

# 2018



Explore  
[thermofisher.com/attune](http://thermofisher.com/attune)

# Cryopreserved Whole Blood for the Quantification of Monocyte, T-Cell and NK-Cell Subsets, and Monocyte Receptor Expression by Multi-Color Flow Cytometry: A Methodological Study Based on Participants from the Canadian Longitudinal Study on Aging

Chris P. Verschoor,<sup>1,2\*</sup> Vikas Kohli<sup>1,2</sup>

<sup>1</sup>Department of Health Research Methods, Evidence and Impact, McMaster University, Hamilton, ON, Canada

<sup>2</sup>Canadian Longitudinal Study on Aging (CLSA), Hamilton, ON, Canada

Received 1 December 2017; Revised 23 January 2018; Accepted 15 March 2018

Grant sponsor: Government of Canada through the Canadian Institutes of Health Research (CIHR) (Canadian Longitudinal Study on Aging [CLSA]), Grant number: LSA 94473

Grant sponsor: Canada Foundation for Innovation

Additional supporting information may be found in the online version of this article.

\*Correspondence to: Chris Verschoor, Assistant Professor, Department of Health Research Methods, Evidence and Impact, McMaster University, 1280 Main St. W, MIP 309A, Hamilton, ON, L8S 4K1, Canada. Email: cversch@mcmaster.ca

Published online 6 April 2018 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23372

© 2018 International Society for Advancement of Cytometry

## • Abstract

Immunophenotyping by multi-color flow cytometry is arguably the best tool to identify and quantify distinct cell lineages from the peripheral blood and other biological fluids/tissues. Effective in both clinical and research settings, it can be used to estimate the frequency of a given cell type or measure its phenotypic or functional properties. Normally, immunophenotyping is performed in fresh or fractionated blood (i.e., PBMCs) the same day, or within 24 hours of collection; however, this may not be feasible for all study designs. We have previously shown that cryopreserved blood, a biospecimen that is simple and inexpensive to prepare, is comparable to fresh blood for the enumeration of major leukocyte cell types. For the following study, we sought to extend these observations to distinct subsets of: monocytes (classical, intermediate, and non-classical), T-cells (CD4/CD8 naïve, central and effector memory, senescent, and terminally differentiated, and regulatory T-cells), and NK-cells (CD56 bright and dim); we also examined the expression of monocyte cell-surface receptors CX3CR1, CCR2, TLR2, and TLR4. Our results indicate that cryopreserved blood is comparable to fresh blood; with exception to relatively rare subsets and lowly expressed receptors, the absolute or relative frequency of cell subsets generally correlated >0.80 between blood types, while monocyte receptor expressed was mostly >0.70. Furthermore, the day-to-day coefficient of variation for most cell subsets and parameters was below 20%. Given these findings, we suggest that cryopreserved peripheral blood be given greater consideration for studies in which the quantification of distinct leukocyte subsets is required. © 2018 International Society for Advancement of Cytometry

## • Key terms

flow cytometry; immunophenotyping; blood; monocytes; T-cells; natural killer cells; regulatory T-cells; Canadian Longitudinal Study on Aging (CLSA)

**THE** use of multi-color flow cytometry for the identification and quantification of distinct cell lineages, commonly known as immunophenotyping, has proven to be a powerful tool in both clinical and research settings. For instance, the quantification of normal and neoplastic plasma cells has been shown to be useful in diagnosing multiple myeloma (1), while the distribution of T-cell subsets (e.g., naïve, memory, and senescent) has been used to better understand the cause of vaccine failure in the elderly (2). This procedure relies on the use of fluorescently conjugated monoclonal antibodies that preferentially bind cell-surface or intracellular markers that are unique to a particular set of cells; for example, antibodies binding cluster of differentiation (CD)-45, CD3, CD4, and CD19 will allow for the enumeration of T-helper cells (CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>) and B-lymphocytes (CD45<sup>+</sup>CD19<sup>+</sup>) in whole or fraction-

ated peripheral blood. Immunophenotyping can also be used to examine the phenotype of a given cell subset, which may give indication of a donor's health status or susceptibility to disease. As an example, the DNA content of hematopoietic cells can be used to detect neoplastic cells in bone marrow (1), while the expression levels of cell-surface CD11b can be used to judge the activation of circulating CD15<sup>+</sup> neutrophils (3).

Normally, immunophenotyping is employed in biological fluids, although it is commonly performed in cellular homogenates derived from tissues as well (4). In both cases, freshly acquired biospecimens tend to yield the most optimal staining quality and therefore the ability to discriminate cell types. However, for certain study designs and research environments, especially in cases when sample collection and flow cytometry facilities are geographically distant, the analysis of fresh biospecimens is not feasible. In such cases, cryopreservation of biospecimens following collection is highly advantageous. Cryopreservation also provides further benefit in that biospecimens can be analyzed in large batches, minimizing overall analytical variability; this is especially important for studies in which collection occurs over a long period of time, or a large number of samples are obtained. Recently, we (5) and others (6) showed that cryopreserved peripheral blood, a biospecimen that is simple and inexpensive to prepare, is comparable to fresh blood for the enumeration of a number of leukocyte types, including monocytes, neutrophils, B-lymphocytes, T-cells, natural killer, and (NK)-cells; our study also showed that these measures were also comparable to that obtained from cryopreserved peripheral blood mononuclear cells (PBMCs) or a hematology analyzer. However, many of these cell types, especially monocytes, T-cells, and NK-cells, are known to represent distinct groups of subsets that have specific roles in maintaining health and preventing disease. For example, we have previously shown that the classical, intermediate, and non-classical subsets of monocytes (delineated by the expression of CD14 and CD16) change with age and exhibit differential cytokine responses to bacterially derived ligands (7). Others have similarly reported for T-cell (central and effector memory, naïve, senescent, and terminally differentiated and regulatory T-cells) (8), and NK-cell (CD56 bright and dim) (9) subsets. Clearly, an investigation into the performance of cryopreserved whole blood for the identification of these distinct subsets is warranted.

In the following study we measured the frequency of distinct monocyte, T-cell (including regulatory T-cells) and NK-cell subsets and the expression of monocyte cell-surface receptors (CCR2, CX3CR1, TLR2, and TLR4) using multi-color flow cytometry, comparing results obtained in fresh, and cryopreserved peripheral blood. We also compared total leukocyte measures in fresh and cryopreserved blood to that obtained from a hematology analyzer, and examined the precision of measures obtained from cryopreserved blood over multiple days. Overall, cryopreserved blood was very comparable to fresh blood: most cell frequency estimates correlated above 0.80, with exception to those subsets that were relatively rare, and monocyte receptor expression mostly correlated above 0.70, with exception to lowly expressed receptors and

receptors expressed on low-frequency subsets. Day-to-day precision was generally below a coefficient of variation (CV) of 20%, with exception to relatively rare cell subsets. This work demonstrates the utility of using cryopreserved peripheral blood for the measurement of distinct leukocyte subsets, which should be given greater consideration for studies in which cryopreservation is necessary and complex biospecimen preparation is not feasible.

## MATERIALS AND METHODS

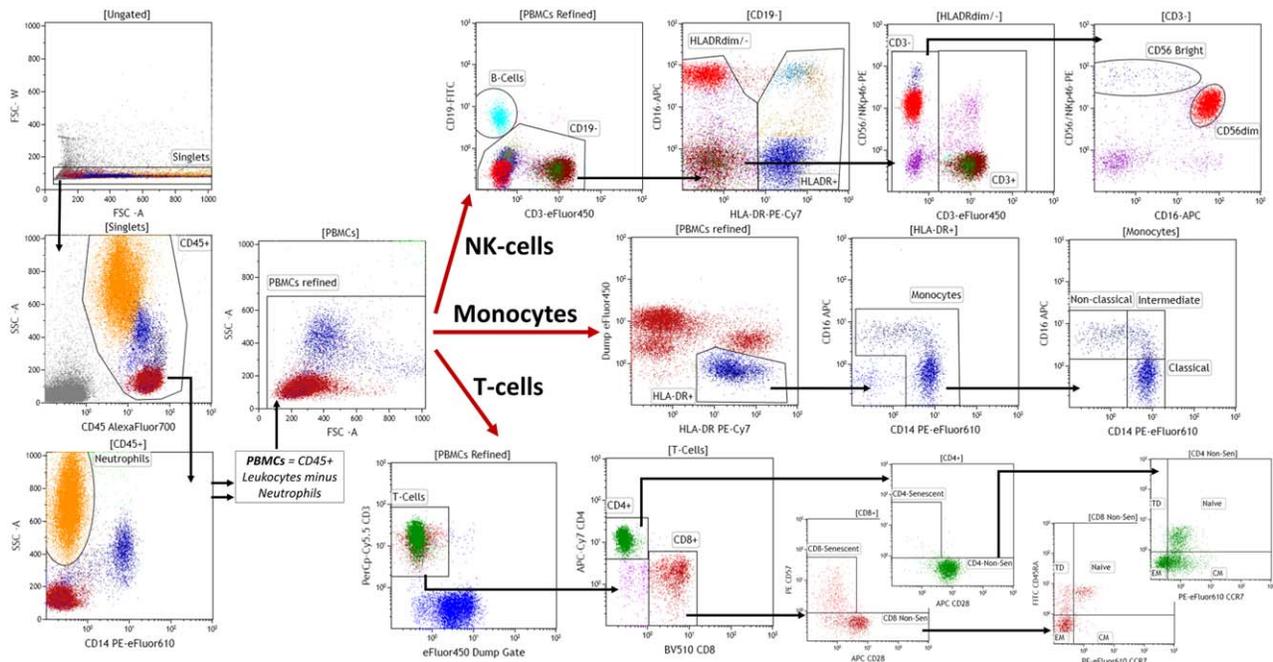
### Sample Collection and Processing

Venous peripheral blood was collected from a total of 36 participants in Hamilton, Ontario, Canada between June and July, 2017, at a data collection site for the Canadian Longitudinal Study on Aging (CLSA) (10) using a standardized collection protocol. All participants were community-dwelling adults between 45 and 85 years old, and written, informed consent for blood collection was provided. Blood employed in immunophenotyping experiments was collected in ACD (acid-citrate-dextrose) anti-coagulant vacutainers and stored at 4°C for up to 6 hours following collection, while complete blood counts were obtained from fresh EDTA (ethylenediaminetetraacetic acid) blood immediately following collection using a Coulter Ac-T diff Analyzer (Beckman Coulter, ON, CA).

ACD blood cryopreservation was performed using a standardized protocol by a trained technician within 2 hours of collection. Briefly, blood was mixed 1:1 with 20% DMSO diluted in RPMI (10% DMSO final), gently inverted, and aliquoted into 0.5 ml screw-top matrix tubes (ThermoFisher, MA, USA). Vials were then placed into a CoolCell controlled-rate freezing container (BioCision, CA, USA) and stored at -80°C for up to one month.

### Leukocyte Immunophenotyping Panels

Four cellular stains were employed in the current study to identify monocyte, T-cell, and NK-cells subsets, as well as regulatory T-cells (Tregs). A complete immunophenotyping protocol including detailed information on the antibodies used in the study and their relative concentrations for each stain can be found in Supporting Information. The monocyte stain included the following conjugated antibodies: CD45-AlexaFluor700, CD14-PE-eFluor 610, CD16-APC, CD3-eFluor450, CD15-eFluor450, CD19-eFluor450, CD56-eFluor450, Nkp46-eFluor 450, and TLR4-PE (eBioscience, CA, USA); and HLA-DR-PE-Cy7, CX3CR1-PerCP-Cy5.5, CCR2-Brilliant Violet (BV) 510, and TLR2-FITC (Biolegend, CA, USA). Monocytes were CD45<sup>+</sup>HLA-DR<sup>+</sup>CD14<sup>+</sup> and lineage (CD3, CD15, CD19, CD56, and Nkp46) negative. Classical, intermediate, and non-classical monocyte subsets were defined as CD14<sup>++</sup>CD16<sup>-</sup>, CD14<sup>++</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup>, respectively. The expression of the cell-surface receptors CX3CR1, CCR2, TLR2 and TLR4 were quantified as the mean fluorescent intensity (MFI) in each monocyte subset minus the MFI for an appropriate lineage negative mononuclear cell population control. A complete gating strategy can be found in Figure 1 and a comparison of gating in cryopreserved and fresh blood in Supporting Information Figure



**Figure 1.** Gating strategies used to identify monocyte, NK-cell and T-cell subsets in fresh and cryopreserved blood. Separate stains were used for each leukocyte subset, and the initial gating (i.e., from singlets to peripheral blood mononuclear cells [PBMCs]) is common for all gating strategies. PBMCs are derived using a Boolean gate, that is, total CD45<sup>+</sup> leukocytes minus neutrophils. For CD4 and CD8 T-cells, naïve, central (CM) and effector (EM) memory, and terminally-differentiated (TD) subsets are gated from non-senescent (non-sen) cells. FSC, front-scatter; SSC, side-scatter. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

S1. Note, this strategy may slightly underestimate (<5%) the frequency of non-classical monocytes due to overlap with neutrophils, which are removed prior to monocyte subset gating. The T-cell stain included the following antibodies: CD45-AlexaFluor700, CD3-PerCP-Cy5.5, CD28-APC, CD45RA-FITC, CCR7-PE-eFluor610, CD14-eFluor450, CD15-eFluor450, CD19-eFluor450, CD56-eFluor450, and Nkp46-eFluor450 (eBioscience, CA, USA); CD4-APC-Cy7 and CD8-BV510 (Biolegend, CA, USA); and CD57-PE (Invitrogen, CA, USA). T-cells were CD45<sup>+</sup>CD3<sup>+</sup>CD4/CD8<sup>+</sup> and lineage (CD14, CD15, CD19, CD56, and Nkp46) negative. T-cells (CD4/CD8) were further subdivided into senescent (CD57<sup>+</sup>CD28<sup>-</sup>), effector memory (CD57<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup>), central memory (CD57<sup>-</sup>CD45RA<sup>-</sup>CCR7<sup>+</sup>), naïve (CD57<sup>-</sup>CD45RA<sup>+</sup>CCR7<sup>+</sup>), and terminally differentiated (CD57<sup>-</sup>CD45RA<sup>+</sup>CCR7<sup>-</sup>). A complete gating strategy can be found in Figure 1 and a comparison of gating in cryopreserved and fresh blood in Supporting Information Figure S2. NK-cell subsets were identified using our previously published comprehensive stain (5), but replacing CD123-AlexaFluor647 with CD16-APC (Biolegend, CA, USA). NK-cells were CD45<sup>+</sup>CD19<sup>-</sup>HLA-DR<sup>-</sup>CD3<sup>-</sup>, and further divided as CD56 Bright and Dim subsets: CD56/NKp46<sup>++</sup>CD16<sup>lo/-</sup> and CD56/NKp46<sup>+</sup>CD16<sup>+</sup>, respectively. As with our previous stain, antibodies for the NK-cell markers CD56 and NKp46 were both conjugated to PE. A complete gating strategy can be found in Figure 1 and a comparison of gating in cryopreserved and fresh blood in Supporting Information Figure S3. Tregs were measured using the Human Regulatory T Cell Staining Kit (eBioscience, CA, USA), which includes conjugated antibodies for

CD4-FITC, CD25-PE, and FoxP3-APC, and a fixable viability dye (eFluor506); we also included CD45-AlexaFluor700 and CD3-eFluor450 in the surface stain. Tregs were viable (eFluor506 negative), positive for CD45, CD3, CD4, CD25 and FoxP3, and low granularity (side-scatter, SSC). A compensation matrix was generated for each stain using OneComp eBeads (eBioscience, CA, USA), and cells are presented as absolute counts (x10<sup>9</sup>/L; see below for our leukocyte count approach), relative to peripheral blood mononuclear cells (PBMCs; low granularity CD45<sup>+</sup> cells without neutrophils), or relative to their respective class (i.e., total monocytes, or CD4/CD8 T-cells).

**Leukocyte Immunophenotyping Procedures**

Cellular staining to identify monocyte, T-cell, and NK-cell subsets was performed as in our previous study (5) and is described in detail in the Supporting Information. Cryopreserved blood was moved to ice from -80°C storage, left for up to 20 minutes until completely thawed and gently inverted three times to mix. A 100 µl of fresh or cryopreserved blood was transferred to a 2 ml snap-top tube and mixed with a 50 µl cocktail of the aforementioned antibodies suspended in FACS Wash (0.5% BSA, 2 mM EDTA, and PBS), and incubated for 30 minutes at room temperature in the dark. Up to 2 ml of 1x Fix/Lyse solution (eBioscience, CA, USA) was then added, followed by inversion and an additional 10 min incubation at room temperature in the dark. Samples were centrifuged at 600 ×g for 5 minutes, supernatants removed, and pellets resuspended in 1 ml chilled FACS Wash. Centrifugation was repeated, supernatants removed, and pellets resuspended

in 250  $\mu$ l of FACSWash. Samples were analyzed immediately using a 10-color Beckman Coulter Gallios flow cytometer (Beckman Coulter, ON, CA), and subsequent gating using Kaluza Analysis v1.3 (Beckman Coulter, ON, CA). The frequency of Tregs were determined in 100  $\mu$ l of cryopreserved or fresh blood using the Human Regulatory T Cell Staining Kit (eBioscience, CA, USA), according to manufacturer's recommendations.

To estimate the absolute count of leukocyte subsets (as opposed to the relative proportions of each subset), we first measured the absolute number of PBMCs in each blood sample using a slightly different cellular staining procedure that does not include centrifugation steps; this procedure and example calculations are provided in Supporting Information. Not including centrifugation steps is important since a significant proportion of granulocytic (neutrophils) cells in cryopreserved blood are lost during centrifugation (5) leading to artificially reduced estimates. The rationale for basing absolute counts on PBMCs, not total leukocytes, is for that very same reason. Proportional estimates of a given leukocyte subset relative to total CD45 expressing cells, as in the staining procedure described above, may be inflated since a significant number of granulocytic cells are lost with centrifugation. Hence, absolute estimates of that leukocyte subset (calculated as the relative proportion multiplied by the total leukocyte count) would also be inflated. Proportional estimates relative to PBMCs would be expected to be more accurate given the reduced cellular loss in the PBMC compartment (as we have shown (5)), which would relate to better accuracy of absolute estimates. To enumerate total PBMCs, cryopreserved blood was thawed on ice for 20 minutes and 100  $\mu$ l was transferred to a 2.0 ml snap-top tube. CD45-PE (eBioscience, CA, USA), diluted 1/50 in 50  $\mu$ l of FACSWash, was added and allowed to incubate in the dark at room temperature for 30 minutes. Afterward, 500  $\mu$ l of 1x Fix/Lyse solution was added, followed by inversion and incubation in the dark at room temperature for 10 minutes. Approximately 25,000 123count eBeads (eBioscience, CA, USA) were then added, followed by inversion and analyzed immediately as above. Total PBMCs were characterized as SSC-low, CD45 expressing leukocytes, while beads were identified as being highly fluorescent in both the FL2 (i.e., PE) and FL10 (i.e., BV510) channels. We also obtained total leukocyte (WBC) counts (i.e., total CD45 expressing leukocytes) in order to compare this approach to a hematology analyzer. The absolute count of PBMCs was calculated as the number of PBMC events divided by the number of bead events, multiplied by the number of beads added ( $\sim$ 25,000) and the dilution factor (amount of blood added relative to 1 ml; 20 for cryopreserved blood in the current procedure). The absolute count of a given leukocyte subset was therefore the frequency of that subset relative to the number of PBMCs multiplied by the total PBMC count.

### Statistics

All statistics were performed in R version 3.2.3 (R Foundation for Statistical Computing). Cell frequencies are compared by Spearman's Rank Correlation and test, and in the

Supporting Information, presented as median (25th–75th percentile), and compared using the Wilcoxon Signed Rank test. Bias was calculated using the R package “BlandAltmanLeh.” The difference in slopes for comparisons of total leukocyte counts by flow cytometry (fresh and cryopreserved blood) to the hematology analyzer was tested using a mixed effects model, fitting an interaction between the fixed effects blood type and flow cytometry estimate, and participant as a random effect. For precision experiments, cryopreserved blood from six donors were analyzed on three different days. The average coefficient of variation (CV) for each day was calculated as the root mean squared; that is, square root of the sum of squared CVs for each sample, divided by the total number of samples. Change across days was tested by repeated measures ANOVA.

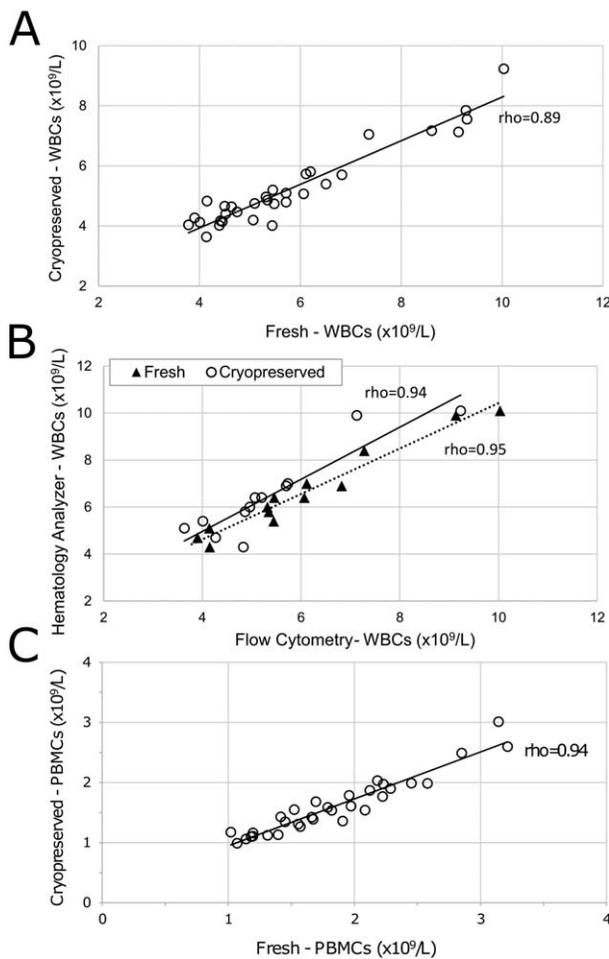
## RESULTS

### Comparing Total Leukocyte Counts Derived from Fresh and Cryopreserved Blood to That Obtained from a Hematology Analyzer

In our initial study, we found that enumerating total leukocytes (CD45<sup>+</sup>) in fresh or cryopreserved blood using a procedure that did not include centrifugation based wash steps was optimal; however, this was only conducted in a small number of samples. To replicate these findings, we compared the leukocyte counts obtained using this approach in fresh and cryopreserved blood from 32 donors, and validated these findings in a subset of donors in which complete blood counts from a hematology analyzer was also available. As previously reported by us and others (5,6), the total leukocyte counts obtained using flow cytometry in fresh and cryopreserved was highly correlative ( $\rho = 0.89$ ,  $P < 0.001$ ), with only slightly lower estimation in cryopreserved blood (%bias = 10.2%) (Fig. 2A). We also found that estimates in blood analyzed by flow cytometry was highly correlative to and only slightly underestimated that obtained from a hematology analyzer: fresh,  $\rho = 0.95$  ( $n = 13$ ,  $P < 0.001$ ), %bias = 8.7%; cryopreserved,  $\rho = 0.94$  ( $n = 12$ ,  $P < 0.001$ ), %bias = 18.7% (Fig. 2B). We tested the difference in slopes for these comparisons using a mixed effects model and found that it was not significant (fresh-cryopreserved =  $-0.181$ ,  $P = 0.17$ ). Given that a substantial proportion of granulocytic cells (neutrophils) in cryopreserved blood may be lost following centrifugation, as we have previously shown (5), our estimates of the absolute count of leukocyte subsets (below) is calculated using the total PBMC count, not total leukocyte. As expected, the absolute count of PBMCs is also highly correlative between fresh and cryopreserved blood ( $\rho = 0.94$ ,  $P < 0.001$ ; Fig. 2C).

### Monocyte and NK-Cell Subset Determination in Cryopreserved and Fresh Blood

We used two separate stains to discriminate classical (CD14<sup>+</sup>CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>CD16<sup>+</sup>) and non-classical (CD14<sup>+</sup>CD16<sup>+</sup>) monocyte subsets, and CD56 bright (CD56/NKp46<sup>+</sup>CD16<sup>lo/-</sup>) and dim (CD56/NKp46<sup>+</sup>CD16<sup>+</sup>) NK-cell subsets in peripheral blood from 26 donors (Fig. 1). Both stains performed similarly in either fresh or



**Figure 2.** Absolute frequency of total white blood cells (WBCs) and peripheral blood mononuclear cells (PBMCs) in whole blood as determined by flow cytometry and hematology analyzer. (A) CD45 expressing leukocytes were measured in cryopreserved and fresh whole blood and compared using Spearman's correlation test ( $n = 32$ ,  $P < 0.001$ ). (B) The number of CD45 expressing leukocytes measured in cryopreserved ( $n = 12$ ) and fresh ( $n = 13$ ) whole blood were compared to total leukocyte counts obtained from a hematology analyzer as in panel A ( $P < 0.001$  for both comparisons). (C) PBMCs (CD45 expressing leukocytes minus granulocytic cells) were measured in cryopreserved and fresh whole blood as in A, and compared using Spearman's correlation test ( $n = 32$ ,  $P < 0.001$ ). Rho indicates the degree of correlation by Spearman correlation and cell counts are presented as  $\times 10^9$  per liter.

cryopreserved blood, with little qualitative differences with regards to fluorescent intensity of each marker (Supporting Information Figs. S1 and S3). However, it was apparent that the detected intensity (MFI) of the cell-surface receptors CCR2, CX3CR1, and TLR4 on each monocyte subset was significantly lower in cryopreserved blood, while TLR2 was significantly higher (Fig. 3; Supporting Information Table S1). That being said, each receptor, with exception to TLR4, correlated significantly between fresh and cryopreserved blood for each subset considered (rho: max = 0.84, min = 0.49). For cell frequency estimates, we compared measures as absolute cell

counts, relative to PBMCs, and for monocyte subsets, relative to total monocytes. The differences in estimates for both monocyte and NK-cell subsets were small between fresh and cryopreserved blood (Fig. 3; Supporting Information Table S1), and all measures correlated significantly between blood preparations types, with little difference in magnitude when considering different frequency units (i.e., absolute counts vs. relative to PBMCs vs. relative to total monocytes). Monocyte subset frequency correlations ranged from 0.78 and 0.95 (with exception to intermediate monocytes relative to PBMCs, 0.54), while NK-cell subset frequencies ranged from 0.68 to 0.96.

### T-Cell Subset Measurements between Different Blood Preparations

For the determination of T-cell frequency, we employed a comprehensive stain that allowed us to discriminate senescent ( $CD57^+CD28^-$ ), effector memory ( $CD57^+CD45RA^-CCR7^-$ ), central memory ( $CD57^-CD45RA^-CCR7^+$ ), naive ( $CD57^-CD45RA^+CCR7^+$ ), and terminally differentiated ( $CD57^-CD45RA^+CCR7^-$ ) cells of either CD4 or CD8 lineage (Fig. 1). Unlike the previous two stains, estimates of T-cell subset frequency tended to be higher in fresh blood (Fig. 4; Supporting Information Table S2). Similarly, however, measurements correlated significantly between blood preparations and did not appear to vary based on the frequency unit considered. Correlations ranged from 0.72 to 0.99, with exception to terminally differentiated  $CD4^+$  T-cells, which correlated 0.64 for absolute counts and frequency relative to PBMCs, respectively (Fig. 4; Supporting Information Table S2).

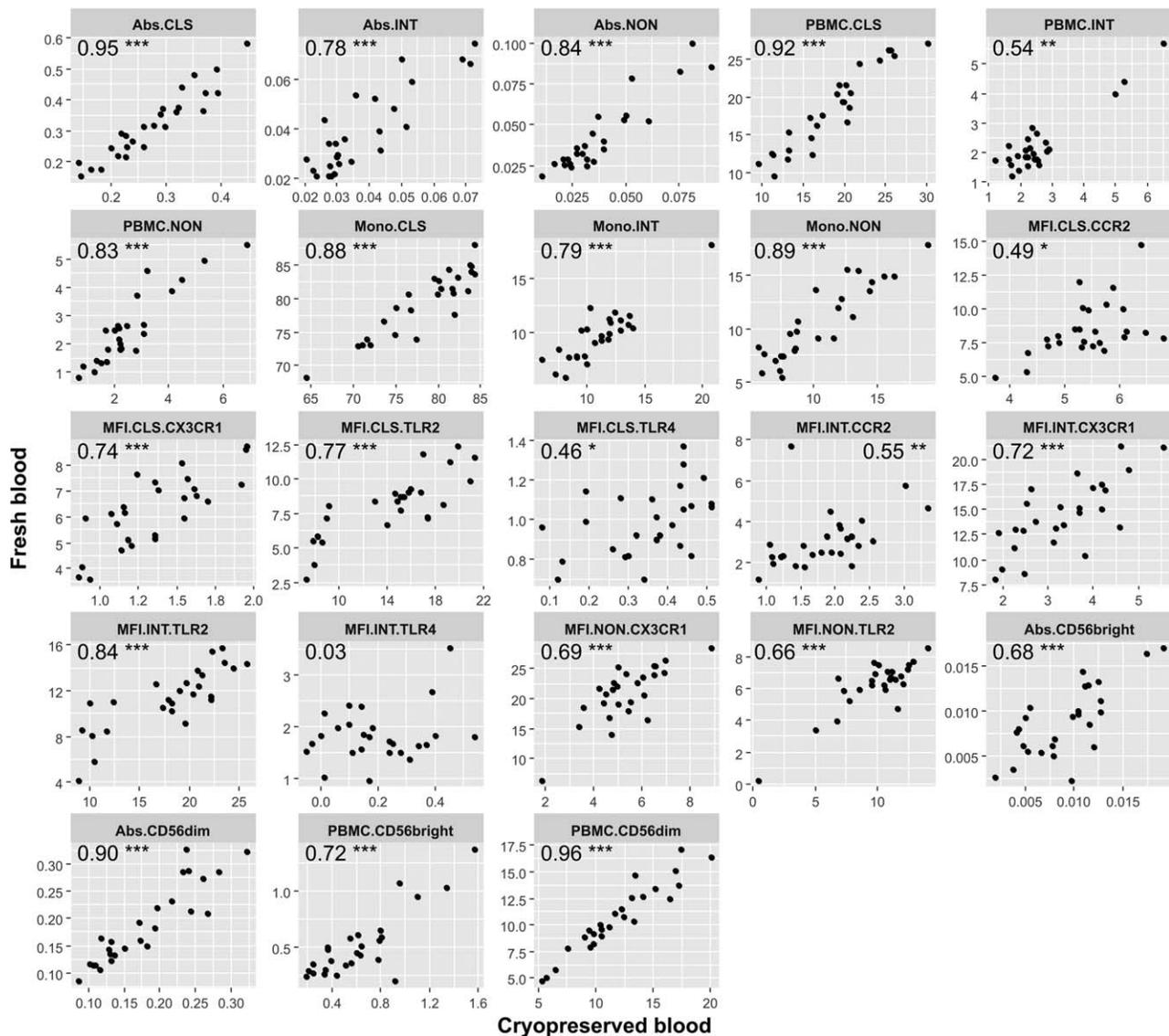
We also had the opportunity to compare fresh and cryopreserved blood for the enumeration of  $CD4^+CD25^+FoxP3^+$  regulatory T-cells (Tregs) in a small subset of donors ( $n = 5$ ). The frequency of Tregs relative to  $CD4^+$  T-cells correlated strongly (rho = 0.90,  $P = 0.037$ ) between the blood preparations (data not shown).

### Interassay Variability for Monocyte, NK-Cell, and T-Cell Subset Stains in Cryopreserved Blood

To determine the interassay variability of our subset stains in cryopreserved blood, we performed each in blood from six randomly chosen donors on three separate days (Supporting Information Table S3). Frequency and monocyte receptor expression estimates were generally not significantly different day-to-day, and the calculated average coefficient of variation (CV) was for the most part  $<20\%$  for all measures. Senescent and terminally differentiated  $CD4^+$  T-cells tended to exhibit higher CVs (between 26 and 41%), which we attribute to their relatively low frequency ( $<2.5\%$  of the  $CD4$  T-cell pool) (Supporting Information Table S3).

### DISCUSSION

The primary goal for this study was to compare the frequency of distinct monocyte, T-cell, and NK-cell subsets, and the cell-surface expression of monocyte receptors between fresh and cryopreserved peripheral blood. Our choice of parameters was based on stains we previously employed to look at the relationship of monocyte (7) and T-cell subsets (2)



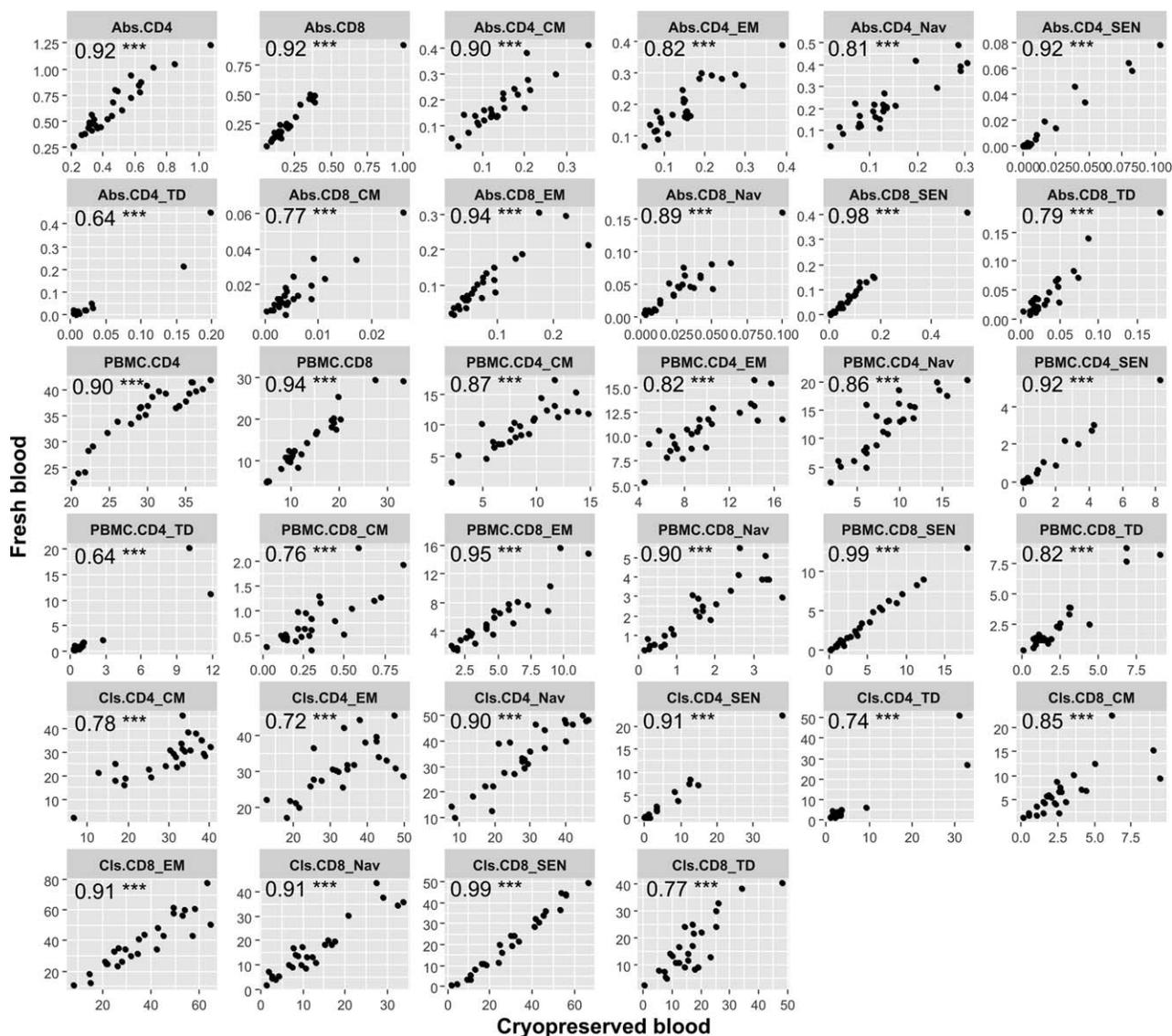
**Figure 3.** Comparison of monocyte and NK-cell subset measures in fresh and cryopreserved blood. Spearman's correlation (Rho) and significance presented in the upper portion of each plot; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . Plot titles indicate the unit (Abs, absolute count; PBMC, % relative to PBMCs; Mono, % relative to total monocytes; MFI, mean fluorescent intensity), cell subset (monocytes: CLS, classical; INT, intermediate; NON, non-classical; NK-cells: CD56bright and CD56dim), and for MFIs, the receptor quantified on each monocyte subset (i.e., CCR2, CX3CR1, TLR2, TLR4). Note: CCR2 and TLR4 on non-classical monocytes not presented due to low/below background expression (as shown in [17]).

with health-related parameters in the elderly; similar studies have been performed for the NK-cell subsets we examined here (11).

In our previous study (5), the vast majority of frequency estimates as absolute counts were derived using a procedure that included cell washing (centrifugation, followed by pellet resuspension) after the addition of lysis buffer. While we were able to determine that this approach was not as accurate as a wash-free approach (flow cytometry analysis immediately following red blood cell lysis) in a small number of replicate samples, we nonetheless sought to confirm these findings in the current study. Here, we show that not only is the estimation of total leukocyte and PBMC counts using the wash-free

approach highly correlative between fresh and cryopreserved blood, both flow cytometric measurements correlate strongly ( $\rho > 0.90$ ) with results obtained from a hematological analyzer, a standard and reliable method for leukocyte counting. Although cryopreserved blood does appear to underestimate total leukocyte counts, which is likely due to the reduced viability and subsequent loss of neutrophils during the cryopreservation and staining procedure (5), the bias is only 10% less than fresh blood and 19% less than the hematology analyzer.

For the enumeration of monocyte, T-cell, and NK-cell subsets, we compared results obtained from fresh and cryopreserved blood as absolute counts and relative to PBMCs or the parental lineage (i.e., total monocytes, or CD4/CD8 T-



**Figure 4.** Comparison of T-cell subset measures in fresh and cryopreserved blood. Spearman's correlation (Rho) and significance presented in the upper portion of each plot; \*\*\*,  $P < 0.001$ . Plot titles indicate the unit (Abs, absolute count; PBMC, % relative to PBMCs; Cls, % relative to parental T-cell class [i.e., CD4 or CD8]); MFI, mean fluorescent intensity; CM, central memory; EM, effector memory; Nav, naïve; SEN, senescent; TD, terminally differentiated).

cells). Interestingly, the unit of measurement appeared to have little effect on the magnitude of correlation between blood types, which was significant at  $P < 0.001$  for nearly all measures. We also found that there was little qualitative difference in staining between blood types, although slightly reduced marker expression was commonly observed for cryopreserved blood. For all subset frequency estimates, correlations were generally above 0.80, with exception to those subsets that are relatively rare; for example, CD56 bright NK-cells, and terminally differentiated CD4 T-cells, both of which representing  $<1\%$  of PBMCs, exhibited correlations between 0.64 and 0.75. Terminally differentiated CD4 T-cells and senescent CD4 T-cells, which actually correlated  $>0.90$ , exhibited the poorest day-to-day precision in cryopreserved blood, between 26 and 41% CV, whereas nearly all other

measures were below 20% CV. This is not substantially different from the between-run precision estimates for the Coulter Act Diff hematology analyzer, which is generally below 15% for measurements of different white blood cell types at a variety of levels (12), and is within the previously recommended benchmark of 25% for immunophenotyping experiments (13). Not surprisingly, the greatest differences were also observed for the relatively rare cell types, namely CD56 bright NK-cells, and senescent and terminally differentiated CD4 T-cells. The expression of cell-surface receptors on monocyte subsets also correlated significantly, with exception to TLR4 on intermediate and non-classical monocytes (TLR4 on classical monocytes was significant but correlated poorly, 0.46); this is most likely due to the inherently low expression of TLR4 on monocytes (14–16). It was noted

that differences were the greatest and most varied in comparisons of monocyte receptor expression, and MFIs were generally higher in fresh blood, although the opposite was observed for TLR2. For this reason and the generally lower correlations observed, we caution, but do not completely discount, the use of cryopreserved blood for the estimation of monocyte receptor expression.

In addition to the analyses described above, we also had the opportunity to produce preliminary data regarding the performance of regulatory T-cell (Treg) staining in cryopreserved blood. The methodological approach to identify Tregs differs from most other subsets in that it requires antibodies specific for the transcription factor FoxP3, which resides in the nucleus, and hence, requires cells to be fixed and permeabilized following staining for cell-surface markers. We found cryopreserved blood to correlate significantly with fresh blood for the enumeration of Tregs ( $\rho = 0.90$ ,  $P = 0.037$ ), however, given our small sample size ( $n = 5$ ), we suggest caution until confirmatory data is available.

In summary, our results indicate that cryopreserved blood is a suitable biospecimen for the enumeration of monocyte, T-cell, and NK-cell subsets and quantification of monocyte cell-surface receptor expression. For most parameters it correlates significantly with fresh blood and exhibits CVs below 20% when measured over multiple days. Given that it is simple and relatively inexpensive to prepare, researchers should give consideration to this biospecimen for studies in which cryopreservation is necessary and/or the minimization of batch-related variability is important.

#### ACKNOWLEDGMENTS

We would like to thank Chetna Naik and data collection site technicians for their assistance in study organization and the preparation cryopreserved blood. This research was made possible using the data/biospecimens collected by the Canadian Longitudinal Study on Aging (CLSA). The CLSA is led by Drs. Parminder Raina, Christina Wolfson and Susan Kirkland.

#### LITERATURE CITED

1. Kumar S, Paiva B, Anderson KC, Durie B, Landgren O, Moreau P, Munshi N, Lonial S, Bladé J, Mateos M-V, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol* 2016;17:e328–e346.
2. Verschoor CP, Lelic A, Parsons R, Eveleigh C, Bramson JL, Johnstone J, Loeb MB, Bowdish DME. Serum C-reactive protein and congestive heart failure as significant predictors of herpes zoster vaccine response in elderly nursing home residents. *J Infect Dis* 2017;216:191–197.
3. Verschoor CP, Loukov D, Naidoo A, Puchta A, Johnstone J, Millar J, Lelic A, Novakowski KE, Dorrington MG, Loeb M, et al. Circulating TNF and mitochondrial DNA are major determinants of neutrophil phenotype in the advanced-age, frail elderly. *Mol Immunol* 2015;65:148–156.
4. Chu DK, Jimenez-Saiz R, Verschoor CP, Walker TD, Goncharova S, Llop-Guevara A, Shen P, Gordon ME, Barra NG, Bassett JD, et al. Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. *J Exp Med* 2014;211:1657–1672.
5. Verschoor CP, Kohli V, Balion C. A comprehensive assessment of immunophenotyping performed in cryopreserved peripheral whole blood. *Cytometry B Clin Cytom*. doi:10.1002/cyto.b.21526.
6. Langenskiöld C, Mellgren K, Abrahamsson J, Bemark M. Determination of blood cell subtype concentrations from frozen whole blood samples using TruCount beads. *Cytometry B Clin Cytom*. doi:10.1002/cyto.b.21390.
7. Verschoor CP, Johnstone J, Millar J, Parsons R, Lelic A, Loeb M, Bramson JL, Bowdish DME. Alterations to the frequency and function of peripheral blood monocytes and associations with chronic disease in the advanced-age, frail elderly. *PLoS One* 2014;9:e104522.
8. Johnstone J, Millar J, Lelic A, Verschoor CP, Walter SD, Devereaux PJ, Bramson J, Loeb M. Immunosenescence in the nursing home elderly. *BMC Geriatr* 2014; 14:50.
9. Miyagi T, Shimizu S, Tatsumi T, Nishio K, Hiramatsu N, Kanto T, Hayashi N, Takehara T. Differential alteration of CD56(bright) and CD56 (dim) natural killer cells in frequency, phenotype, and cytokine response in chronic hepatitis C virus infection. *J Gastroenterol* 2011;46:1020–1030.
10. Raina PS, Wolfson C, Kirkland SA, Griffith LE, Oremus M, Patterson C, Tuokko H, Penning M, Balion CM, Hogan D, et al. The Canadian longitudinal study on aging (CLSA). *Can J Aging Rev Can Vieil* 2009;28:221–229.
11. Al-Attar A, Presnell SR, Peterson CA, Thomas DT, Lutz CT. The effect of sex on immune cells in healthy aging: Elderly women have more robust natural killer lymphocytes than do elderly men. *Mech Ageing Dev* 2016;156:25–33.
12. Persaud M, Clancy T, Sheridan B, Quinsey P. An Evaluation of the AcT 5diff Cap Pierce Hematology Analyzer. *Lab Hematol* 2001;7:130–139.
13. O'Hara DM, Xu Y, Liang Z, Reddy MP, Wu DY, Litwin V. Recommendations for the validation of flow cytometric testing during drug development: II assays. *J Immunol Methods* 2011;363:120–134.
14. Sabroe I, Jones EC, Usher LR, Whyte MKB, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: A critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol* 2002;168:4701–4710.
15. Guzzo C, Ayer A, Basta S, Banfield BW, Gee K. IL-27 Enhances LPS-induced proinflammatory cytokine production via upregulation of TLR4 expression and signaling in human monocytes. *J Immunol* 2012;188:864–873.
16. Juarez E, Nuñez C, Sada E, Ellner JJ, Schwander SK, Torres M. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir Res* 2010;11:2.
17. Cros J, Cagnard N, Woollard K, Patey N, Zhang S-Y, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 2010;33:375–386.