 48 hours vs. 10.1% for immediate processing; p=0.0001) while naïve B cells showed a corresponding decrease in cells processed after 24 and 48 hours (61.3% at 24 hours and 57.7% at 48 hours vs. 66.5% for immediate processing; p=0.0001) but these changes were not statistically significant at 72 hours (Table 4A). There was also a marginal linear trend for increase in IgD– memory B cells with delayed cell processing (p for trend = 0.05) and a significant trend of decrease in naïve B cells with delayed cell processing (p for trend = 0.02). Though none of the pairwise comparisons were significant, NK cells showed a significant increase with delayed processing (p for trend = 0.001) (Figure 2A, Table 4A). The percentage of DCs did not change substantially with delayed cell processing but the percentage of DC-P cells consistently decreased with delayed cell processing with DC-P accounting for 9.7% of DCs in cells that were processed immediately to 3.6% of DCs in cells that were processed after a delay of 72 hours (p<0.0002) (p for trend <0.001)(Table 4A). This was accompanied by a corresponding increase in DC-M cells (p for trend = 0.006) (Table 4A). The total number of monocytes did not change with cell processing delay (Figure 2A).

Cryopreservation alone had minimal effect on immunophenotyping

There was no difference in cell viability between cells immunophenotyped immediately (WB-D0) and cells cryopreserved immediately after collection (PBMC-D0) (Tables 3B). We found no substantial differences in percentage of most cell subsets identified by immunophenotyping when comparing the cryopreserved cells (PBMC-D0) to cells that were processed without cryopreservation (WB-D0) (Figure 2B) with the exception of CD4+ central memory T cells which decreased significantly with cryopreservation (33.7% vs. 41.5%; p<0.007) and CD8+ effector T cells which increased significantly with cryopreservation (35.8% vs. 21.1%; p<0.007) (Table 3B). Though there were no changes in percentage of total NK cells, the percentage of NK CD56 low subset was significantly higher when cells were cryopreserved as compared to freshly processed cells (87.9% vs. 76.8%; p<0.0002) (Table 4B).

Combination of delay in cell processing and cryopreservation impacted immunophenotyping of several cell subsets

Cell viability was significantly lower among cells cryopreserved after a delay of 24 to 72 hours as compared to cells processed immediately (WB-D0) (p for trend <0.0001) (91.3% for PBMC-D24, 73.4% for PBMC-D48 and 58.9% for PBMC-D72 vs. 93.3% for WB-D0; p 0.003 all pairwise comparisons) (Table 3B). When cells were cryopreserved after a delay of 72 hours there was a significant increase in percentage of total T cells (73.1% vs. 85.3%; p<0.0001) (Figure 2C). In addition, the percentage of CD4+ T cells increased (63.1% vs. 69.3%; p=0.007) and was accompanied by a corresponding decrease in the percentage of CD8+ T cells (28.3% vs. 23.3%; p=0.009) when cells were cryopreserved after a 72 hours

delay (Table 3B). In addition, there was also a significant linear trend towards increase in CD4⁺ T cells (p for trend = 0.008) and decrease in CD8⁺ T cells (p for trend = 0.004) with a combination of delayed cell processing and cryopreservation (Table 3B). There was a significant increase in naïve CD8⁺ T cells over time (p for trend = 0.003) (Table 3B). The increase in percentage of T cells over 72 hours was accompanied by a corresponding decrease in percentage of NK cells (9.7% vs. 2.1%; $p < 0.0001$ and p for trend < 0.0001) when cells were cryopreserved after a delay of 72 hours (Figure 2C). None of the NK cell subsets were substantially affected by cryopreservation and delayed cell processing (Table 4B) though there was a significant trend towards decreased percentage of CD56HI NK cell subset (p for trend = 0.001). However, the CD16⁺ monocytes showed a significant increase with delay in cell processing and cryopreservation (p for trend = 0.004) (Table 4B). The percentage of B cells showed a significant increase when cells were cryopreserved after a delay of 24 hours or 48 hours as compared to cells processed immediately, but this change was not significant when cells were cryopreserved after 72 hours (Figure 2C). Among B cell subsets, percentage of IgD⁺ memory B cells reduced with delayed processing and cryopreservation (p for trend = 0.01). Pairwise comparisons showed a significant reduction when cells were cryopreserved 48 hours and 72 hours after collection (12.3% after 48 hours and 13.9% after 72 hours vs. 17.6% when processed immediately; $p = 0.0002$) while changes in other B cell subsets did not reach statistical significance (Table 4B).

Blood collected in CPT tubes had higher T cells and B cells as compared to blood collected in EDTA tubes

Total T cells and B cells were both marginally lower in blood collected and processed immediately in CPTTM tubes as compared to blood collected and processed in EDTA tubes (67.02% vs. 75.34% for T cells; $p = 0.04$ and 5.52% vs. 8.85% for B cells; $p = 0.01$) (Supplementary Table 1). Expressed as a percentage of T cells, cytotoxic T cells (CD8⁺) were significantly higher (36.30% vs. 31.42%; $p = 0.003$) and helper T cells (CD4⁺) were significantly lower (52.62% vs. 60.56%; $p = 0.002$) in blood collected and processed immediately in CPTTM tubes as compared to blood collected and processed in EDTA tubes (Supplementary Table 1). In addition, CD8⁺ effector T cells expressed as a percentage of CD8⁺ T cells (50.68% vs. 39.34%; $p = 0.006$) and IgD⁺ memory B cells expressed as a percentage of B cells were higher (19.88% vs. 12.99%; $p = 0.03$) in CPTTM tubes vs. EDTA tubes (Supplementary Table 1). The overall proportions of dendritic cells, NK cells and monocytes were not significantly different between CPTTM and EDTA tubes. However the percentage of NK CD56high subset was marginally higher in CPTTM tubes vs. EDTA tubes (3.69% vs. 1.60%; $p = 0.03$) (Supplementary table 1).

DISCUSSION

We evaluated the effect of both delayed cell processing and cryopreservation on immunophenotyping of immune cell subsets. The combined effect of both cryopreservation and delayed cell processing up to 48 hours had a minimal impact on the distribution of several T-cell subsets and dendritic cells while there were substantial differences in several immune cell distributions with a 72 hour delay in cell processing and cryopreservation. B

cells, monocytes and NK cells were substantially affected by a combination of cryopreservation delay in cell processing for as little as 24 hours.

Several previous studies have shown no effect of cryopreservation in the enumeration of T cells, helper CD4+ and cytotoxic CD8+ T cells^{2, 7, 8, 11, 12}. These results are largely consistent with the results of our study where we did not find any pairwise differences in percentage of total T cells unless cells were cryopreserved after a delay in cell processing for 72 hours.

However, we did find a significant trend towards decrease in CD8+ T cells, increase in CD8+ naïve T cells and increase in CD4+ T cells with delayed cell processing and cryopreservation. These findings were in contrast to a previous study that showed significant decrease in naïve CD8+ T cells and a corresponding increase in CD8+ effector T cells with cryopreservation¹ and another study that showed a decrease in total T cells and CD4+ T cells after cryopreservation⁵. Limieux et al showed that though there were significant differences in several T cell subsets when immunophenotyping was performed on cryopreserved PBMCs without resting the cells, the differences in the proportion of naïve, central memory, effector, effector memory, Th1 and Th2 as well as activated subsets within the helper CD4+ and cytotoxic CD8+ T cell populations were no longer significant after a rest period of 1 to 24 hours⁵. In addition a one hour rest period also showed greater concordance of helper CD4+ and cytotoxic CD8+ T cell subsets with the fresh sample than the sample that was rested for 24 hours or not rested at all⁵. Limieux et al used negative selection of T cells prior to cryopreservation instead of cryopreserving PBMCs from whole blood (as is commonly done in other studies that evaluated the effect of cryopreservation) and this may account for the differences seen in their study as compared to other published findings⁵. This study used whole blood collected in EDTA tubes as the reference in this study while other studies used Ficoll separated PBMCs as the reference. Comparison of blood collected by Ficoll separation (CPT™ tubes) and EDTA showed that cells collected by Ficoll separation had decreased CD8+ T cells, increased CD4+ T cells and increased CD8+ effector T cells. While most of the observed changes in T cell subsets between fresh blood collected in CPT™ and EDTA tubes were in the opposite direction to what was observed with cryopreservation, using blood collected in EDTA tubes as a reference may account for some of the discrepancies observed between studies. Under the conditions described in our study, we found that T cells, remained stable under most experimental conditions except when cell processing was delayed for 72 hours prior to cryopreservation. Even when cell processing was delayed for up to 72 hours before cryopreservation the proportion of several (but not all) T cells subsets were still comparable to the freshly processed sample as there were no consistent differences in various T cell subsets.

A previous study showed B cells increase in cryopreserved blood as compared to samples that were processed immediately without cryopreservation⁸. The increase in B cells in cryopreserved PBMCs remained unchanged by either a 1 or 24 hours rest period⁵. However, other studies showed no effect of cryopreservation on B cell distribution^{12, 13}. This study found the percentage of IgD+ memory B cells was significantly reduced by a combination of delay in cell processing and cryopreservation but delay in processing alone showed a significant trend towards increase in B cells and a decrease in naïve B cells. Previous studies

have reported that NK cells are lower in cryopreserved PBMCs but still highly correlated to fresh blood^{5, 8} and the lower count did not improve after a rest period⁵. In contrast, other studies that included no rest period after thawing found that NK cells enumeration was not affected by cryopreservation of PBMCs compared to fresh blood^{2, 12}. In this study, we observed a significant trend towards increasing percentage of NK cells with delay in cell processing but a significant decrease in percentage of total NK cells and the NK CD56HI subset after a combination of delayed processing and cryopreservation. In addition, though overall NK cell proportions remained unchanged after cryopreservation alone, we found higher percentages of CD56low subset in samples cryopreserved immediately as compared to samples that were processed immediately without cryopreservation. These data suggest that cryopreservation has a significant impact on immunophenotyping of NK cells. Two previous studies showed that monocytes count was more elevated in cryopreserved blood or cryopreserved PBMCs compared to fresh blood^{2, 8}. In addition, another study showed cryopreservation did not affect total number of monocytes but monocyte subsets (defined by expression of CD16) were affected by cryopreservation¹⁴. However, none of these studies allowed the cells to rest prior to staining. Proportion of monocytes in cryopreserved PBMCs was similar to fresh blood after 1 and 24 hours of rest after thawing⁵. Our study findings are consistent with these previous studies in that cryopreserved monocytes were comparable to fresh monocytes only when a rest period was included. We found that the proportion of cryopreserved monocytes that did not have a one hour rest period after thawing were substantially different from the proportion of monocytes in fresh blood (data not shown). Pairwise comparisons showed that the percentage of overall monocytes and CD16– monocytes were affected only when there was a delay of up to 72 hours in processing cells prior to cryopreservation though the CD16– monocytes showed trend towards a significant decrease with delayed processing and cryopreservation. While the dendritic cells retain their function after cryopreservation, previous studies have shown that they increase in numbers following cryopreservation^{2, 8}. Ficoll separation regardless of cryopreservation status has also shown to increase the ratio of plasmacytoid DCs to myeloid DCs⁴. Our study confirmed that there was a significant trend towards increase in plasmacytoid DCs and a non-significant trend towards decrease in myeloid DCs after a combination of cell delay and cryopreservation. Interestingly, this pattern was reversed when evaluating DC subsets after delayed cell processing alone.

Studies that have evaluated the effect of thawing procedures have, in general, shown that a short rest period of 1 hour post thawing generally improves comparability of various immune subsets in cryopreserved cells and fresh cells⁵. An 18 hour rest period also reduced the number of apoptotic cells in thawed PBMCs and improved lymphocytes functionality⁹ while another study showed that a rest period of upto 8 hours did not affect PBMC viability¹⁶. However, a longer rest period of up to 24 hours results in higher proportion of activated T cells in the cryopreserved sample⁷. We incorporated a one hour rest period in our thawing protocol to improve the comparability of various cell subsets. Since our goal was to perform immunophenotyping the same day the samples were thawed, we did not evaluate the effect of longer rest periods on immune cell distributions.

The effect of delayed cell processing on immunophenotyping has been more sparsely documented. Several studies have shown that delayed cell processing has a substantial

functional impact on the number of cell subsets. Specifically storing cells overnight at 4°C reduced the percentage of all T cells and helper CD4+ T cells in particular³ though there was no change in T cell percentages when cells were stored at room temperature. Other studies have also confirmed that large variations in temperature observed during sample shipment can result in reduced yield, cell viability and T cell function as compared to delayed cell processing when samples are stored at room temperature⁶. Two other studies have also reported that storing samples at room temperature is better as compared to storing cells at 4°C^{17, 18}. A previous study also showed that processing PBMCs within 8 hours of collection prior to cryopreservation resulted in greater cell viability as compared to PBMCs processed after a delay of 24 hours¹⁰. Our study found that several (but not all) cell subsets stored at room temperature actually remain stable for immunophenotyping up to 72 hours. However, a combination of both delay in cell processing and cryopreservation significantly affects some cell subsets. In addition to functional changes in lymphocytes, at least, one study has reported that there were no changes in percentage of CD4+, CD8+ and CD56+ after blood samples were stored overnight at room temperature⁶. We also evaluated the effect of transport and shipment on immunophenotyping on a subset of 10 samples and these results were very similar to the results presented in this manuscript (data not shown).

There are several limitations to this study. This study did not evaluate functional subsets of T cells (e.g. Tregs, Th1, Th2, Th17 subsets) and the effect of delayed cell processing and cryopreservation on these subsets needs to be evaluated in future studies. The inclusion of blood collected in EDTA tubes as the reference may account for some discrepancies in conclusions between this and other studies. However, comparison of fresh blood collected in CPT™ and EDTA tubes show comparable results for a majority of cell subsets evaluated in this study. Though several sources of variability in immunophenotyping including type of anticoagulant, temperature of sample shipment, delay between sample collection and processing, cryopreservation methods and thawing methods have all been documented, we describe the systematic evaluation and standardization of various aspects of sample collection and processing to enable immunophenotyping efforts in large scale epidemiological studies. This study showed that a delay of up to 48 hours between sample collection and processing, followed by cryopreservation and a short incubation at 37°C for one hour after thawing gives immunophenotyping results that is comparable to freshly processed whole blood for a majority of T cell subsets, NK cells, monocytes and dendritic cells. However, percentage of B cells was increased by both delay in cell processing and cryopreservation. Though we have not evaluated all possible sources of variation in this validation study, we have evaluated common sources of variation in epidemiological studies and used these experiments to develop standardized protocols for reliable measurement of mononuclear cell subsets using cryopreserved PBMCs in the Health and Retirement Study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:
Overview of study design to evaluate the effect of delay in cell processing and cryopreservation on proportion of 31 cell subsets identified by immunophenotyping.

Figure 2A: Effect of delayed cell processing on immune cell subsets.

The figure shows average cell percentages of T cells, B cells, NK cells, monocytes and dendritic cells (DC) at time 0 (WB-D0) to average cell percentages of fresh blood processed at times 24, 48 and 72 after collection (WB-D24, WB-D48 and WB-D72 respectively). Cell percentages are expressed as percent lymphocytes for B and T cells and as percent PBMCs for DC, NK and monocytes.

● indicates statistically significant difference ($p < 0.007$) in proportion of cells detected at particular time point versus WB-D0 (reference).

WB-D0 = Whole blood processed immediately, WB-D24 = Whole blood processed after a delay of 24 hours, WB-D48 = Whole blood processed after a delay of 48 hours and WB-D72 = Whole blood processed after a delay of 72 hours.

Figure 2B: Effect of cryopreservation on immune cell subsets.

The figure shows average cell percentages of T cells, B cells, NK cells, monocytes and dendritic cells for cryopreserved samples at time 0 (PBMC-D0) to average cell percentages of fresh blood at time 0 (WB-D0). Cell percentages are expressed as percent lymphocytes for B and T cells and as percent PBMC for monocytes, DC, and NK cells.

● indicates statistically significant difference ($p < 0.007$) in proportion of cells detected at particular time point versus WB-D0 (reference).

WB-D0 = Whole blood processed immediately, PBMC-D0 = Peripheral Blood Mononuclear Cells cryopreserved immediately.

Figure 2C: Combined effect of delay in cell processing and cryopreservation on immune cell subsets.

The figure shows average cell percentages of T cells, B cells, NK cells, monocytes and dendritic cells for cryopreserved samples at time 24, 48 and 72 hours (PBMC-D24, PBMC-D48, PBMC-D72 respectively) to average cell percentages in whole blood processed immediately (WB-D0). Cell percentages are expressed as percent lymphocytes for B and T cells and as percent PBMCs for DC, NK and monocytes.

● indicates statistically significant difference ($p < 0.007$) in proportion of cells detected at particular time point versus WB-D0 (reference).

WB-D0 = Whole blood processed immediately, PBMC-D24 = Peripheral Blood Mononuclear Cells cryopreserved after a delay of 24 hours, PBMC-D48 = Peripheral Blood Mononuclear Cells cryopreserved after a delay of 48 hours and PBMC-D72 = Peripheral Blood Mononuclear Cells cryopreserved after a delay of 72 hours.

Table 1:

List of antibodies and fluorochromes used to characterize all cell subsets

Antibody	Clone	Fluorochrome	Provider (cat #)	Panels	Titration
brilliant stain buffer	NA	NA	BD (659611)	1,2	NA
viability dye	NA	FVS 570 (PE)	BD (564995)	1,2	NA
CD3	UCHT1	APC	BD (555335)	1,2	1:5
HLA-DR	G46-6	PE-CF594	BD (562331)	1,2	1:5
CD19	SJ25C1	PE-Cy7	BD (557835)	1,2	1:5
CD27	O323	FITC	Biolegend (302806)	1	1:2.5
CD8	RPA-T8	BUV395	BD (563796)	1	1:5
IgD	IA6-2	BUV737	BD (564687)	1	1:5
CCR7	G043H7	BV421	Biolegend (353208)	1	1:2..5
CD28	CD28.2	BV510	Biolegend (302936)	1	1:2.5
CD95	DX2	BV605	Biolegend (305628)	1	1:2.5
CD45RA	HI100	BV711	Biolegend (304138)	1	1:2.5
CD4	RPA-T4	APC-Cy7	BD (557871)	1	1:2.5
CD11c	B-ly6	BB515	BD (564490)	2	1:5
CD20	2H7	BUV395	BD (563781)	2	1:5
CD16	3G8	BUV737	BD (564433)	2	1:5
CD56	NCAM16.2	BV421	BD (562751)	2	1:5
CD14	MOP9	BV510	BD (563079)	2	1:5
CD123	9F5	BV711	BD (563161)	2	1:5
CD45	2D1	APC-Cy7	BD (560178)	2	1:10
CD11c	B-ly6	BB515	BD (564490)	2	1:5
CD20	2H7	BUV395	BD (563781)	2	1:5
CD16	3G8	BUV737	BD (564433)	2	1:5

Table 2:

Immunophenotypic characterization of 31 cell subsets

CELL TYPE	MARKERS
T cells	CD3+ CD19–
Helper T cells	CD3+ CD19– CD8– CD4+
Helper T cells: Central Memory (CM)	CD3+ CD19– CD8– CD4+ CD45RA– CCR7+ CD28+ CD95+
Helper T-cells: Effector (EFF)	CD3+ CD19– CD8– CD4+ CD45RA+ CCR7–
Helper T cells: Effector memory (EM)	CD3+ CD19– CD8– CD4+ CD45RA– CCR7–
Helper T cells: Naïve	CD3+ CD19– CD8+ CD4+ CD45RA+ CCR7+ CD95– CD28+
Cytotoxic T cells	CD3+ CD19– CD8+ CD4–
Cytotoxic T cells: Central Memory (CM)	CD3+ CD19– CD8+ CD4– CD45RA– CCR7+ CD28+ CD95+
Cytotoxic T cells: Effector (EFF)	CD3+ CD19– CD8+ CD4– CD45RA+ CCR7–
Cytotoxic T cells: pre-Effector (pE)	CD3+ CD19– CD8+ CD4– CD45RA+ CCR7– CD27– CD28–
Cytotoxic T cells: pre-Effector 1 (pE1)	CD3+ CD19– CD8+ CD4– CD45RA+ CCR7– CD27+ CD28+
Cytotoxic T cells: pre-Effector 2 (pE2)	CD3+ CD19– CD8+ CD4– CD45RA+ CCR7– CD27+ CD28–
Cytotoxic T cells: Effector Memory (EM)	CD3+ CD19– CD8+ CD4– CD45RA– CCR7–
Cytotoxic T cells: EM1	CD3+ CD19– CD8+ CD4– CD45RA– CCR7– CD27+ CD28+
Cytotoxic T cells: EM2	CD3+ CD19– CD8+ CD4– CD45RA– CCR7– CD27+ CD28–
Cytotoxic T cells: EM3	CD3+ CD19– CD8+ CD4– CD45RA– CCR7– CD27– CD28–
Cytotoxic T cells: EM4	CD3+ CD19– CD8+ CD4– CD45RA– CCR7– CD27– CD28+
Cytotoxic T cells: Naïve	CD3+ CD19– CD8+ CD4– CD45RA+ CCR7+ CD95– CD28+
B cells	CD3– CD19+
IgD+ memory B cells	CD3– CD19+ IgD+ CD27+
IgD– memory B cells	CD3– CD19+ IgD– CD27+
Naïve B cells	CD3– CD19+ IgD+ CD27–
Natural Killer (NK) cells	CD3– CD19– CD20– CD14– CD16+ CD56+
NK Cells: CD56HI	CD3– CD19– CD20– CD14– CD16+ CD56HI
NK Cells: CD56LO	CD3– CD19– CD20– CD14– CD16+ CD56LO
Monocytes	CD3– CD19– CD20– CD14+
CD16– monocytes	CD3– CD19– CD20– CD14+ HLA-DR+ CD16–
CD16+ monocytes	CD3– CD19– CD20– CD14+ HLA-DR+ CD16+
Dendritic cells	CD3– CD19– CD20– CD14– HLA-DR+
Myeloid Dendritic cells (DC-M)	CD3– CD19– CD20– CD14– HLA-DR+ CD11c+ CD123–
Plasmacytoid Dendritic cells (DC-P)	CD3– CD19– CD20– CD14– HLA-DR+ CD11c– CD123+

Table 3A:

Effect of delayed cell processing on immunophenotyping of T cell subsets using EDTA whole blood

	Reference (WB-D0)	Delayed cell processing				P for trend
Cell Subsets [#]	0hr Mean (95% CI)	24hr Mean (95% CI)	48hr Mean (95% CI)	72hr Mean (95% CI)		
Cell Viability	93.0 (91.9, 94.1)	81.1 *** (75.9, 86.3)	80.4 *** (77.4, 83.3)	88.3 *** (85.8, 90.8)		0.07
T cells	73.1 (69.6, 76.7)	75.5 (71.1, 79.9)	74.6 (70.5, 78.6)	71.0 (65.9, 76.1)		0.11
Helper T cells	63.1 (56.2, 69.9)	64.2 (58.9, 69.6)	65.4 (60.2, 70.6)	62.7 (56.5, 68.9)		0.88
Helper T cells: CM	41.5 (36.2, 46.8)	39.3 (33.1, 45.4)	40.2 (34.5, 45.8)	39.9 (31.8, 48.0)		0.09
Helper T cell s: EFF	0.4 (0.0, 0.8)	0.5 (0.1, 0.9)	0.4 (0.0, 0.7)	0.8 (0.1, 1.5)		0.1
Helper T cells: EM	18.6 (13.8, 23.3)	22.8 (16.1, 29.4)	20.7 (16.6, 24.7)	21.3 (12.7, 29.9)		0.29
Helper T cells: Naïve	36.3 (28.8, 43.7)	34.2 (26.3, 42.1)	36.4 (29.0, 43.8)	34.6 (25.3, 43.9)		0.94
Cytotoxic T cells	28.3 (23.7, 33.0)	28.6 (23.9, 33.3)	28.6 (24.0, 33.3)	31.0 (25.3, 36.7)		0.14
Cytotoxic T cells: CM	9.5 (5.7, 13.3)	10.4 (6.5, 14.2)	10.8 (6.7, 14.9)	11.9 (7.8, 16.0)		0.15
Cytotoxic T cells: EFF	21.1 (9.9, 32.3)	24.1 (13.6, 34.6)	25.4 (14.8, 35.9)	27.7 (15.4, 40.0)		0.01
Cytotoxic T cells: pE	49.5 (38.1, 70.0)	52.3 (41.5, 63.0)	51.1 (40.3, 61.9)	49.8 (38.7, 60.8)		0.38
Cytotoxic T cells:pE1	16.9 (10.1, 23.6)	15.5 (11.1, 20.0)	14.5 (9.8, 19.3)	14.1 (9.1, 19.1)		0.17
Cytotoxic T cells:pE2	31.3 (22.3, 40.4)	29.5 (20.8, 38.3)	31.9 (23.1, 40.8)	33.7 (24.9, 42.4)		0.92
Cytotoxic T cells: EM	39.2 (29.9, 48.5)	37.3 (28.4, 46.3)	35.3 (26.4, 44.3)	33.8 (23.9, 43.6)		0.002
Cytotoxic T cells:EM1	62.7 (54.0, 71.4)	62.7 (54.1, 71.2)	60.0 (52.1, 67.8)	55.0 (45.1, 64.8)		0.001
Cytotoxic T cells: EM2	12.2 (9.7, 14.6)	11.7 (9.1, 14.3)	13.1 (10.2, 16.0)	16.1 *** (13.3, 19.0)		0.001
Cytotoxic T cells: EM3	18.8 (10.6, 27.0)	18.0 (10.1, 26.0)	18.7 (10.9, 26.5)	21.4 (11.8, 31.0)		0.09
Cytotoxic T cells: EM4	6.3 (4.6, 8.1)	7.6 (5.3, 9.9)	8.3 (6.5, 10.0)	7.5 (5.3, 9.7)		0.06
Cytotoxic T cells:Naïve	20.9 (13.8, 28.0)	21.0 (14.1, 27.9)	21.2 (14.6, 27.9)	19.5 (11.2, 27.9)		0.84

*** Indicates statistically significant pairwise comparisons ($p < 0.007$) between WB-D0 (reference) and the individual time points.

⁺ Blood collected in EDTA tube and processed at time 0 (WB-D0) was used as the reference for all comparisons

[#] CD4⁺ and CD8⁺ subsets of T-cells were expressed as percentage of T-cells. Subsets of CD4⁺ and CD8⁺ cells (effector, effector memory, central memory and naïve cells) were expressed as percentage of CD4⁺ and CD8⁺ cells respectively. The cytotoxic pE, pE1 and pE2 subsets were

expressed as a percentage of cytotoxic effector cells. Cytotoxic EM1, EM2, EM3 and EM4 subsets were expressed as a percentage of cytotoxic effector memory cells.

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Table 3B:

Effect of cryopreservation alone and combined effect of delayed cell processing and cryopreservation on immunophenotyping of T cell subsets using blood collected in CPT™ tubes

	Reference (WB-D0)	Cryopreser- vation ⁺ (PBMC-D0)	Delayed cell processing and cryopreservation ⁺			P for trend
Cell subsets [#]	0hr Mean (95% CI)	0hr Mean (95% CI)	24hr Mean (95% CI)	48hr Mean (95% CI)	72hr Mean (95% CI)	
Cell Viability	93.0 (91.9, 94.1)	94.5 (93.5, 95.4)	91.3 (89.0, 93.5)	73.4 ^{***} (67.5, 79.3)	58.9 ^{***} (54.1, 63.7)	<0.0001
T cells	73.1 (69.6, 76.7)	73.3 (68.3, 78.3)	67.4 ^{***} (63.3, 71.5)	71.0 (66.9, 75.2)	85.3 ^{***} (82.2, 88.3)	<0.0001
Helper T cells	63.1 (56.2, 69.9)	58.1 (51.5, 64.7)	58.7 (52.4, 64.9)	62.1 (55.4, 68.7)	69.3 (64.4, 74.2)	0.008
Helper T cells: CM	41.5 (36.2, 46.8)	33.7 ^{***} (27.5, 39.9)	34.9 (29.5, 40.3)	38.0 (30.7, 45.2)	40.5 (33.6, 47.5)	0.98
Helper T cells: EFF	0.4 (0.0, 0.8)	1.0 (0.2, 1.9)	1.0 (0.2, 1.7)	0.5 (0.2, 0.8)	0.5 (0.1, 0.9)	0.74
Helper T cells: EM	18.6 (13.8, 23.3)	23.0 (16.9, 29.1)	22.4 (16.9, 28.0)	16.6 (11.7, 21.5)	21.5 (15.5, 27.6)	0.66
Helper T cells: Naïve	36.3 (28.8, 43.7)	38.3 (31.1, 45.5)	37.7 (30.2, 45.1)	41.0 (32.1, 50.0)	33.0 (24.2, 42.0)	0.47
Cytotoxic T cells	28.3 (23.7, 33.0)	32.2 (26.7, 37.7)	32.0 (26.4, 37.7)	27.2 (22.4, 32.0)	23.3 ^{***} (19.3, 27.3)	0.004
Cytotoxic T cells: CM	9.5 (5.7, 13.3)	7.3 (2.6, 11.9)	7.4 (2.5, 12.2)	9.4 (5.5, 13.3)	10.4 (6.0, 14.7)	0.26
Cytotoxic T cells: EFF	21.1 (9.9, 32.3)	35.8 ^{***} (22.0, 49.6)	36.1 ^{***} (22.9, 49.3)	23.7 (12.8, 34.6)	22.1 (12.0, 32.1)	0.49
Cytotoxic T cells: pE	49.5 (38.1, 70.0)	53.1 (41.8, 64.4)	55.6 (44.7, 66.5)	54.0 (42.4, 65.5)	48.9 (37.3, 60.4)	0.70
Cytotoxic T cells:pE1	16.9 (10.1, 23.6)	15.3 (9.4, 21.3)	13.7 (8.6, 18.7)	15.2 (8.9, 21.5)	21.4 (13.0, 29.7)	0.08
Cytotoxic T cells:pE2	31.3 (22.3, 40.4)	30.0 (21.7, 38.4)	29.1 (20.5, 37.8)	28.8 (20.3, 37.2)	27.9 (19.4, 36.4)	0.10
Cytotoxic T cells: EM	39.2 (29.9, 48.5)	32.3 (22.3, 42.2)	31.7 (21.4, 42.0)	30.8 (22.0, 39.7)	34.8 (24.9, 44.7)	0.19
Cytotoxic T cells:EM1	62.7 (54.0, 71.4)	62.1 (54.6, 69.7)	59.1 (52.2, 66.0)	58.1 (52.4, 63.9)	65.2 (58.1, 72.3)	0.48
Cytotoxic T cells: EM2	12.2 (9.7, 14.6)	12.4 (9.1, 15.6)	12.9 (9.3, 16.6)	12.6 (8.9, 16.3)	10.7 (6.7, 14.8)	0.23
Cytotoxic T cells: EM3	18.8 (10.6, 27.0)	17.8 (10.7, 24.8)	17.8 (11.1, 24.5)	17.4 (11.8, 23.0)	14.7 (9.1, 20.3)	0.08
Cytotoxic T cells: EM4	6.3 (4.6, 8.1)	7.8 (5.2, 10.3)	10.1 (7.1, 13.2)	11.5 ^{***} (9.3, 13.8)	9.4 ^{***} (7.8, 11.0)	0.01
Cytotoxic T cells: Naïve	20.9 (13.8, 28.0)	18.8 (11.9, 25.6)	18.7 (13.1, 24.3)	28.2 ^{***} (19.2, 37.1)	25.3 (17.2, 33.5)	0.003

*** Indicates statistically significant pairwise comparisons (p<0.007) between WB-D0 (reference) and the individual time points

⁺ Blood collected in EDTA tube and processed at time 0 (WB-D0) was used as the reference for all comparisons

[#]CD4⁺ and CD8⁺ subsets of T-cells were expressed as percentage of T-cells. Subsets of CD4⁺ and CD8⁺ cells (effector, effector memory, central memory and naïve cells) were expressed as percentage of CD4⁺ and CD8⁺ cells respectively. The cytotoxic pE, pE1 and pE2 subsets were expressed as a percentage of cytotoxic effector cells. Cytotoxic EM1, EM2, EM3 and EM4 subsets were expressed as a percentage of cytotoxic effector memory cells.

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Table 4A:

Effect of delayed cell processing on immunophenotyping of subsets of B cells, NK cells, dendritic cells and monocytes using EDTA whole blood

	Reference (WB-D0)	Delayed cell processing ⁺			
Cell Subsets [#]	0hr Mean (95% CI)	24hr Mean (95% CI)	48hr Mean (95% CI)	72hr Mean (95% CI)	P for trend
B Cells	6.8 (5.4,8.1)	7.9 (6.2,9.5)	8.8 *** (7.0,10.5)	9.1 (6.6, 11.5)	0.01
IgD+ memory B cells	17.6 (13.4, 21.9)	19.1 (13.5, 24.7)	14.1 (10.8, 17.3)	20.3 (15.2, 25.3)	0.92
IgD– memory B cells	10.1 (7.7, 12.6)	12.5 *** (9.6, 15.4)	17.2 *** (13.8, 20.6)	11.18 (8.3, 14.1)	0.05
Naïve B cells	66.5 (60.3, 72.8)	61.3 *** (55.2, 67.4)	57.7 *** (52.5, 63.0)	63 (55.1, 70.9)	0.02
NK Cells	9.7 (7.7,11.6)	10.8 (8.6,12.9)	10.7 (8.0,13.4)	13.0 (10.1, 15.9)	0.001
NK cells CD56HI	2.0 (1.4, 2.5)	1.9 (1.4, 2.5)	2.1 (1.3, 2.9)	1.6 (1.1, 2.1)	0.39
NK cells CD56LO	76.8 (67.3, 86.3)	76.4 (67.7, 85.0)	76.1 (67.1, 85.2)	78.2 (69.0, 7.3)	0.34
Dendritic Cells	1.9 (1.3,2.6)	1.8 (1.3,2.3)	1.7 (1.2,2.1)	1.8 (1.3, 2.3)	0.27
DC-M	79.9 (76.8,83.0)	82.2 (79.4, 85.0)	82.1 (78.3, 85.9)	84.1 (80.4, 87.7)	0.006
DC-P	9.7 (7.9, 11.5)	7.1 *** (5.4, 8.8)	4.3 *** (3.0, 5.6)	3.6 *** (2.8, 4.4)	<0.001
Monocytes	5.9 (3.0,8.9)	5.5 (2.9,8.2)	5.5 (3.3,7.8)	5.1 (3.6, 6.7)	0.68
CD16– Monocytes	92.5 (90.9, 94.0)	92.7 (91.6, 93.7)	91.9 (90.4, 93.5)	91.5 (89.9, 93.1)	0.14
CD16+ Monocytes	6.5 (5.1, 7.9)	6.0 (5.1, 7.0)	6.3 (5.1 7.6)	6.6 (5.4, 7.9)	0.72

*** Indicates statistically significant pairwise comparisons ($p < 0.007$) between WB-DO (reference) and the individual time points

⁺ Blood collected in EDTA tube and processed at time 0 (WB-D0) was used as the reference for all comparisons

[#] Subsets of B cells, monocytes, DCs and NK cells were expressed as a percentage of monocytes, DCs and NK cells respectively.

Table 4B:

Effect of cryopreservation alone and combined effect of delayed cell processing and cryopreservation on immunophenotyping of subsets of B cells, NK cells, dendritic cells and monocytes using blood collected in CPT™ tubes

	Reference (WB-D0)	Cryopreser- vation (PBMC- D0) ⁺	Delayed cell processing and Cryopreservation ⁺			
Cell Subsets [#]	0hr Mean (95% CI)	0hr Mean (95% CI)	24hr Mean (95% CI)	48hr Mean (95% CI)	72hr Mean (95% CI)	P for Trend
B cells	6.8 (5.4, 8.1)	5.3 (4.5, 6.2)	11.8 ^{***} (9.6, 13.9)	15.0 ^{***} (11.4, 18.6)	7.1 (4.8, 9.5)	0.46
IgD+ memory B cells	17.6 (13.4, 21.9)	15.0 (12.1, 17.8)	12.0 ^{***} (9.5, 14.5)	12.3 ^{***} (9.7, 14.8)	13.9 ^{***} (10.7, 17.2)	0.01
IgD- memory B cells	10.1 (7.7, 12.6)	11.7 (7.5, 15.9)	9.6 (5.6, 13.5)	9.9 (6.7, 13.1)	9.7 (7.1, 12.2)	0.77
Naïve B cells	66.5 (60.3, 72.8)	68.2 (62.0, 74.4)	71.7 (65.6, 77.8)	68.4 (62.3, 74.4)	66.1 (59.4, 72.9)	0.40
NK cells	9.7 (7.7, 11.6)	9.1 (6.5, 11.6)	6.5 ^{***} (4.6, 8.4)	4.0 ^{***} (2.6, 5.3)	2.1 ^{***} (1.1, 3.1)	<0.0001
NK cells CD56HI	2.0 (1.4, 2.5)	1.6 (0.9, 2.4)	1.4 (0.9, 1.9)	1.5 (0.8, 2.2)	0.9 ^{***} (0.4, 1.4)	0.001
NK cells CD56LO	76.8 (67.3, 86.3)	89.5 ^{***} (85.2, 93.7)	90.4 ^{***} (85.7, 95.1)	72.8 (63.3, 82.3)	73.5 (64.0, 83.0)	0.05
Dendritic cells	1.9 (1.3, 2.6)	2.0 (1.6, 2.2)	3.2 (2.4, 4.0)	3.4 (1.9, 4.9)	1.6 (1.3, 2.0)	0.84
DC-M	79.9 (76.8, 83.0)	77.7 (68.8, 86.6)	79.1 (73.5, 84.7)	79.1 (74.2, 84.1)	73.5 (66.0, 81.1)	0.06
DC-P	9.7 (7.9, 11.5)	12.2 (8.3, 16.1)	14.9 (10.5, 19.2)	13.6 (9.6, 17.7)	16.6 ^{***} (12.1, 21.0)	0.002
Monocytes	5.9 (3.0, 8.9)	9.2 (6.7, 11.8)	14.5 ^{***} (10.5, 18.5)	14.5 ^{***} (10.0, 19.0)	6.4 (4.1, 8.7)	0.85
CD16- Monocytes	92.5 (90.9, 94.0)	93.9 (92.7, 95.1)	96.3 ^{***} (95.7, 96.9)	89.8 (84.7, 95.0)	83.4 (74.2, 92.5)	0.004
CD16+ Monocytes	6.5 (5.1, 7.9)	4.7 (3.7, 5.6)	2.5 ^{***} (2.1, 3.0)	5.0 (1.76, 8.4)	10.7 (2.3, 19.1)	0.13

*** Indicates statistically significant pairwise comparisons (p<0.007) between WB-D0 (reference) and the individual time points.

⁺ Blood collected in EDTA tube and processed at time 0 (WB-D0) was used as the reference for all comparisons.

[#] Subsets of B cells, monocytes, DCs and NK cells were expressed as a percentage of monocytes, DCs and NK cells respectively.