

RESEARCH ARTICLE

Comparing PBMC isolation approaches and assessing epigenetic immune cell quantification as quality control for PBMCs

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OPEN ACCESS

Citation: Heller L, Janack I, Schildknecht K, Baron U, Olek S (2026) Comparing PBMC isolation approaches and assessing epigenetic immune cell quantification as quality control for PBMCs. PLoS One 21(3): e0341547. <https://doi.org/10.1371/journal.pone.0341547>

Editor: Suprabhat Mukherjee, Kazi Nazrul University, INDIA

Received: June 12, 2025

Accepted: January 8, 2026

Published: March 11, 2026

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Data availability statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Funding: The author(s) received no specific funding for this work.

Abstract

Ex vivo stability of whole blood is limited. To conserve the mononuclear cell fraction, PBMCs are isolated, stored and serve as substrate for downstream analyses. The quality of PBMCs depends on the purification procedure. Thereby, anti-coagulants, collection tubes, storage temperature and time before isolation, as well as the separation matrix and protocols during purification may impact quality of the resulting PBMC product. However, only a limited number of key quality indicators (KPIs) are currently used to characterize PBMC isolates focusing on viability and overall cell count. Here, the effect of the relevant purification parameters on those KPIs was assessed, and epigenetic immune cell analysis was introduced to provide an additional performance indicator. This method was also compared to flow cytometric analysis, currently the gold standard tool for immune cell quantification. Sample quality differed significantly depending on tube type/anticoagulant, pre-purification temperature and times. The choice of the purification procedure, including the separation matrix, had a minor effect and freshly processed blood yielded good processing results independent of the protocol. Unsurprisingly, the loss of sample integrity that occurred prior to purification cannot be rescued by downstream isolation protocols. The longer the time from blood draw to PBMC purification, the more granulocyte contamination was observed impairing PBMC purity. With given tubes and temperatures, PBMC quality is chiefly dependent on time-to-process, arguably the parameter most difficult to control in sample logistics and clinical trials. The often-used key performance indicators focusing only on cell viability and recovery are of limited value, as they may be falsified by potential granulocyte contamination. Thus, as additional quality parameter, we propose "true recovery", i.e., recovery of lymphocytes and monocytes only. These subfractions can be measured easily by qPCR-based epigenetic immune cell counting. Such an approach may provide better comparability across sample analyses in clinical trials.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Quantitative, qualitative and functional analyses of immune cells constitute important parameters in medical research and in clinical trials. Current key technologies for this type of investigation are dependent on physiologically intact cells. In whole blood outside the body, however, stability and viability of leukocytes is limited. Therefore, it is key to ensure that biological properties of leukocytes are not affected by post-physiological influences. To protect the integrity of cells from peripheral blood *ex vivo*, the mononuclear cell fraction (PBMC) is isolated, usually by density gradient centrifugation. PBMCs are retrieved as low-density layers separated from denser structures such as granulocytes, cell debris and red blood cells as well as from plasma. Washed and resuspended in suitable matrices, PBMCs can be frozen and stored in liquid nitrogen preserving their integrity and viability. After thawing, such PBMCs often serve as substrates for biomedical research and data generation.

A significant source of inconclusive laboratory data may be resulting from differences in sample handling. To better understand and control the quality of generated PBMCs, key (purification) performance indicators (KPIs) have been established.

The frequently assessed KPIs for PBMC purification include cell viability and count [1], but their restricted significance is also accepted. Viability is rarely under 90%, which marks a standard acceptance limit. Beyond this, differences in viability carry little information. The cell count is a blurred boundary due to varying original cell numbers and an unknown granulocyte contamination, which artificially increases the apparent yield of mononuclear cells. A low number of PBMCs may well indicate problems with the separation method or the cell integrity but may also just report a low leukocyte count in the original blood sample.

Granulocytes can undergo morphological changes in anticoagulated whole blood over time [2]. Eventually, such low-density granulocytes (LDGs) resemble mononuclear cells, rendering their separation from PBMCs incomplete. As a result, purification of longer stored samples leads to significantly higher granulocyte contamination. A higher cell count after purification may therefore either indicate a high original PBMC count or result from cell purification from aged blood. Moreover, LDGs have previously been associated with T cell inhibition and may thus interfere with downstream cell-based assays [3].

Cell viability and count are only unprecise indicators of the quality of the technical process. Downstream sample analysis may yield different results not owing to biological causes but to heterogeneous sample purifications. It is therefore important to further investigate and better understand the impact of various technical parameters during the time between blood draw and PBMC preparation and possibly minimize or mediate the observed effects.

Immediate PBMC purification from fresh blood is usually not feasible in multicentric clinical trials. Therefore, time-to-processing is a key parameter as it impacts the quality of a blood sample. Minimizing *ex vivo* exposure time is always preferable since it determines the extent of all exogenous influences. At the same time, this is not easy to control. The temperature, at which whole blood samples are kept prior to the PBMC preparation, also affects the quality of the PBMC product. Collection tubes

containing varying anticoagulants and stabilizing buffers as well as all steps taken during different purification methods also influence the resulting cell product quality. The purification protocol itself, however, may allow better or worse separation between different cell fractions, but can only be as good as the prior blood treatment allows.

With limited quality assessment provided by viability and cell count alone, PBMC preparations may benefit from further tests to verify their utility in sensitive downstream assays. However, understanding influences on the sample processing does not improve the quality of existing samples or necessarily allow ways to optimize the production in future. The main benefit of novel or extended analytical means would therefore be their incorporation in experimental design and data interpretation.

Here, we introduce epigenetic immune cell counting [4,5] as a quantitative method to provide further insight into the quality of a PBMC sample. The method allows “freezing” the immune cell count at blood draw unaffected by downstream handling. It also permits analysis of the exact same parameters after PBMC purification. This is feasible as epigenetic immune cell counting is a DNA-based technology and applies to a wide variety of different substrates and conditions, such as fresh, frozen or dried whole blood, tissues and isolated cells including PBMCs. Further, DNA stability ascertains unchanged information when DNA is directly isolated from whole fresh blood [6]. Most apparently, granulocyte content for fresh whole blood and contamination of LDGs after PBMC purification can be identified and quantified. On top of this, the analysis of various cell lineage markers (for T, B, and NK cells) can provide information about the general immune cell status.

Epigenetic immune cell quantification relies on cell type-specific differential DNA methylation. In specific genomic loci, DNA is uniquely unmethylated in one cell type, whereas all other cells are methylated in the same locus. Under the assumption that each cell contains a given number of gene copies, such marker regions can be methylation-specifically amplified and the cell count can be deduced from that information. For this, the genomic DNA in the sample needs to be treated with bisulfite, which alters unmethylated cytosines to uracil whereas methylated cytosines remain unchanged. This way, epigenetic, i.e., DNA methylation marks are translated into sequence information. Since uracil base pairs with adenine whereas cytosine with guanine, qPCR reactions are specific to the genomic methylation status and report the number of unmethylated or methylated DNA copies [7].

For the markers used in this study, i.e., CD3, CD4, CD8, OSBL5 (NK cells), LRP5 (B cells), LRKK1/LCN1 (granulocytes) and PARK2 (monocytes), we have previously shown high concordance between analysis with flow cytometric and epigenetic (DNA methylation) analysis in fresh frozen and dried blood samples [7,8].

This study aims at identifying critical steps and describing the ideal processes between blood collection, cell preservation and subsequent use of processed PBMCs. For this, various tube types, temperatures and times-to-processing were monitored. Moreover, different purification processes were compared, focusing on potential differences in the quality of the PBMC products. Acknowledging the limited information for PBMC quality obtained from standard performance indicators of cell viability and count, the cellular composition was analyzed chiefly by epigenetic immune cell counting.

In summary, the quantitative assessment of granulocyte contaminations provides an important, easy-to-measure parameter to define the quality of PBMC isolates. This information allows to amend the determined standard recovery rate, and we therefore propose the term “true recovery” as a meaningful KPI, which now defines the recovery of mononuclear cells only.

Materials and methods

Blood samples

Blood was collected from voluntary, clinically unsuspecting donors between 07/01/2022 and 06/08/2024 and accessed for research purposes on 08/08/2024. Donors consented in writing (ethical commission approval A2/028/13) and blood collection was done using vacutainers with EDTA or Sodium Heparin as anti-coagulants (Becton Dickinson). It was stored at room temperature (RT), 4°C or 37°C for up to 96 hours.

PBMC isolation, cell counting

SepMate: 6 ml whole blood were mixed with 7 ml PBS and added into SepMate-tubes, pre-filled with 15 ml Lymphoprep (Stemcell) as density gradient medium and centrifuged at 1200 x g for 15 minutes. The PBMC layer was carefully extracted, diluted with PBS to yield a total volume of 50 ml and centrifuged at 500g for 10 minutes. The supernatant was removed, and the cell pellet was washed with 50 ml PBS. Finally, the cells were resuspended in 2 ml to 5 ml medium (depending on pellet size) (Gibco RPMI 1640, Fisher Scientific) containing 5.6% human serum albumin (HSA, Octapharma). Cells were counted as described below. Ficoll overlay: 6 ml whole blood was mixed with 7 ml PBS and layered on 15 ml Ficoll-Paque PLUS (VWR) medium in pre-filled 50 ml Falcon tubes. Gradient centrifugation was performed at 740 x g for 30 minutes with centrifuge breaks switched off. The PBMC layer was carefully removed and diluted with wash medium (i.e., RPMI-1640 plus L-Glutamine (Fisher Scientific) with a final concentration of 1 x Ficoll Overlay-Wash-Supplement (CTL Europe)) to a total volume of 50 ml in a Falcon tube followed by centrifugation at 330g for 10 minutes. The cell pellet was washed once in 50 ml wash medium, and the cells were resuspended in a pellet-dependent volume between 2- and 5-ml wash medium. Cells were counted as described below.

Cell counting

Counting of cells was performed using 20 μ l of cell suspension mixed with 20 μ l acridine orange/ propidium iodide stain (AO/PI, Cenibra) in a Nexcelom BioScience Cellometer (Spectrum-101–0021).

Antibody-based cell staining and flow cytometry

500,000 freshly isolated PBMCs were pelleted and resuspended in 50 μ l PBS. For whole blood, 50 μ l sample were stained with 1 μ l human anti-CD45 antibody (BUV395-conjugated, 1:100 diluted, BD Bioscience) and 0.4 μ l live/dead dye (Invitrogen, 1:250 diluted) in 48.6 μ l PBS for 15 minutes at RT. Following a washing step with 4 ml PBS (500g, 5 min), 500 μ l FACS Lysis Buffer (prewarmed at 37°C; Fisher Scientific) were added and incubated overnight at 4°C. Then, samples were centrifuged (500g, 5 min., RT), resuspended in 200 μ l PBS and acquired on a LSR-Fortessa X-20 (BD Bioscience) and analyzed using FlowJo (version 10.8.1, BD Bioscience).

Cell lysis, automated direct bisulfite conversion and epigenetic qPCR

75 μ l of whole blood or 500,000 PBMCs in PBS (75 μ l) were thoroughly mixed with 49 μ l lysis binding buffer (Invitrogen), 18 μ l Proteinase K (Sigma-Aldrich) and gently shaken (900rpm) at 56°C for 60 min. Then, 270 μ l ammonium bisulfite solution (68–72%; TIB Chemicals) and 90 μ l tetrahydrofurfuryl alcohol (Merck) were added and incubated for 55 min at 80°C. Converted DNA was isolated with Dynabeads (Invitrogen) following manufacturer's recommendations. For qPCR set up, samples were pipetted as triplicates and cycling was performed in a Roche LightCycler 480 II following previously published protocols and using the according primers and probes [6,7]. Percentages of specific cell populations were calculated, and quality control was conducted according to reference literature [6].

Statistical analysis

All calculations were done using GraphPad Prism 10.5.0. The nonparametric Mann Whitney U test or, for paired samples, the Wilcoxon signed rank sum test were used to test for median difference between groups. Power calculations were based on a two-sided t-test. To detect changing efficiency of PBMC purification depending on pre-purification time, the experimental design was made under the assumption of a power of 80% and an alpha level of 5%. Under these parameters, 20% difference in purification efficiency can be detected with a minimum n=8, based on a worst-case standard deviation derived from a maximum CV of 15%. These sample numbers were then used in the selected time course experiments with EDTA- and Heparin-anticoagulated blood. Change point detection was computed using the PELT algorithm

(via the “ruptures” Python library) to identify structural breaks in the time series. The model employed a least-squares (L2) cost function with a penalty parameter of 3 and a minimum segment size of 15 to filter out transient noise [9].

Results

Anticoagulation tubes

17 unpaired EDTA- and Heparin- anticoagulated blood samples were kept under controlled conditions at RT for 0, 6, 12, 24, 48, 72 or 96 hours and subsequently purified using the SepMate protocol.

Unsurprisingly, samples that were freshly prepared or stored for not more than 12 hours showed a viability of approx. 99%, which was reduced to 98.4–97.7% and 96.0–96.15% after 24 and 48 hours, respectively. Viability dropped further to 91.2–92.3% after 72 hours and 82.1–82.35% after 96 hours. The viability of PBMCs freshly isolated from EDTA-anticoagulated blood was slightly, but significantly higher (in average 1%; Mann-Whitney U test $p < 0.05$) than from blood collected in Heparin tubes (Table 1). For PBMCs prepared 0 hours and 24 hours after blood draw, EDTA anticoagulated samples resulted in a median of $1.39 \times 10^6/\text{ml}$ and $1.21 \times 10^6/\text{ml}$, whereas Heparin-coagulated blood yielded $0.52 \times 10^6/\text{ml}$ and $1.0 \times 10^6/\text{ml}$ PBMCs, respectively. Upon whole blood-aging for 48 and 96 hours, the cell count for EDTA-anticoagulated samples decreased to $1.12 \times 10^6/\text{ml}$ and $0.89 \times 10^6/\text{ml}$, whereas it increased to $0.89 \times 10^6/\text{ml}$ and $1.44 \times 10^6/\text{ml}$ for Heparin-anticoagulated PBMCs, respectively (Table 1). Overall, anticoagulants had a significant effect on the cell yield with a median of $1.18 \times 10^6/\text{ml}$ (EDTA) and $0.84 \times 10^6/\text{ml}$ (Heparin) cells when combining the analysis over all time points ($n = 63$; $p = 0.038$).

In addition to the samples described above, 104 matched blood samples were analyzed. These samples were collected under less controlled conditions, with exact temperature and time-to-processing unknown. However, parallel collections from the same patients and time points were obtained in both EDTA- and Heparin- anticoagulated tubes. Subsequent analysis of these samples confirmed significant differences in PBMC viability and cell counts, depending on the anticoagulant used. Viability was slightly, but stably higher when anticoagulated with EDTA compared to Heparin (median 95.2% vs. 94.7%, $p = 0.0005$). PBMC counts per ml correlated strongly between EDTA- and Heparin-anticoagulated samples (Fig 1a; Spearman $\rho = 0.87$, $p = 0.0001$). However, EDTA tubes yielded a higher median count ($0.66 \times 10^6/\text{PBMCs/ml}$) compared to Heparin ($0.46 \times 10^6/\text{PBMCs/ml}$). This difference was statistically significant (Wilcoxon matched pairs signed rank test, $p = 0.0001$) and corresponds to a methodical bias of 40.4% (95% CI: 32.37–48.47) (Fig 1b).

Next, we conducted epigenetic and flow cytometric immune cell counting verifying equivalence between the two methods. Analyzing 117 observations of analytical marker pairs for granulocytes, monocytes and lymphocytes, correlation analysis indicated highly significant Spearman ρ of 0.91 (CI95% 0.87–0.94, $p < 0.01$). A bias of 5.9% (SD 30.2%) and limits of agreement from 53.2% to 65.1% between the two techniques were observed following the approach of Bland-Altman applying relative percent difference over the mean observation over all cell types (S1 Fig).

Table 1. Calculated median cell counts and viabilities of PBMCs isolated from blood samples.

	Median		Median		Median		Median		Median		Median		Median	
Performance indicator	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]	Count [mil/ml]	Viability [%]
Storage time prior to PBMC isolation	0 h		6 h		12 h		24 h		48 h		72 h		96 h	
Anticoagulant: EDTA (n = 9)	1.39	99.50	1.16	99.60	1.24	98.80	1.21	98.40	1.12	96.00	1.18	91.20	0.89	82.10
Anticoagulant: Heparin (n = 8)	0.52	99.25	0.55	99.30	0.67	99.10	1.00	97.70	0.89	96.15	1.65	92.30	1.44	82.35

<https://doi.org/10.1371/journal.pone.0341547.t001>

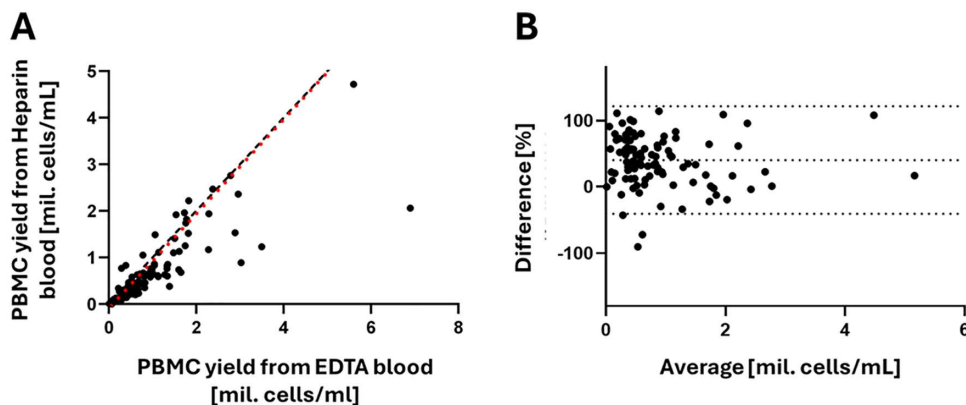


Fig 1. PBMC analysis in matched pairs collected in different anticoagulants. 104 randomly selected, matched blood samples were Heparin- and EDTA-anticoagulated followed by PBMC isolation. A) shows scatterplot of cell numbers and B) displays a Bland-Altman plot indicating the percentage bias between the two coagulants.

<https://doi.org/10.1371/journal.pone.0341547.g001>

The frequency and number of lymphocytes (i.e., the sum of T, B and NK cells), monocytes and granulocytes were analyzed on purified PBMC along the controlled time course, which were expected to be in the range of 70–90%, 10–20% and 0–2%, respectively [10].

For EDTA anticoagulated blood, the mean lymphocyte count in purified PBMCs was around 80% when the purifications were performed at 0h, 6h or 12 hours after blood draw. When purification was conducted 24 hours or later (i.e., 48, 72 or 96 hours), variations among single donors were higher, and the mean lymphocyte count dropped to approximately 58% to 65%. Conversely, granulocyte counts were at 0–5% for the first three points and increased to a mean count of approx. 20% when purification was performed 24 hours or later. The change point between 12 and 24 hours was confirmed by linearly penalized segmentation analysis (PELT). For PBMCs purified from Heparin-anticoagulated blood, mean lymphocyte count remained at around 78% after 0, 6, 12 and 24 hours, indicating stability for at least 24 hours. Accordingly, granulocytes remained consistently low (0–5%) between 0 and 24 hours and PELT analysis further supported the higher stability of purification in Heparin-based matrices. Across both anticoagulants, epigenetically assessed monocyte frequencies showed a mild decline over time (Fig 2).

Epigenetic and flow cytometric assessment of aging blood samples

Flow cytometric and epigenetic qPCR measurements were compared for lymphocyte (TBNK), granulocyte and monocyte cell counts (Original data are shown in S1 Table). In Figs 3–5, data points are color-coded to indicate their position relative to the PELT-detected change points: black dots represent time points before, and green dots represent time points after the change points. Spearman rho correlation for lymphocyte counts was at 0.84 (0.71 to 0.89) in PBMCs from EDTA- and 0.89 (0.79 to 0.94) for Heparin-anticoagulated blood samples ($p < 0.0001$ for both) with relative method biases of -8.37 (LoA = -31.7 to 14.96%) and -3.13 (-27.18 to 20.92%), respectively (Fig 3).

Relative counts for lymphocytes were visibly shifting towards lower shares for both methods and anticoagulants. However, the methods performed substantially equivalently at early and late time points, with no significant differences between the methodical biases prior and after the change point. When assessing granulocyte content with the two methods, Spearman rho was at 0.95 in PBMCs from EDTA- and 0.93 for Heparin-anticoagulated samples ($p < 0.0001$ for both) with mean percentage biases of -1.99 and 37.59 , respectively (Fig 4).

Analysis of monocytes showed a moderate correlation between epigenetic qPCR and flow cytometry (Spearman rho = 0.52, $p < 0.0001$) for PBMCs that were purified from blood drawn into EDTA tubes as shown in Fig 5. Although the

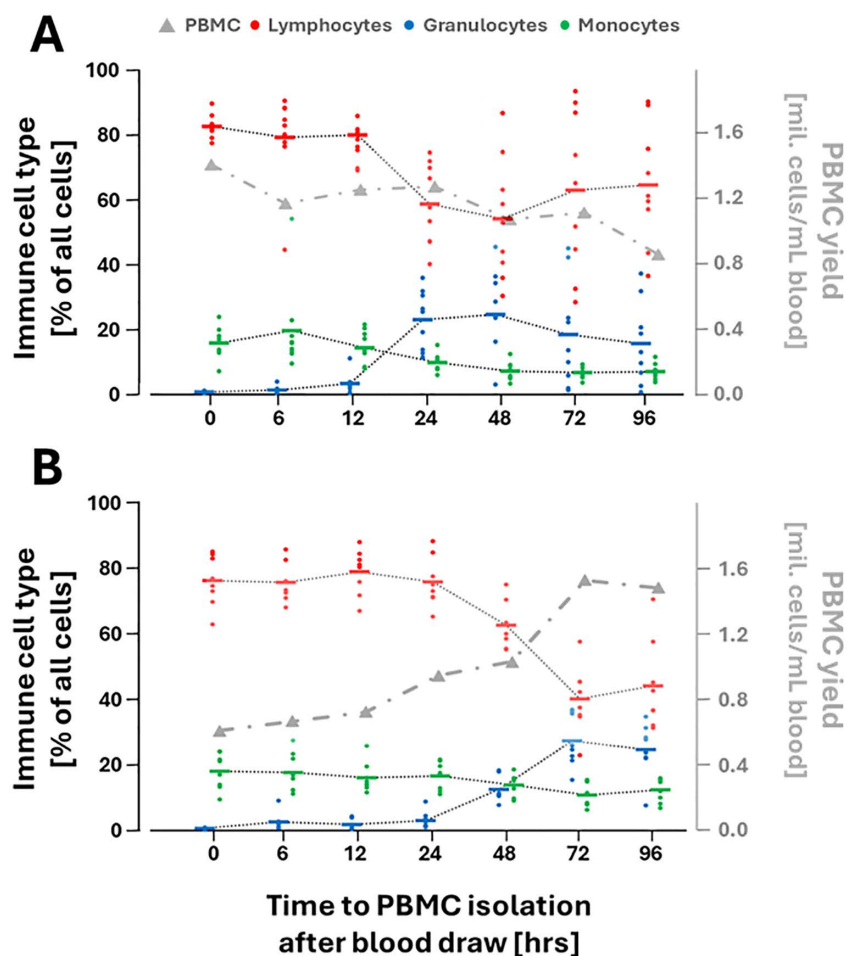


Fig 2. Frequency of defined immune cells in isolated PBMCs assessed by epigenetic qPCR. Lymphocyte frequencies (left Y-scale) and numbers (right Y-scale) are depicted in red and result from adding T, B and NK cell counts. Granulocytes (blue) and monocytes (green) are also assessed over time. Overall, the absolute cell count is shown in grey. All analyses were performed with PBMCs isolated from (A) EDTA- and (B) Heparin- anticoagulated blood. Medians for each cell type are displayed at each time point. Per cell type medians were connected to display trend over time.

<https://doi.org/10.1371/journal.pone.0341547.g002>

overall correlation appeared stable before and after change point, a significant shift in median monocyte counts was observed (+5% before and -1% after the change point; Mann Whitney U test, $p < 0.0001$), indicating an altered association. For blood drawn into Heparin tubes, correlation between technologies was strong before the 24 hours change point (Spearman rho=0.9, $p = 0.0001$), but no correlation was observed afterward (Spearman rho=0.04). Across all samples, the mean bias was -23.31% (ranging from -103.5 to 56.85%). Prior to the change point, mean differences were minimal (0.2%) indicating equivalence. However, after PBMC aging, equivalence was lost, with the average mean difference increasing to -60%, reflecting significantly higher cell counts detected by flow cytometry.

Short term storage temperature of Heparin-anticoagulated blood

To analyse the influence of storage temperature of whole blood on PBMC purification, Heparin-anticoagulated blood aliquots from four different donors were stored at 4°C, 22°C and 37°C. PBMCs were isolated 0, 12, 24 and 48 hours after blood draw using SepMate. Sample aging led to higher granulocyte contamination for all temperatures as shown by a

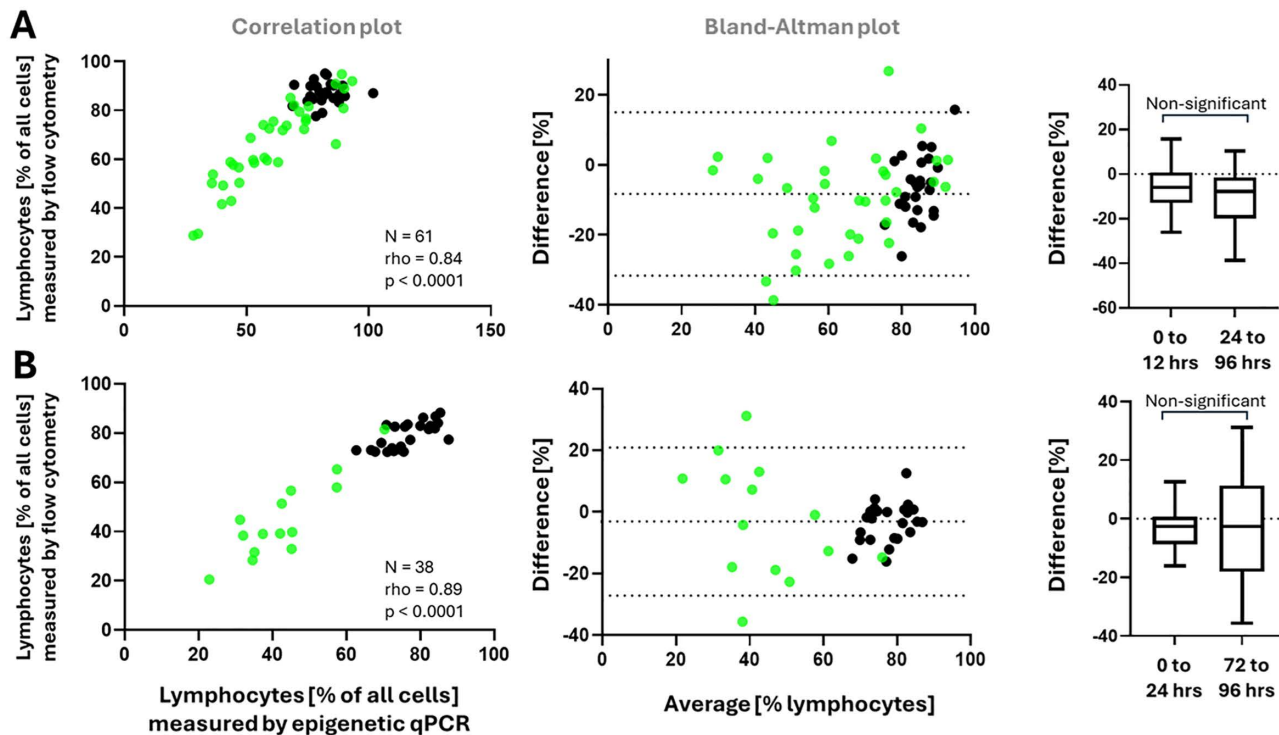


Fig 3. Lymphocyte analysis by Flow cytometry and epigenetic qPCR on aging PBMC preparations. PBMC samples from blood drawn into EDTA and Heparin are shown in the upper and lower row, respectively. In the left column, lymphocyte counts as assessed by flow (y-axis) and epigenetics (x-axis). In the middle column, the respective Bland-Altman plot, displaying relative differences (y-axis) over average of each measurement (x-axis), is shown. Samples purified before and after the changepoint are indicated in black and green, respectively. On the right-hand side, Box-Plots of the percent difference for all data assessed prior to and after the changepoint, determined by PELT are given. Box-Plots according to Tukey, outlier not shown.

<https://doi.org/10.1371/journal.pone.0341547.g003>

slope of linear regression tested against a slope equal to zero ($p=0.0025$ for RT, $p=0.017$ for 4°C , $p=0.0038$ for 37°C). After 12 hours of storage, samples kept at 4°C and 37°C showed high levels of granulocyte contamination (means of 28.16% and 17.65%, respectively), whereas samples stored at ambient temperature exhibited substantially lower contamination levels (mean of 4.14%) (Fig 6). These findings were consistent over time: after 24 hours and 48 hours of storage at 4°C , mean granulocyte contamination remained elevated at 44.25% and 38.78%, respectively. Similarly, when whole blood was stored at 37°C prior to PBMC isolation, co-purified granulocytes reached 26.62% and 33.94% at the same time points (i.e., 24 and 48 hours). Contamination levels were significantly lower, though not absent, when blood was stored at room temperature, with means of 11.43% and 12.86% after 24 and 48 hours, respectively. Despite these differences, cell viability remained high under all conditions: After 12 hours, median viability was 98.6%, 95.15%, and 99.7%; after 24 hours of blood storage, viability was at 98.5%, 95.6% and 98.6% and even after 48 hours, viability was at 97.7%, 92.4% and 95.2% for 4°C , 37°C , and at RT, respectively.

PBMC purification methods

To test the influence of purification methods on the quality of PBMC samples, whole blood from nine normal donors was drawn into Heparin tubes, processed freshly or kept at ambient temperatures for 72 hours. Each sample was split and then processed in parallel with two isolation methods, SepMate and Ficoll overlay (CTL). When testing all samples at both time points together in this matched pair approach, median total cell count was at 1.23×10^6 vs. 1.28×10^6 cells per

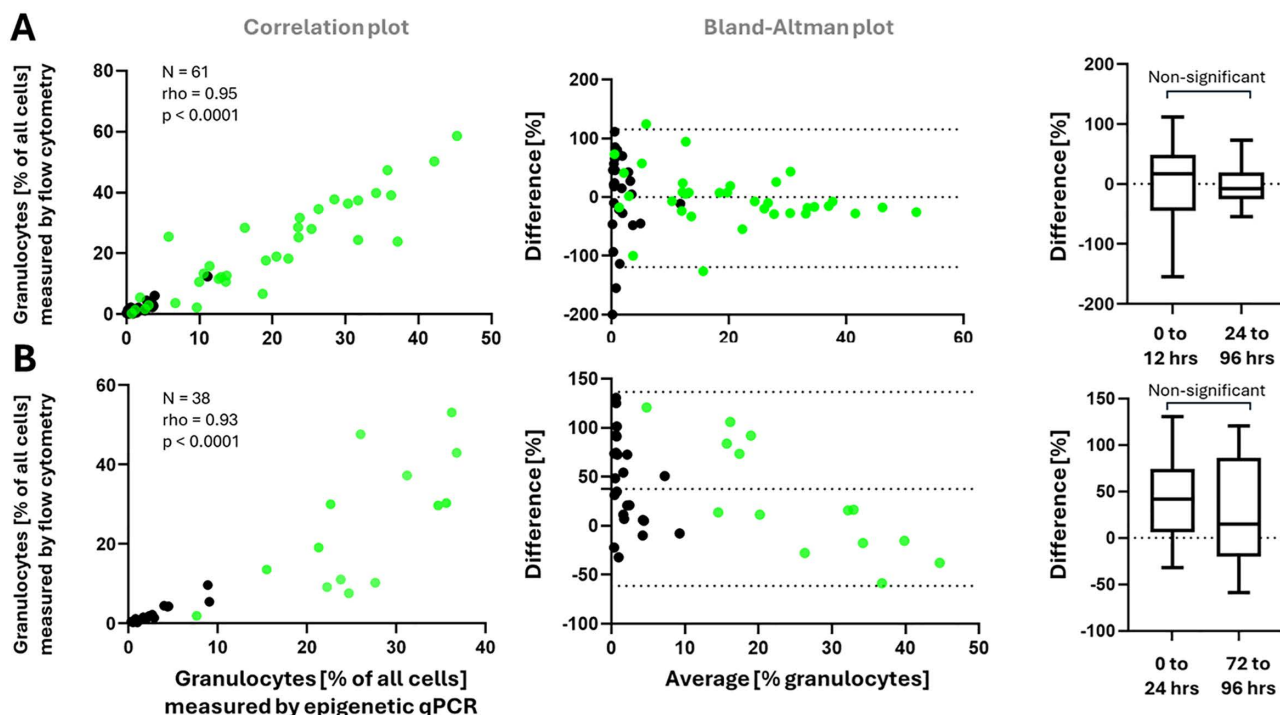


Fig 4. Granulocyte analysis by Flow cytometry and epigenetic qPCR in aging PBMC preparations. PBMC samples from blood drawn into EDTA and Heparin are shown in the upper and lower row, respectively. In the left column, granulocytes were assessed by flow (y-axis) and epigenetics (x-axis). In the middle column, the according Bland-Altman plots display relative differences (y-axis) over average of each measurement (x-axis). Samples purified before and after the changepoint are indicated in black and green, respectively. The very low granulocyte content in early vs the relatively high counts after prolonged incubation are a challenge for the method comparison. On the right-hand side, boxplots of the percentage difference for all data assessed prior to and after the PELT changepoint are given. Box-Plots according to Tukey, outlier not shown.

<https://doi.org/10.1371/journal.pone.0341547.g004>

ml blood (Wilcoxon rank sum test, $p=0.73$) for the two methods, respectively (Fig 7). Mean viability was slightly higher for SepMate (98%) than Ficoll overlay (95.4%). These differences were not statistically significant (Wilcoxon rank sum test, $p=0.09$). Corroborating prior findings, cell counts were higher and viability lower after 72 hours when compared to freshly purified samples (1.37×10^6 vs. 1.13×10^6 cells per ml, $p=0.07$ and 90.5% vs. 97.5%, $p<0.0001$). When time points within the purification methods were analyzed, those trends could be confirmed: for both SepMate and CTL, cell counts trended to be higher when PBMCs were purified after 72 hours compared to freshly purified PBMCs and viability was significantly higher for freshly isolated samples.

Confirming the concordance between both methods, 48 non-paired samples from healthy donors were purified with either of the two methods. A similar recovery was observed and viability for samples purified with SepMate (median=98.5%) was higher than those purified with Ficoll overlay (96.6%; $p=0.0001$).

Granulocyte contamination and True count of PBMCs

Whole blood samples from 8 normal donors drawn into Heparin were forwarded to epigenetic immune cell counting directly or upon PBMC purification from blood stored at ambient temperature after 0, 12, 24, 48, 72 or 96 hours (Table 2).

The epigenetic cell counts in fresh whole blood served as reference measurement. Unpurified fresh whole blood sample (0h) yielded a mean of 28.1% CD3+T cells, 9.7% monocytes (MOC), 3.9% NK cells and 58.3% neutrophil granulocytes (nGRC), when assessed as ratio of the individual cell count relative to the sum of all populations. Changes over the

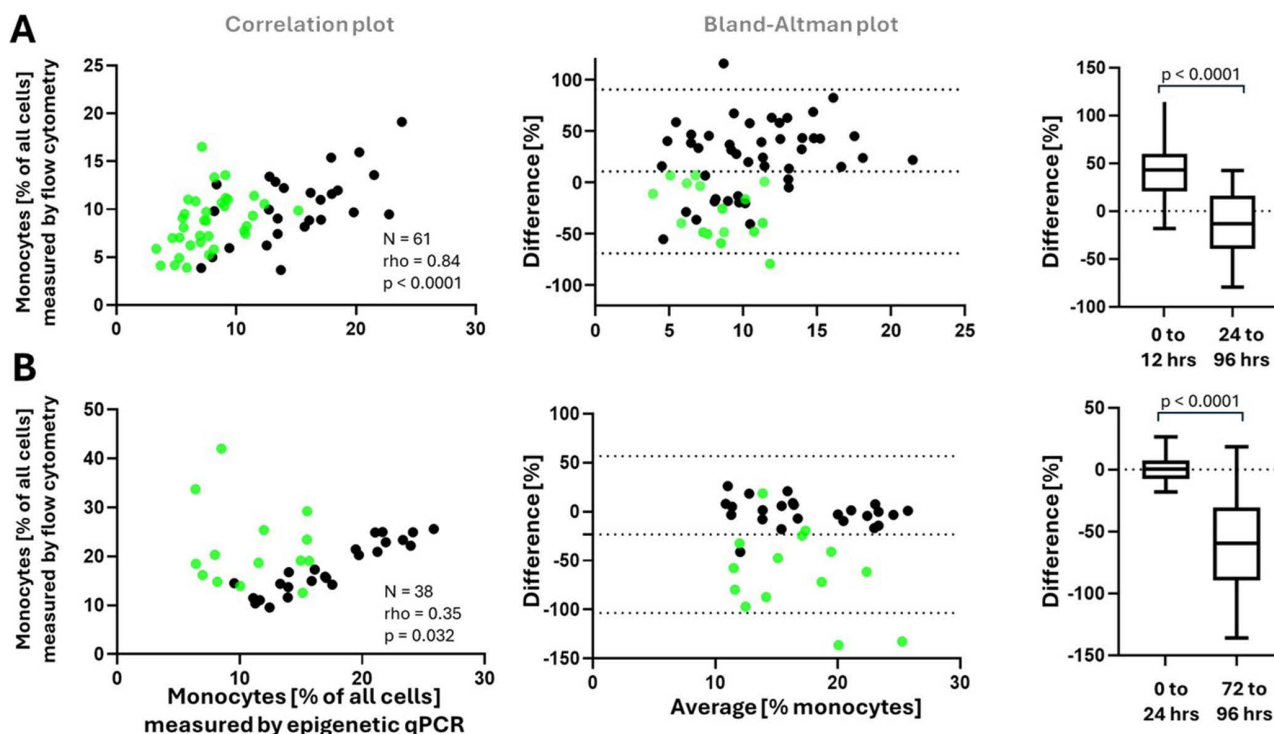


Fig 5. Monocyte analysis by Flow cytometry and epigenetic qPCR on aging PBMC preparations. PBMC samples from blood drawn into EDTA and Heparin are shown in the upper and lower row, respectively. In the left column, monocytes were assessed by flow cytometry (y-axis) and epigenetic qPCR (x-axis). In the middle column, the according Bland-Altman plots display percentage differences (y-axis) over average of each measurement (x-axis). Samples purified before and after the changepoint are indicated in black and green, respectively. On the right-hand side, Box-Plots of the percent differences for all data assessed prior to and after the PELT changepoint are given. Box-Plots according to Tukey, outlier not shown.

<https://doi.org/10.1371/journal.pone.0341547.g005>

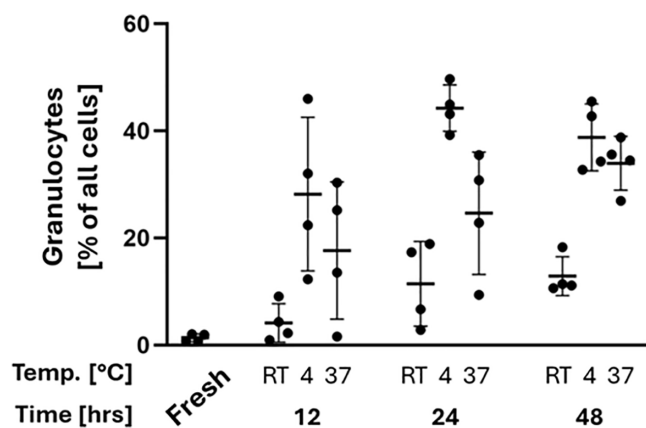


Fig 6. Granulocyte presence in PBMC isolates measured by epigenetic qPCR. SepMate was used to purify PBMCs after initial storage of Heparin anticoagulated whole blood from four donors at 4°C, room temperature and 37°C for 12, 24 or 48 hours. All four data points are shown, and means are indicated; whisker added to visually separate category.

<https://doi.org/10.1371/journal.pone.0341547.g006>

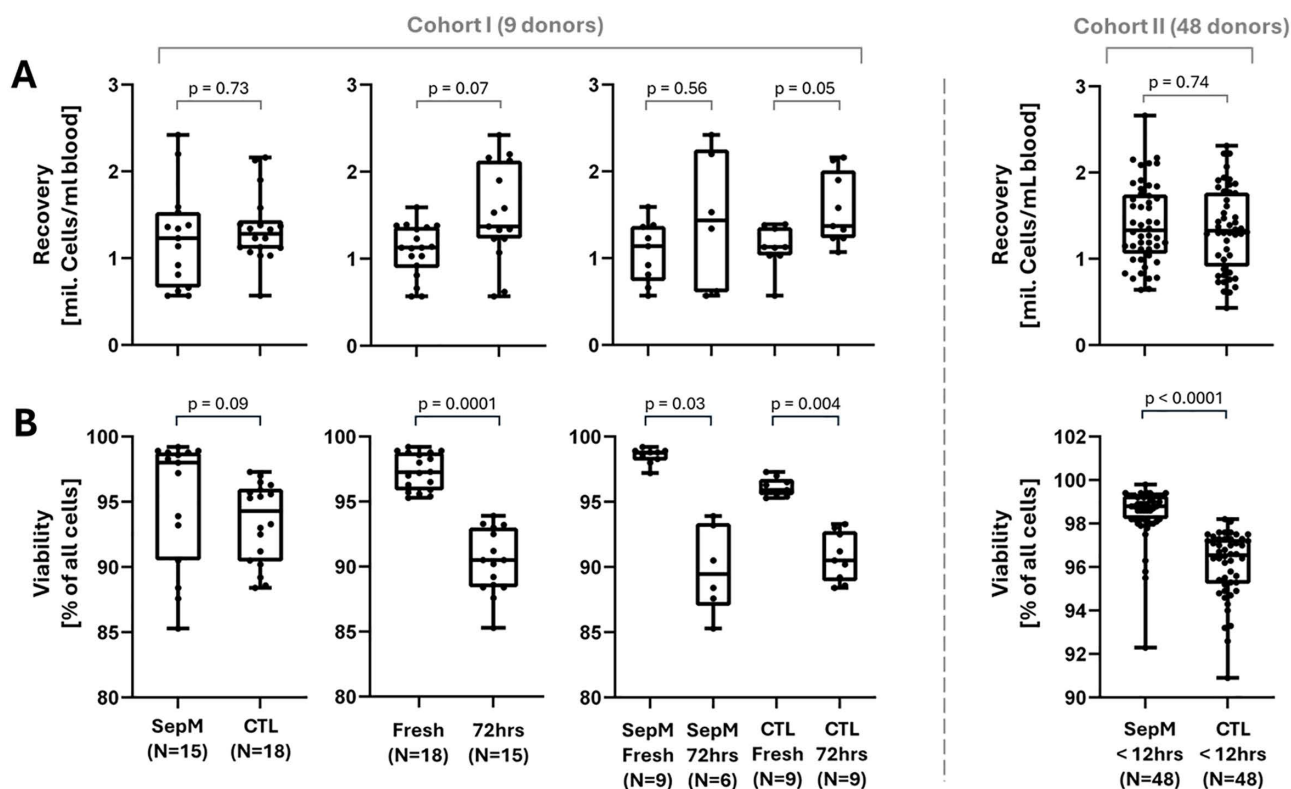


Fig 7. PBMC quality dependence on isolation protocol and matrix. Standard performance indicators, cell count (recovery) and viability, were tested. For a matched sample cohort with 9 donors, PBMCs were isolated 0 and 72 hours after blood draw using the Sepmate protocol (SepM) or the Ficoll overlay method (CTL). On a second, non-matched cohort, PBMCs from 48 donors were prepared within 12 hours of blood draw. Boxes are given by the range of the third (Q3) to the first quartile (Q1). The median is displayed in the middle of the box. The whiskers extend to the max (min) observation or 1.5 times interquartile range above Q3 (below Q1). Observations displayed as jittered scatter in their respective category.

<https://doi.org/10.1371/journal.pone.0341547.g007>

Table 2. Epigenetic immune cell counting in PBMCs isolated from aged blood.

Material	Time before processing or analysis	PBMC count [mil cells per mL whole blood] (mean)	T cells		Monocytes		NK cells		Neutrophils	Total PBMCs
			[mean %] of T, NK, Moc, nGr (Acceptance Range)	[mean %] of T, NK, Moc (Acceptance Range)	[mean %] of T, NK, Moc, nGr (Acceptance Range)	[mean %] of T, NK, Moc (Acceptance Range)	[%] of T, NK, Moc, nGr (Acceptance Range)	[%] of T, NK, Moc (Acceptance Range)	[%] of T, NK, Moc, nGr (Acceptance Range)	
whole blood (n=8)	0 h	na	28,1 (22,5 - 33,7)	67,4 (53,9 - 80,9)	9,7 (7,8 - 11,6)	23,2 (18,6 - 27,8)	3,9 (3,1 - 4,7)	9,4 (7,5 - 11,3)	58,3 (46,6 - 70,0)	na
	24 h		27.6	66.5	9.9	23.9	4.0	9.5	58.5	
	48 h		26.6	66.5	9.6	24.1	3.8	9.4	60.0	
	72 h		27.2	66.3	9.1	22.2	4.7	11.5	58.9	
	96 h		27.9	69.6	8.3	20.6	4.0	9.9	59.9	
PBMC (n=8)	0 h	0.56	69,7 (55,8 - 83,6)	70.2	20,5 (16,4 - 24,6)	20.7	9,0 (7,2 - 10,8)	9.1	0,8 (0,6 - 1,0)	0.55
	24 h	0.91	67.2	69.7	19.6	20.3	9.6	10.0	3.6	0.88
	48 h	1.02	58.7	67.0	18.0	20.5	11.0	12.5	12.3	0.89
	72 h	1.52	44.0	70.1	14.7	23.5	4.0	6.4	37.3	0.96
	96 h	1.47	45.7	68.7	16.8	25.2	4.1	6.1	33.5	0.98

<https://doi.org/10.1371/journal.pone.0341547.t002>

different time points after collection appeared to be within a 20% range of the freshly collected PBMC sample. Together, this data suggests good stability of the epigenetic measurement of whole blood stored for up to 96 hours at ambient temperature. However, when whole blood samples were stored under those conditions and then forwarded to PBMC purification, results showed a significant and directional change. Whereas freshly purified PBMCs contained a mean of 69.7% CD3+T cells, 20.5% monocytes, 9.0% NK cells and only residual number (0.8%) neutrophil granulocytes, prolonged incubation led to decreasing CD3+T cells, monocyte and NK cell counts and increasing neutrophil granulocyte counts. Moderate changes were seen at 24 and 48 hours, where CD3+T cells decreased to 67.2% and 58.2%, monocytes changed to 19.6% and 18.0%, respectively. NK cells changed more randomly but neutrophils increased for these time points to 3.6 and 12.3%, respectively. The ability to separate PBMCs from neutrophil granulocytes decreased further at 72 hours and remained low with some variations at 96 hours, yielding 44.0% and 45.7% CD3+T cells, 14.7% and 16.8% monocytes, driven by the inversely proportional increased number of neutrophils at 37.3 and 33.5%, respectively. The count of NK cells also varied significantly but indicated no trend.

Next, hypothetically attributing the observed changes to co-purification of neutrophil granulocytes, the stability of populations was assessed without including granulocyte counts, both for non-purified blood and purified PBMCs. The relative number of CD3+T cells (mean 67.4% vs. 70.2%), monocytes (23.2% vs. 20.7%) and NK cells (9.4% vs. 9.1%) were well comparable between whole blood and purified PBMCs. The ranges over all points were small. CD3+T cell counts ranging between 66.3% and 69.6% (3.3% variation) and 67.0% – 70.2% (3.2%) for whole blood and PBMCs, respectively. Monocytes varied between 20.6% – 24.1% (3.5%) and 20.3% – 25.2% (4.5%) for the two substrates, respectively. NK cells ranged between 9.4% and 11.5% in whole blood but showed higher variation of 6.1% and 12.5% (6.4%) in PBMCs. Considering the percentage of the granulocyte/neutrophil count, the adjusted “(true) recovery” of PBMCs/ mononuclear cells is specifically reduced after prolonged storage times. For PBMCs isolated directly after blood draw, very little adjustment is needed, and the original cell count of 0.56×10^6 cells per ml remains stable. For later time points, the adjusted recovery is significantly lower than the original cell counts leading to a recovery of between 0.88 and 1.0 million cells for the latter time points compared to 0.9 to 1.52 million cells when not adjusted for neutrophil contamination.

Together, while there is a hint for higher variations of PBMC recovery data compared to fresh blood and increasing with the aging of blood samples, the data appear well reproducible if granulocyte contamination is known.

Discussion

Lack of extracorporeal stability of blood samples is an acknowledged challenge in immune diagnostics and clinical trials. Current mitigation efforts often include isolation of peripheral blood mononuclear cells (PBMCs) after blood drawing and subsequent PBMC storage in liquid nitrogen prior to analysis. However, this process harbors its own challenges, influencing read-out of subsequent analytical procedures [3,11]. In this study, common parameters and challenges of PBMC isolation were revisited.

Those challenges with blood as analyte begin with its extraction from the veins and end with the analytical results. Decisions must be made with respect to anticoagulation, temperature and time between blood drawing and further processing. Effects of these parameters are permanent and unequivocally influence analytical results. Tube type, i.e., the anticoagulant and associated buffer conditions, can be determined freely. Temperature can be kept stable and monitored, but it is often a challenge to control and monitor time-to-analysis, especially when PBMC preparation involves central laboratories.

Anticoagulation of whole blood with EDTA reliably yields approx. 40% higher overall cell counts, especially in fresher samples, and shows small (i.e., 0.5%), but significantly, higher cell viability than other anticoagulation agents. However, molecular (i.e., DNA methylation-based) markers detect increasing numbers of granulocytes inside the PBMC layer upon gradient centrifugation as early as 12 hours after blood draw. This is suggestive for rapidly declining cell integrity, particularly for granulocytes, in EDTA-anticoagulated blood, confirming prior reports [12]. At the same time, however, cell count

upon PBMC purification decreased over time, surprisingly and contra-intuitive with an increasing granulocyte intrusion. It remains unclear, why despite increasing contamination, the overall cell count is reduced. A possible explanation is a general loss of cell integrity and therefore reduction in yield. When Heparin-anticoagulated blood was purified after different incubation times and despite lower cell counts and slightly lower viability, the purification appears to be stable for a longer period, i.e., at least up to 24 hours. While remaining under 10%, contamination is steadily increasing after 6 hours. Granulocyte shares increase to more than 10% after approx. 24 hours along with the overall cell count. Generally, this trend was independent of incubation conditions, including ambient storage, which resulted in the highest cellular integrity and viability over time, when compared to 4°C or 37°C. Whereas viability over time was highest at 4°C concurring with previous publications [13], it coincided with early and high granulocyte contamination. Physiological body temperature of 37°C does not preserve extracorporeal whole blood well with both varying cell counts and low viability [14,15]. The selected purification method, as tested here with SepMate and Ficoll overlay procedures, indicates higher viability and recovery for SepMate [1], possibly owing to shorter processing times and less complicated handling in the separation process. More generally, our data suggests that purification results chiefly reflect the initial incubation conditions, i.e., time, tube and temperature of the unprocessed whole blood, whereas the purification methods influence the outcome only moderately and are highly dependent on the former.

When assessing the different cell populations within PBMCs, comparison between molecular markers using epigenetic and morphological properties in flow cytometric analysis yielded concordance between all markers for fresh blood before the change point. This concordance was lost mainly for monocytes when PBMC purification was performed on aged blood samples. The number of cells with monocytic morphology increased significantly in flow cytometric assessment compared to epigenetic markers. At this point, supported by PELT change point selection, sideward/forward scatter selection in flow cytometry, otherwise a reliable separator for healthy cells, loses correlation with the molecular monocyte marker. Also, this observation went along with the increased detection of granulocyte contamination when assessing with molecular markers only. Together, higher cell counts, lack of separation of granulocytes from PBMCs, the increasing number of cells with molecular signatures of granulocytes as well as previous reports of the existence of – under physiological conditions – highly proinflammatory low density granulocytes [2,16] suggest ongoing changes in the composition of PBMCs.

Before reaching the change point for monocytes and granulocytes, good correlation and concordance of epigenetic and flow cytometric data are observed. This was the case for all cell types and time points, when antigen-specific markers were used, which corroborated previous comparisons between epigenetic and flow markers [7]. Epigenetic markers remained unchanged in the read-out between 0 and 96 hours showing high stability for all cell types in aging whole blood samples.

Given high reproducibility of the epigenetic assays and unstable, i.e., time-dependent blood purification, epigenetic immune monitoring was tested for improved PBMC characterization. Over time, the relative count of T cells, monocytes and NK cells were reduced, with granulocytes increasing inversely proportionally. With no measurement in place for assessing the purity of PBMCs, determining cell count for the individual cell counts is not possible. For example, the T cell count moves from an average of 69.7% for freshly prepared to 44% when PBMCs are purified after 72 hours. Granulocyte contaminations in PBMCs are low-density granulocytes as they co-segregate with monocyte in gradient centrifugation. It is unclear, if these in vitro developing LDGs maintain their highly proinflammatory profile, as has been described for LDS in healthy donors and autoimmune patients [17]. However, in case proinflammatory LDGs pose acute problems that PBMCs have changed drastically from their in vivo status. Using epigenetic immune monitoring therefore provides an easy additional tool to detect and quantify PBMC composition including but not limited to the granulocyte contamination. This assessment may then be a simple quality tool flagging PBMCs where results may be not trustworthy. In addition, the method allows an initial cell count with the best possible back traceability to the fresh whole blood sample as it captures all relevant cell populations, including granulocytes, which have been reported to disintegrate after thawing and prior to flow cytometric immune cell counting (Table 2). Naturally, with increasing granulocyte contamination, the cell count increases.

However, these cells no longer only represent the target PBMC population but include neutrophils and render the yield a dubious quality parameter. When considering the number of detected granulocytes, the true PBMC cell count can be adjusted painting a more realistic count of purified PBMC.

Conclusions

In summary, we assessed multiple parameters influencing PBMC purification and evaluated epigenetic immune monitoring as a quality control method for resulting PBMC preparations. Pre-purification factors – such as storage time, temperature, and choice of anticoagulation tube – were found to be the primary determinants of PBMC quality, whereas differences in gradient centrifugation methods are of limited impact. Based on our data, we recommend using Heparin-anticoagulated blood in combination with the SepMate method for PBMC isolation. Storage conditions (time and temperature) strongly affect PBMC quality and can lead to granulocyte co-purification. Epigenetic immune monitoring proved to be an efficient and meaningful approach for PBMC quality assessment and may help reduce ambiguous analytical results arising from suboptimal PBMC preparations.

Supporting information

S1 Table. Original data of Flow cytometry and epigenetic qPCR measurements. The table shows cytometric and epigenetic immune cell quantification of lymphocytes, granulocytes and monocytes in PBMCs derived from blood in Heparin and EDTA anticoagulation tubes after increasing storage times. Lymphocyte percentage was calculated from T-, B- and NK cell numbers.

(DOCX)

S1 Fig. Correlation and Bland-Altman Analysis for all cell types. On the left-hand side, correlation between Flow cytometric and epigenetic cell typing is shown for granulocytes (black dots), lymphocytes (grey rectangles) and monocytes (light grey triangles). Spearman correlation for all cell Types is shown in the table on the right-hand side. The relative percent difference over the average calculated from the two methods is shown in the Bland-Altman plot. Indicating a 5.94%. The data indicate substantial equivalence of the methods.

(TIF)

Acknowledgments

We would like to thank Matthias Eckelmann, Alkmini Filippou, Susanne Strahlendorff-Herppich and Ciro Novaes. Udo, thank you for many years of scientific partnership. You, Ivana and Alex most significantly influenced what turned out to be a cool journey through epigenetics and epigenomics.

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