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Validation of an apoptosis assay for extracorporeal photopheresis

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Abstract

Objectives: This validation study investigated a flow cytometric apoptosis assay according to good manufacturing practice (GMP).

Background: Extracorporeal photopheresis (ECP) is a treatment for various immunological diseases and cutaneous T-cell lymphomas. It is based on the induction of apoptosis by 8-methoxypsoralene and ultraviolet A light. The quantification of apoptosis is therefore essential for ECP improvements. However, despite numerous publications on apoptosis, validated technical details are lacking.

Methods and materials: Mononuclear cells were collected by apheresis and treated by ECP or camptothecin. Samples taken before and after ECP were cultured for 24, 48 and 72 h and analysed for apoptosis and viability of T cells and monocytes by flow cytometry with Annexin V and 7-AAD staining. Accuracy of the assay, intra- and inter-assay precision and the pre-analytical and analytical stability of the analytes were the investigated parameters.

Results: Our data indicate that the median intra- and inter-assay precision coefficient of variation for T cells was 3.86% and 4.80%, respectively. Pre-analytical stability of T cells and monocytes was ensured during short-term storage for up to 2 h on ice. After staining, analytical stability was limited to 30 min, likely because of ongoing apoptosis and loss of monocytes due to plastic adhesion.

Conclusion: The results of this validation study show that the assay is GMP-compliant and that its reliability, accuracy and precision are acceptable. While pre-analytical stability of the cells was compatible with on-site procedures, our analytical stability data indicate that this assay is not suited for batch mode analysis of ECP products.

KEYWORDS
apoptosis, ECP, extracorporeal photopheresis, validation

1 | INTRODUCTION

Extracorporeal photopheresis (ECP) has been an established therapy for cutaneous T-cell lymphoma, graft-versus-host-disease (GvHD), rejection after solid organ transplantation and various autoimmune diseases for over a decade.[1-4] In this therapeutic procedure, autologous leukocytes are collected by apheresis, treated with 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) light ex vivo, undergo covalent DNA

strand linkage by 8-MOP and are reinfused back to the patient. This serves to prevent transcription and replication, leading to cell inactivation and apoptosis.[5,6] Assays for apoptosis can therefore be used as a surrogate parameter for pharmacological potency.[7]

Apoptosis is an organised cell death process consisting of different intrinsic and extrinsic apoptosis pathways. Both pathways lead to the activation of proteolytic enzymes (caspases 3, 6, 7) and to DNA fragmentation, DNA budding and chromatin condensation.[8] The mechanism by which apoptotic cells elicit an immunosuppressive effect is very well understood.[9,10] Rapid clearance of apoptotic cells by macrophages and dendritic cells (DCs) results in up-regulation of immunosuppressive factors (e.g., Transforming growth factor beta-β, Interleukine-10) and down-regulation of costimulatory molecules. Such 'tolerized' macrophages and DCs again suppress T-cell effector activity and support regulatory T-cell function.[11,12]

Several techniques are available for the detection of different stages of apoptosis.[13] In particular, flow cytometry is able to discriminate vital from apoptotic cells at the single-cell level in a complex mixture of cells[14,15] Double staining with Annexin V, which binds phosphatidylserine on the surface of apoptotic cells, and 7-AAD as a membrane-impermeable dye for the DNA of dead or damaged cells offers the possibility to simultaneously detect apoptotic and dead cells. Despite its widespread established use,[16,17] data on the robustness and limits of operation of this assay are lacking. We therefore designed this study to characterise the validity of this assay according to the guideline of good manufacturing practice of the European Union (GMP), which ensures quality standards for drug production.[18]

2 | MATERIAL AND METHODS

2.1 | Patients

Data were obtained in 2017/2018 according to the EU Guidelines for GMP.[18] All participants gave their informed consent and received offline ECP as medically indicated. The study included a total of nine patients (eight male, one female), five (56%) with acute GvHD and one each with chronic GvHD, Sézary syndrome, cutaneous T-cell lymphoma and cellular rejection. This study protocol was approved by the local ethics committee (16-101-0046).

2.2 | Photopheresis

Apheresis was carried out with a Spectra Optia (Terumo BCT) apheresis system in cMNC mode. The ECP procedures were set up to yield leukocyte and plasma volumes of 90 and 110 ml, respectively, after cell harvesting. The resulting 200-ml cell suspension was sterilely transferred to a UVA-PIT (PIT Medical Systems GmbH) bag system, consisting of a recirculation bag and a UV-permeable ethylene vinyl acetate irradiation bag. Next, 2 ml of 8-MOP 20 mg/L (Uvadex, Therakos, West Chester, PA) was injected into the bag (final 8-MOP concentration before irradiation: 200 ng/ml). Subsequently, UVA radiation (2 J/cm^2) was delivered through the UVA-PIT system. The treated cell suspension (product) was then immediately administered to the patient.

2.3 | Sample preparation

ECP product samples were taken before the addition of 8-MOP (pre) and after irradiation (post).

Control samples were obtained from healthy blood donors. Mononuclear cells (MNCs) were separated by gradient centrifugation (Biocoll separating solution, Merck) and washed with 2 ml of dulbecco's phosphate-buffered saline (DPBS) buffer (Sigma Aldrich) at 300*g*, 5 min.

Apoptosis testing was carried out 24, 48 and 72 h after incubation in a TexMACS GMP medium (Miltenyi, Bergisch-Gladbach, Germany) with 1% Glutamax (Gibco) at 37°C and 5% CO_2. Cells incubated with camptothecin (Sigma; 200 μM, 4–6 h, 37°C, 5% CO_2) served as positive controls for gate adjusting.[19]

2.4 | Analytics

2.4.1 | Cell count

Cell concentrations were measured undiluted on an XN-550 Automated Hematology Analyzer (Sysmex, Kobe, Japan).

2.4.2 | Flow cytometry

Briefly, 1×10^6 MNCs/tube were stained after treatment with 10 μl of FcR Blocking Reagent (Miltenyi) in 90-μl Cell Staining Buffer (Beckman Coulter). Immunophenotyping of leukocytes was performed with commercially available antibodies from Beckman Coulter (BC): CD45-KrOrange, CD33-PE-Cy7 and CD3-ECD; CD56-APC-AF750, CD20-APC-AF750, CD19-APC-AF750 and CD66b-APC-AF750 were used for lineage exclusion. All primary antibodies were added to the cell suspensions and incubated for 20 min at 4–8°C in the dark. Subsequently, cells were washed with 2 ml of DPBS (300 g, 5 min). The Annexin V/7-AAD kit from BC was used for apoptosis detection. Therefore, cells were suspended in 100 μl of Binding-Buffer, and 10 μl of Annexin V and 20 μl of 7-AAD were added. After an incubation time of 15 min at 4–8°C (dark), 300 μl of Binding-Buffer were added. The following BC antibodies were used as isotype controls: mouse IgG1-ECD for CD3 and mouse IgG1-PC7 for CD33. All antibodies were titrated to obtain an optimal concentration.

Flow cytometric analyses were performed with the Navios Ex and Navios flow cytometers running Cytometry List Mode Data Acquisition Software, versions 2.0 and 1.3, respectively, and Kaluza Analysis Software from Beckman Coulter, version 2.1. The cells were gated as illustrated in Figure 1. T cells (CD3) and monocytes (CD33) were

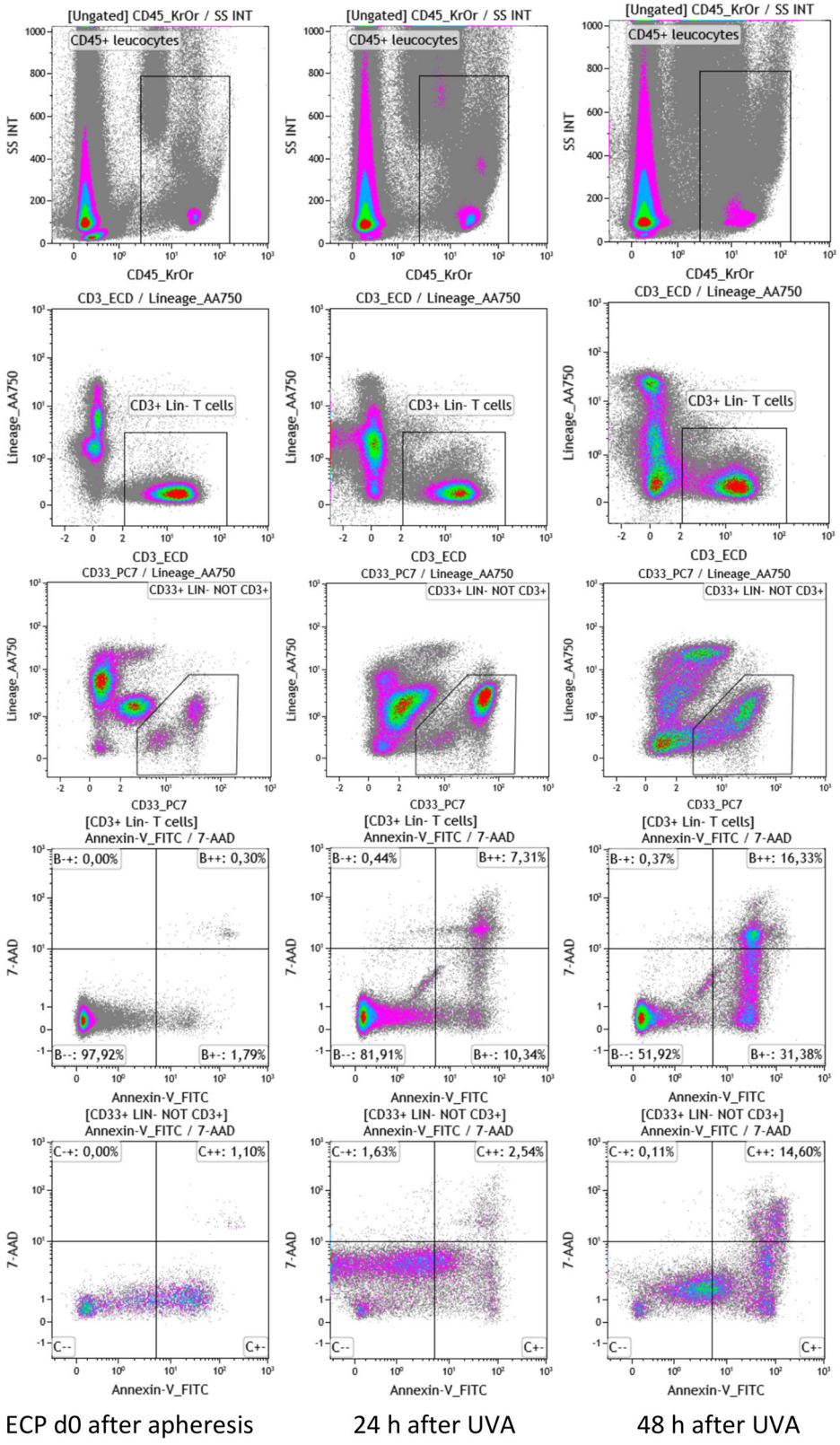

FIGURE 1 Gating strategy in fresh (left column) and incubated extracorporeal photopheresis samples (middle and right column). First, leukocytes were selected (first line), and T cells (CD3) and monocytes (CD33) were analysed by excluding granulocytes, natural killer cells and B cells (second and third lines). Annexin V^+ and 7-$AAD^{+/-}$ cells were sub-gated from T cells (fourth line) and $CD33^+$ monocytes (fifth line). Annexin V exhibited non-apoptosis binding of $CD33^+$ cells; therefore, analysis was limited to Annexin V^+ 7-AAD^+ monocytes [Color figure can be viewed at wileyonlinelibrary.com]

analysed by excluding granulocytes (CD66b), natural killer cells (CD56) and B cells (CD19, CD20). Annexin V^+ and 7-$AAD^{+/-}$ cells were sub-gated from T cells and $CD33^+$ monocytes. Annexin V exhibited non-apoptosis binding of $CD33^+$ cells; therefore, analysis was limited to Annexin V^+ 7-AAD^+ monocytes. Gates were set with isotype controls and Fluorescence Minus One (FMO) control using fresh samples. Aged cells and debris displayed some degree of autofluorescence in channels 3 and 5. We did not exclude these signals in order to capture all cellular material and because results were reported as the difference between treated and untreated samples.

Specific acceptance criteria for specificity, precision and robustness were set. The cut-offs were set such that the unspecificity threshold was ≤5%.

2.5 | Statistical analysis

Microsoft Excel 2010, R and IBM SPSS Statistics 25 were used to collect data; generate figures; and to determine the median, mean and standard deviation. Correlation was determined using the Pearson test. *p*-values below 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Accuracy

First, gating boundaries were verified by using isotype controls or FMOs. We achieved unspecificity values (n = 4) of 0.20%–4.11% for CD3, 0.00%–0.10% for $CD14^+CD16^-$ and 0.49%–4.25% for $CD33^+$. Regarding apoptotic (Annexin V^+/7-AAD^-) or dead cells (Annexin V^+/7-AAD^+), the specification requirement of ≤5% unspecific binding for T cells and monocytes was met; values range from 0.01% to 2.67% (n = 8).

3.2 | Robustness

When analysing a batch of samples (n = ~~6~~10) consecutively by flow cytometry, we observed a trend: T-cell fractions slightly increased, whereas monocyte fractions decreased continuously within less than 20 min (Figure 2). In this time, the tubes were situated in the carousel of the cytometer at room temperature. As a consequence of this, we investigated the analytical stability of the samples after staining. Samples were analysed immediately after staining and after up to 52 min of storage at room temperature and on ice (Figure 3, data combined for both temperatures, n = 14). The reduction of monocytes was clearly visible during storage at room temperature within as little as 30 min (36%), and the decrease was greater at room temperature (48%–63%) than on ice (33%–50%). The content of T cells was almost constant at both temperatures, with a maximum 3.4% increase after 52 min.

In addition to the analytical stability of stained samples, we analysed the pre-analytical stability of analytes stored at room temperature (n = 6) or on ice (n = 8). Clear changes in MNC vitality were observed during storage (Figure 4). Apoptotic and dead T-cell fractions increased by 19% and 81%, respectively, after 4 h of storage on ice. The change was even more pronounced at room temperature (median: 88% and 263%, respectively). In contrast, apoptosis of monocytes was not observed in samples stored for up to 4 h at room temperature or on ice.

Regarding changes in monocyte content during storage, there was no significant reduction of cells stored for up to 4 h. Even incubating the cells from the ECP product for up to 72 h at 37°C in polystyrene tubes resulted in no relevant decrease in monocyte content (data not shown). This seems to be in contrast to our previous analytical stability tests of stained samples, where an approximately 60% decrease in monocytes was detected following storage for up to 52 min after staining. In pre-analytical stability testing of samples stored for up to 4 h before the apoptosis assay, there was no obvious decrease in monocyte content, even at room temperature.

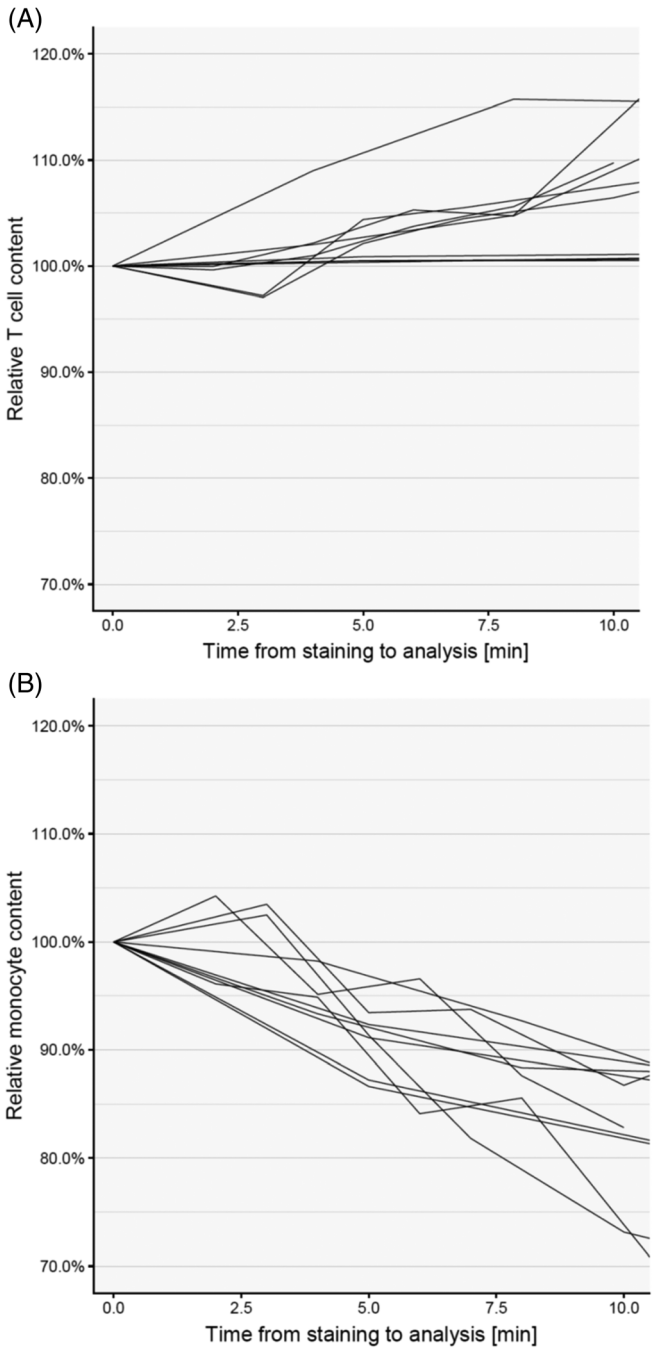

FIGURE 2 Analytical stability influences content of T cells (A) and monocytes (B) at room temperature. The varying content of T cells and monocytes in relation to leukocytes in stained photopheresis samples is regarded over a period of 10 min

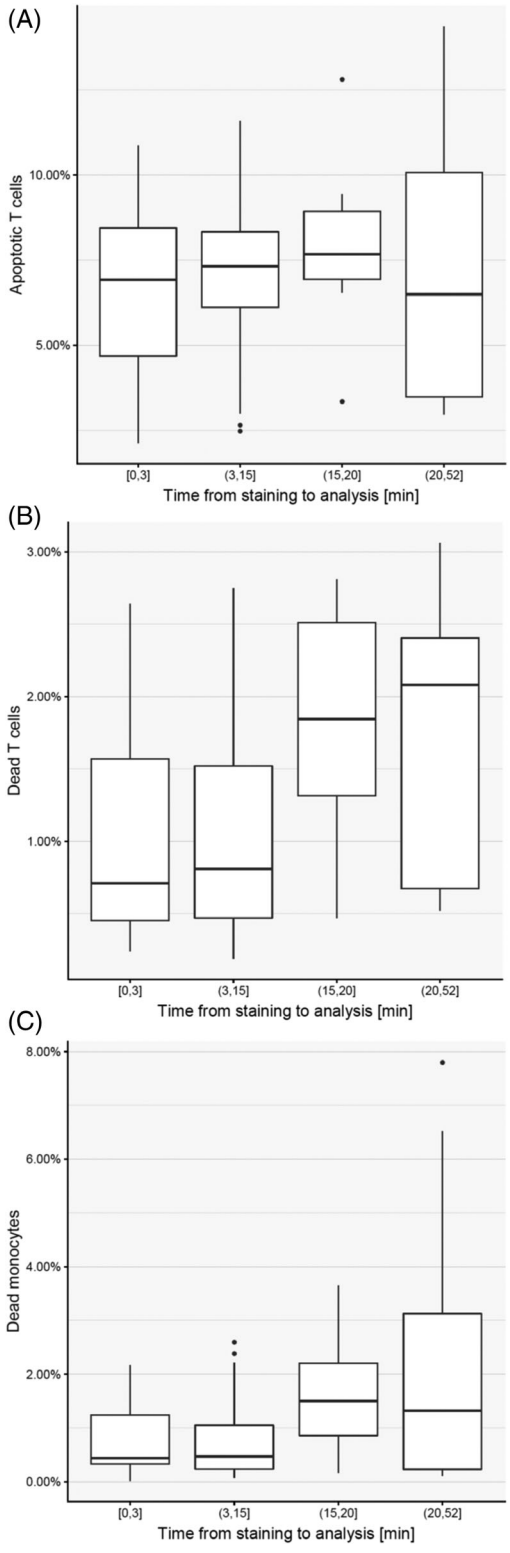

FIGURE 3 Time from staining to analysis (min) in the flow cytometer is shown as analytical stability of apoptosis in T cells (A, B) and monocytes (C). After 0–3 min (n = 22), there were 6.93% apoptotic T cells, 0.71% dead T cells and 0.44% dead monocytes. After 3–15 min (n = 37), 7.32% apoptotic T cells, 0.81% dead T cells and 0.47% dead monocytes were detected. After 15–20 min (n = 12), there were 7.67% apoptotic T cells, 1.85% dead T cells and 1.50% dead monocytes. After 20–52 min (n = 26), 6.50% apoptotic T cells, 2.08% dead T cells and 1.30% dead monocytes were analysed (median values)

However, the intended apoptosis of MNCs was obtained by incubating the cells for up to 72 h at 37°C and 5% CO_2. Apoptosis (in post/pre samples, each n = 3) was induced after 48 h (rates: 47%–163% for $CD3^+$ Annexin V^+ 7-AAD^+, 69%–336% for $CD3^+$ Annexin V^+ 7-AAD^- and 68%–115% for $CD33^+$ Annexin V^+ 7-AAD^+) and continued for up to 72 h.

3.3 | Precision

For repeatability assessment, samples (n = 6) were stained and analysed sixfold by the same person. The median coefficient of variation (CV) for $CD3^+$, $CD33^+$ and $CD3^+$ Annexin V^+ 7-AAD^- cells was 3.86%, 6.9% and 7.08%, respectively. Cell populations with small (<5%) dead T-cell and $CD33^+$ cell fractions had larger CV values.

As T cells exhibited high analytical stability over the analytical period, this cell population was suitable for comparative analysis of the two cytometers. Inter-operator variability was detected on both cytometers (n = 6): median CVs for T cells were 4.8% (0.01%–13.27%), 6.02% (5.86%–6.18%) and 1.01% (0.12%–8.68%), and deviation was <20%.

Comparative analysis revealed that the size of the $CD3^+$ fraction was 25.7%–55.7% (pre) and 24.1%–56.0% (post) according to Navios and 28.3%–48.5% (pre) and 26.5%–45.9% (post) according to Navios Ex. The maximum CV for T cells was 4.79% with Navios Ex and 10.77% with Navios. Accordingly, the overall CV was 4.8% (median), which indicated high comparability between operators on both cytometers. Regarding $CD33^+$ cells, the post samples had a CV of 16.75%, which confirmed the analytical instability of the monocytes, as was already described.

Obviously, cell counts determined using Navios Ex showed lower variation than those obtained with Navios (Figure 5). The new and more precise cuvette design of the flow chamber of the Navios Ex could be the reason for this. Both systems met the specification requirement of CV ≤15%. The condition for comparability of Navios Ex and Navios was met, as demonstrated by the correlation coefficient (r) of r = 0.974 for $CD3^+$ values and r = 0.778 for $CD33^+$ values (p < 0.01).

4 | DISCUSSION

ECP could be performed with the so-called "in-line" technique where apheresis and irradiation are performed by the same device or "offline" with separate machines for apheresis and irradiation. Offline photopheresates are medicinal products[20] that require quality control. This may include cellular composition, haematocrit and residual 8-MOP and should comprise a functional assay that relates to ECP's potency. As the apoptosis of lymphocytes is a well-known mechanism of photopheresis,[17] we designed the present study to evaluate the validity and GMP compliance of an apoptosis assay for MNCs based on flow cytometry with Annexin V and 7-AAD staining.

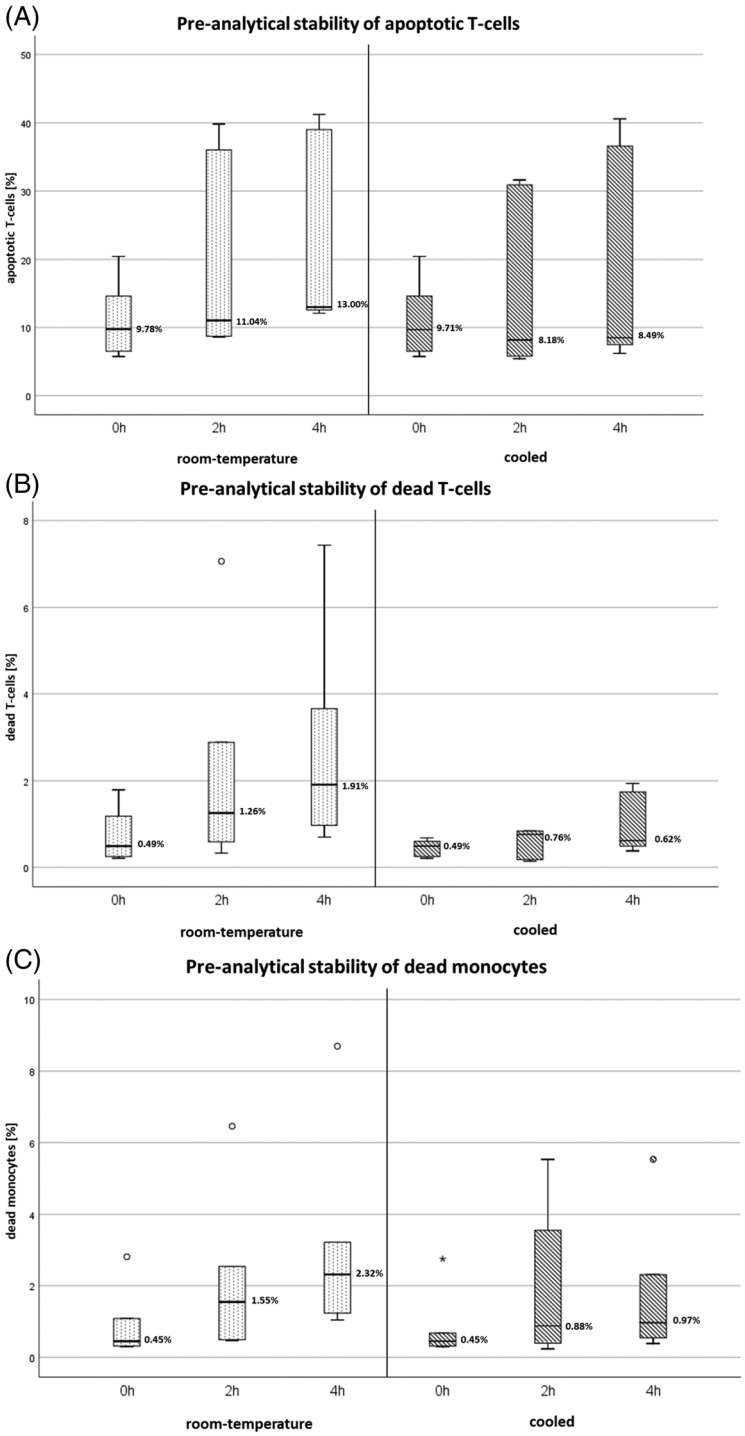

FIGURE 4 Pre-analytical stability of T cells (A, B) and monocytes (C) (%) is shown at room temperature (n = 6) and cooled on ice (n = 8). Stability of photopheresis samples was analysed at two temperatures before staining. There was no significant difference between both temperatures (p > 0.05)

A panel of antibodies developed for this purpose was used to analyse the apoptosis of T cells and monocytes. Isotype controls have been used traditionally as gating controls, and FMO controls are used when gating real positive events.[21,22] Gating boundaries for this assay were set up with fresh samples. This excludes autofluorescence signals with fresh cells. However, increased autofluorescence signals of aged cells were partially included in T-cell and monocyte gating. This strategy, designed to prevent loss of dead cell material, could have resulted in false high apoptosis signals. However, because we reported the results as the difference between treated and untreated cells, confounding as a result of this should have been marginal. In addition, Annexin V exhibited unspecific binding of myeloid cells, possibly because of platelets sticking to monocytes with a high affinity to Annexin V.[23-25] Thus, we limited the analysis to Annexin V^+ 7-AAD^+ monocytes.

In the course of validation testing, we determined that the analytical stability of the photopheresis samples was a crucial parameter. After analysing a batch of stained samples, we noticed that changes in cell content had occurred. A loss of monocytes was observed over the

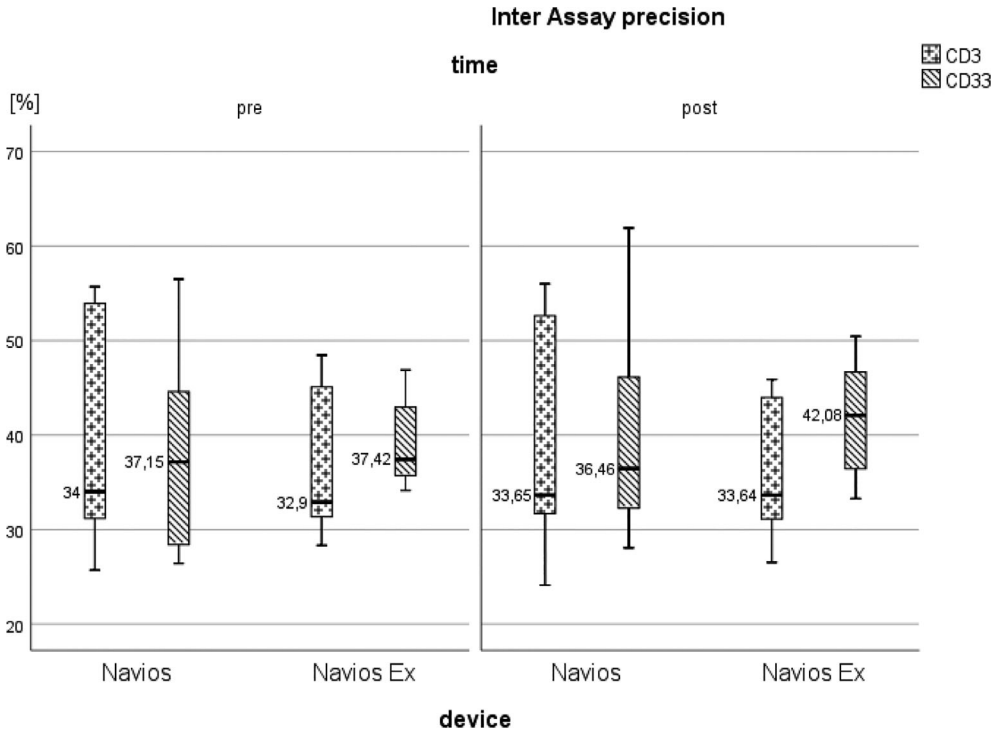

FIGURE 5 Comparison of CD3 and CD33 values (%) of fresh photopheresis samples (pre and post) determined using the Navios versus Navios Ex flow cytometers

analytical period of up to 52 min, including stand-by time in the flow cytometer. This loss was likely a result of monocyte adhesion to plastic material. Furthermore, stained cells might be susceptible to activation by agonistic antibodies, making them less robust during storage. Hence, stained samples should be analysed immediately after staining, and stand-by time in the machine should be taken into account. Considering the stained ECP samples prior to isotype controls in the flow cytometer could avoid cell changes and be appropriate for routine analysis. Regarding pre-analytical stability, proceeding apoptosis was found. Thus, staining and analysis should be performed immediately, and samples should be stored on ice for limited periods only.

For precision testing, $CD3^+$ cells were preferentially used because of their stability over the analytical period. Regarding intra-assay precision, the CV for CD3 cells was 3.86%, which meets the internal specification of <15%. Comparison of the two flow cytometers showed that Navios Ex achieves lower variability. This is possibly due to the improved cuvette design of the flow chamber of the Navios Ex compared with that of the Navios. Considering differences in personnel, flow cytometers and pre- and post-ECP samples, our validation study results demonstrated that both systems achieve a CV of <15% and a deviation of <20% and confirmed their high-intermediate precision.

The onset of apoptosis following ECP could be detected after 48 h of incubation. Cultivation of cells for more than 48 h resulted in no relevant increase in apoptosis. This is in accordance with the findings of other groups[16] that considered an apoptosis induction of >15% at an incubation time of 24–48 h to be a successful ECP.

In this assay, we considered both T-cell and monocyte apoptosis. The latter is considered controversial as monocytes seem to be less sensitive for apoptosis induction. It is believed that irradiated monocytes may differentiate into DCs. Monocytes could therefore be detracted from apoptosis calculation.

In summary, flow cytometry with Annexin V and 7-AAD staining detected the vitality of MNCs at the GMP level and yielded valid results as determined in this validation study considering the pre-analytical and analytical stability of samples kept under different storage conditions for different times. For routine quality control, we focussed on $CD3^+$ T-cell apoptosis after 24–48 h as a convenient potency marker.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Viola Hähnel, Frauke Dormann and Norbert Ahrens conceptualized the study; Viola Hähnel, Frauke Dormann, Katharina Kronenberg and James A Hutchinson analysed the data; Viola Hähnel, Ralph Burkhardt and Norbert Ahrens wrote the manuscript; and all authors edited the manuscript.

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