CD71 surface analysis of T cells: a simple alternative for extracorporeal photopheresis quality control

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Background Extracorporeal photopheresis (ECP) is a leukapheresis-based cellular therapy that is used with increasing frequency worldwide to treat various T-cell-mediated diseases. Currently, the inhibition of T-cell proliferation after photopheresis is analysed frequently using time-consuming assays including radioactive thymidine assays or carboxyfluorescein succinimidyl ester (CFSE) staining. We investigated whether simple surface T-cell staining using surrogate markers of T-cell proliferation can replace time-consuming measurement of T-cell proliferation in ECP quality control.

Study design and methods T-cell activation markers were investigated by flow cytometry after ECP. Candidates were validated by direct comparison with the classical CFSE T-cell proliferation inhibition test and apoptosis staining. Finally, surface T-cell staining was performed in patient samples in comparison with classical methods.

Results CD71 expression exhibited the fastest and most robust upregulation, which was detectable as early as 6–8 h after T-cell stimulation and almost completely abrogated by ECP. In a direct comparison with the CFSE T-cell proliferation assay, suppression of CD71 expression after ECP was almost identical and detectable as early as 16 h after stimulation in peripheral blood mononuclear cells of healthy donors. Furthermore, in direct comparison with classical apoptosis staining, the inhibition delta of CD71 after ECP was significantly higher. Moreover, in patients under T-cell suppressive therapy, T-cell-dependent CFSE and CD71 assays exhibited decreased sensitivity to detect ECP treatment and were inferior in comparison to apoptosis staining.

Conclusion Surface CD71 analysis represents a very simple quality control alternative to detect ECP-mediated T-cell proliferation inhibition in normal PBMC samples devoid of T-cell suppressive drugs.

Key words: extracorporeal photopheresis, CD71, flow cytometry.

Introduction Extracorporeal photopheresis (ECP) is an immunomodulatory cellular therapy that has been used successfully for the first-line treatment of erythrodermic cutaneous T-cell lymphoma (CTCL) [1–3] for more than 30 years. Several studies demonstrated that ECP promotes different immunomodulatory effects including induction of regulatory T cells as well as modulation of dendritic cell activation [4,5]. Since UVA-associated apoptosis induction is a central component of the various clinically used photopheresis methods, reliable and simple quality control is essential [6]. Over time, the range of indications has been continuously expanded, so that ECP is now performed in other T-cell-mediated diseases, including chronic graft-versus-host disease (GvHD) [3,7,8] and allograft rejection after organ transplantation [9–14]. The ECP procedure is
a combination of leukapheresis followed by cell exposure to 8-methoxypsoralen (8-MOP), followed by UVA. It involves the ex vivo collection of leucocytes by apheresis, exposure to the photosensitizing agent 8-MOP plus UVA light and finally, reinfusion into the patient [15]. Although ECP is currently being performed in over 200 centres worldwide [16], there is no consensus on standardized cell-based quality control [17]. Since 2003, various methods for ECP quality control have been described, such as analysis of proliferation inhibition by using $^3$H-thymidine [6,18,19] and CFSE (5,6-carboxy fluorescein diacetate succinimidyl ester) [6,18,20] or measurement of ECP-induced cell apoptosis [20,21]. Due to its use of radioactive material, more recent studies avoided the $^3$H-thymidine assay [20,21]. CFSE-based proliferation assays require labelling of leucocytes and a minimum period of 3 days after T-cell stimulation and are therefore relatively complex and time-consuming methods. The principal disadvantage of T-cell proliferation assays is the possible impaired proliferation capacity of T cells in patients receiving immunosuppressants or other antiproliferative drugs [18,21]. These aspects demonstrate that no ideal test procedures are currently available to analyse T-cell proliferation inhibition after ECP. However, apoptosis quantification could represent a possible alternative, as indicated in recent studies [20,21]. Here, we have investigated a novel approach: analysis of the suppression of surrogate markers for T-cell proliferation after ECP. Furthermore, we compared the results with classical ECP quality control assays including the CFSE T-cell proliferation test and apoptosis quantification. Finally, to address the possible confounding impact of immunosuppressive drugs or other patient-related factors, we performed our analyses in peripheral blood mononuclear cells (PBMCs) from both healthy donors and clinical patient samples.

Materials and methods

**PBMC isolation**

Peripheral blood mononuclear cells were derived by density gradient centrifugation [22] (Biocoll separating solution, Biochrom) from buffy coats (BCs) of blood donors after informed consent (ethics approval file No. 05/00). PBMC (2 x 10$^6$/ml) was cultivated in RPMI 1640 (Anprotect) supplemented with heat-inactivated foetal bovine serum (FBS; 10%, Biochrom), non-essential amino acids (1 x, Sigma-Aldrich Chemie), sodium pyruvate (1 mm, Thermo Fisher Scientific), HEPES (10 mm, Thermo Fisher Scientific) and penicillin/streptomycin (50 Units/ml and 5 μg/ml, Thermo Fisher Scientific).

**Experimental photopheresis**

Peripheral blood mononuclear cells were incubated for 30 min (37°C) with 8-MOP (300 ng/ml, prepared by the hospital pharmacy) and exposed to UVA light (2 J/cm$^2$) in an irradiation chamber (Bio-LINK® BLX-365, Vilber Lourmat) in a 12-well plate in 1 ml. UVA dose was controlled independently by using a calibrated UVA dosimeter (RM-21, Dr. Gröbel UV-Elektronik). The interval between irradiation and cell stimulation was 2 h unless otherwise stated. Results were validated on PBMCs of 11 healthy blood donors. In all samples, haematocrit was <0.2%; mean lymphocyte content was 71% (range: 36.5%–97.4%).

**Patient photopheresis**

Patient ECP was carried with either the Spectra-Optia apheresis system (Terumo BCT) or the MCS + System (Haemonetics) with the corresponding tube systems. Apheresate cells were diluted with saline and incubated with 8-MOP (200 ng/ml) for 15 min and UVA-irradiated (MacoGenic G2, Macopharma) with 2 J/cm$^2$. Patient samples were examined after ethics approval (file No: 136/10) and the patients’ informed consent. ‘Pre-ECP’ (no 8-MOP/ UVA) and ‘Post-ECP’ samples (after irradiation) were washed twice with PBS and cultured (2 x 10$^6$ PBMC/ml) for 26 h in cell culture medium (‘rest period’) before stimulation in an incubator at 37°C with 5% CO$_2$ and 95% relative humidity. Patients (10 ECP procedures; 6 different patients) were either diagnosed with chronic lung transplant rejection or GvHD. All patients received two or more different immunosuppressive including prednisolone, Tacrolimus (calcineurin inhibitor), mycophenolate mofetil or Everolimus (mTOR inhibitor). Mean haematocrit in the tested samples was 1.5% (range: 0.5%–4.9%), and mean lymphocytes content was 43% (range: 30.9%–60.4%).

**Carboxyfluorescein succinimidyl ester staining and T-cell analysis**

Peripheral blood mononuclear cells were centrifuged to remove any culture medium residues. The cell pellet was resuspended in 1 ml PBS, mixed with CFSE (0.5 μM final concentration) and incubated in a water bath for 10 min at 37°C with gentle mixing. For patient samples, a higher CFSE concentration (1 μM) was chosen due to lower dye uptake. Labelling was blocked by addition of warm culture medium (37°C) supplemented with 10% FBS. The samples were centrifuged (at 400 g for 5 min), and warm culture medium was added for 15 min in a water bath at 37°C. Staining was monitored 1 day later in
unstimulated CFSE-stained cells by flow cytometry. The determination of the percentage of division (PD) of all CFSE-stained cells was quantitated with the following formula reported by Faivre et al. [6] (Fig. 1a):

\[
PD(\%) = 100 \times \frac{DIV(\text{events})}{DIV(\text{events}) + UnDIV(\text{events})}
\]

to block nonspecific binding. Monoclonal antibody staining was carried out with CD4, CD8 and CD71 (BioLegend) unless stated otherwise, for 15 min at RT in the dark. Cells were washed and either analysed or prepared for apoptosis staining. To quantify the effect of photopheresis on lymphocyte activation, the difference between pre- and post-photopheresis samples was analysed by calculating the change in expression (\(\Delta\) Expr) between pre- and post-photopheresis values.

**T-cell stimulation**

T-Cell stimulation was performed in 96-well plates (flat bottom, Sarstedt Cell+) coated with CD3 (Clone UCHT1, Bio Legend) and soluble CD28 (Clone CD28.2, BioLegend; each 1 \(\mu\text{g/ml}\) in 200 \(\mu\text{l}\) or with PHA (4 \(\mu\text{g/ml}\), Sigma), Con A (2.5 \(\mu\text{g/ml}\), Sigma), when indicated. To samples from healthy blood donors, 100 U/ml IL-2 (Novartis Pharma) was added. After stimulation, cells and unstimulated controls were incubated for up to 4 days, depending on the experimental set-up.

**FACS analysis**

Cells were analysed with a FACS Canto II Flow Cytometer (BD Bioscience). Samples (125 \(\mu\text{l}\)) were transferred into FACS tubes (Sarstedt) and incubated with 10% vol/vol immunoglobulin G for 10 min at room temperature (RT) to block nonspecific binding. Monoclonal antibody staining was carried out with CD4, CD8 and CD71 (BioLegend) unless stated otherwise, for 15 min at RT in the dark. Cells were washed and either analysed or prepared for apoptosis staining. To quantify the effect of photopheresis on lymphocyte activation, the difference between pre- and post-photopheresis samples was analysed by calculating the change in expression (\(\Delta\) Expr) between pre- and post-photopheresis values.

**Apoptosis quantification**

Apoptotic cells were quantified by staining with Annexin-V (BioLegend) and SYTOX Dead Cell Stain (Thermo Fisher Scientific) (Fig. 1b) in 100 \(\mu\text{l}\) of Annexin binding buffer containing calcium and 1 \(\mu\text{l}\) of Annexin-V (stock 4 \(\mu\text{g/ml}\)) for 10 min at RT. SYTOX was added 5 min prior to FACS analysis, and Annexin-V+ cells were referred to as ‘early apoptotic cells’. Annexin-V+/SYTOX+ or Annexin-V+/SYTOX+ were referred to as ‘late apoptotic cells’. Total apoptotic cells were determined by summing the early and late apoptotic cells.

**Statistics**

Analyses were performed using GraphPad Prism software (version 5.02). Figures show mean values and standard

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Fig. 1 Gating strategy for CD71 staining (a) and apoptosis analysis (b) of early (Sytox\textsuperscript{neg} Annexin\textsuperscript{pos}) and late (Sytox\textsuperscript{neg} Annexin\textsuperscript{pos}) apoptotic cells in T-cell subsets.
errors (SEM). Significances were tested with a two-sided t-test, one-way ANOVA or two-way ANOVA. Subsequent post-tests are indicated in the respective legends. A probability of $P < 0.05$ was considered significant.

Results

Ki-67 suppression by photopheresis

Ki-67 is a well-known cell proliferation marker that is used for different cell populations, including tumour cells [23]. Regarding Ki-67 upregulation, we analysed different T-cell stimulants, including phytohaemagglutinin (PHA), CD3/28 and concanavalin A (Con A; Fig. 2a). CD3/28 and Con A stimulation induced the strongest Ki-67 upregulation ($P < 0.001$ vs. unstimulated control) (Fig. 2a). Next, we investigated the suppression of Ki-67 upregulation by photopheresis. Photopheresis significantly impaired Ki-67 expression 22 h after stimulation in CD4$^+$ and CD8$^+$ T cells. (Fig. 2b/c). Furthermore, no notable Ki-67 upregulation was observed in unstimulated controls. These data suggest that Ki-67 expression analysis might represent, first and foremost, a suitable and rapid quality control marker for ECP. However, one drawback of Ki-67 expression analysis for ECP quality control is the relatively low and slow upregulation in stimulated samples. After combined Con A 2.5/CD3/CD28 stimulation, less than 10% of the T cells were positive for Ki-67 within 22 h.

CD71, CD25 and CD69

Since Ki-67 expression analysis was not ideal for ECP quality control, we investigated other mid- to early-expressed markers of T-cell activation, such as CD25, CD69 and CD71 [24,25]. For this purpose, PBMCs from blood donors underwent experimental photopheretic treatment with subsequent stimulation, and the kinetics of CD71, CD25 and CD69 upregulation were determined. Our data show a strong and rapid upregulation of these markers both in CD4$^+$ and CD8$^+$ T cells within 22 h ($P < 0.001$), indicating their suitability as quality control markers for photopheresis (Fig. 3a–d).

As described in detail [24–26], CD69 is rapidly upregulated after T-cell stimulation. After only 4 h of stimulation, pronounced CD69 expression of CD4$^+$ and CD8$^+$ T cells was detectable (Fig. 3a). In addition, markedly reduced CD69 expression in UVA/8-MOP-treated CD4$^+$ and CD8$^+$ T cells was already recognizable 4 h after stimulation ($P < 0.001$; Fig. 3a). However, further analyses revealed the inferior robustness of CD69 expression with respect to environmental effects (variation in the serum added to the medium) when compared with CD71. Our experiments revealed a marked dependency and variation of CD69 expression with respect to medium composition and UVA exposure in unstimulated T cells (Fig. 3e). A change of the serum manufacturer (serum 1 versus serum 2) led to a marked increase in CD69 expression in UVA-treated but unstimulated CD4$^+$ T cells ($P < 0.01$). Also, in serum-free medium (vs. medium containing serum 1), a marked increase in CD69 expression after irradiation was observed ($P < 0.05$). In contrast, CD71 expression was neither affected by differences in serum manufacturers nor by the omission of serum from the culture medium (Fig. 3f). These data suggest that CD69 expression is affected by cell culture additives and is therefore not optimal for quality control purposes.

In a direct comparison with CD71 and CD69, CD25 exhibited slower upregulation in stimulated CD4$^+$ cells.

Fig. 2 Ki-67 expression after different T-cell stimulation modes (a) and suppression after experimental photopheresis (b/c). Interval between irradiation and cell stimulation was 30 min; cultivation period of 24 h (a) and 22 h (b/c); (a): One-way ANOVA with Dunnett’s post-test, $P$ value vs. unstim; (b/c): Two-way ANOVA with Bonferroni post-test; unstim, unstimulated; PHA, Phythamagglutinin; Con A, Concanavalin A; MFI, mean fluorescence intensity; exP, experimental photopheresis.
An obvious effect of photopheresis was visible 22 h after stimulation ($P < 0.001$). In contrast, irradiated CD8$^+$ T cells expressed higher CD25 baseline levels than controls up to 8 h after stimulation. The best results were obtained with the surface marker CD71 due to its consistency and reliability. Over the entire culture period, CD71 expression was very high in stimulated controls, in contrast with the low or nearly absent CD71 expression observed in UVA/8-MOP-treated CD4$^+$/CD8$^+$ T cells (Fig. 3c). As early as 6–8 h after stimulation, stimulated control CD4$^+$ cells showed a marked increase in CD71 expression with a dominant and marked inhibitory effect of photopheresis ($P < 0.001$; Fig. 3c). Expression of CD71 by CD8$^+$ T cells was slightly delayed after stimulation, whereas a pronounced difference from the irradiated group was detectable after 22 h of stimulation ($P < 0.001$). To determine the earliest possible time-point and a late time-point at which CD71 could be suitable as a quality control marker for photopheresis, we analysed CD71 expression 7 h, 16 h and 4 days after stimulation. These experiments revealed strong suppression of CD71 as early as 16 h after stimulation in photopheresis-treated CD4$^+$ and CD8$^+$ T cells ($P < 0.001$; Fig. 3d), remaining stable for up to 4 days ($P < 0.001$) (Fig. 3d). CD71 expression was also not affected by culture medium components (Fig. 3f). In contrast, CD69 expression exhibited marked variability under different culture medium conditions (Fig. 3e).

**Validation of the CD71 assay and comparison with the CFSE proliferation assay**

CD71-based measurement as a marker of proliferation inhibition after experimental photopheresis was validated using samples from 11 blood donors. For each column (pre- and post-exP), proliferation levels after CD3/CD28/IL-2 stimulation are displayed as relative values (Delta PD) (Table 1). Absolute values (percentage of division; PD) were also collected but are not displayed. Our results demonstrated marked suppression of CD71 expression in all tested samples of stimulated PBMCs after experimental photopheresis. CD4$^+$ T cells showed a mean $\Delta{\text{Expr}}$ of 45.3% (range: 36.4%–54.9%) after a stimulation period of 16 h (Table 1/Fig. 4a), which increased to 70.8% (range: 40.6%–88.3%) 4 days after stimulation (Table 1/Fig. 4b). As in preliminary experiments, CD8$^+$ T cells showed a slightly slower $\Delta{\text{Expr}}$ after experimental photopheresis. The mean $\Delta{\text{Expr}}$ was 34.0% (range: 16.1%–70.8%), increasing to a mean of 61.7% (range: 36.1%–81.0%) 4 days after stimulation. Inhibition of T-cell proliferation after
### Table 1 Comparison of absolute and relative inhibition of CD71/CFSE expression in CD4⁺/CD8⁺ T cells of blood donors

|   | CD71 Assay (CD4⁺) | CFSE Assay (CD4⁺) | CD71 Assay (CD8⁺) | CFSE Assay (CD8⁺) |
|---|-------------------|-------------------|-------------------|-------------------|
|   | 16 h after stimulation | 4 days after stimulation | 16 h after stimulation | 4 days after stimulation |
|   | Pre-ExP | Post-ExP | Δ Expr | Inh (%) | Pre-ExP | Post-ExP | Δ Expr | Inh (%) | Pre-ExP | Post-ExP | Δ Expr | Inh (%) | Pre-ExP | Post-ExP | Δ Expr | Inh (%) |
| n | PD (%) | PD (%) | Δ Expr | Inh (%) | PD (%) | PD (%) | Δ Expr | Inh (%) | PD (%) | PD (%) | Δ Expr | Inh (%) | PD (%) | PD (%) | Δ Expr | Inh (%) |
| 1 | 82.7 | 28.6 | 54.1 | 65.4 | 85.2 | 8.3 | 76.9 | 90.3 | 77.0 | 6.9 | 70.1 | 91.1 |
| 2 | 54.5 | 12.9 | 41.6 | 76.3 | 91.2 | 2.9 | 88.3 | 96.8 | 79.6 | 11.2 | 68.4 | 85.9 |
| 3 | 79.7 | 26 | 53.7 | 67.4 | 86.2 | 8.5 | 77.7 | 90.1 | 73.4 | 11.6 | 61.8 | 84.2 |
| 4 | 72.6 | 36.2 | 36.4 | 50.1 | 89.7 | 4.4 | 85.3 | 95.1 | 80.7 | 8.3 | 72.4 | 89.7 |
| 5 | 87 | 38.6 | 48.4 | 55.6 | 86.4 | 5.1 | 81.3 | 94.1 | 81.9 | 7.3 | 74.7 | 91.1 |
| 6 | 90.4 | 35.5 | 54.9 | 60.7 | 91.3 | 16.4 | 74.9 | 82.0 | 89.3 | 9.9 | 79.4 | 88.9 |
| 7 | 54.9 | 14.3 | 40.6 | 74.0 | 43.8 | 3.2 | 40.6 | 92.7 | 40.6 | 3.1 | 37.5 | 92.3 |
| 8 | 85.5 | 46.6 | 38.9 | 45.5 | 68.4 | 11.4 | 5.07 | 83.3 | 62.8 | 13.9 | 48.9 | 77.9 |
| 9 | 74.8 | 30.7 | 44.1 | 59.0 | 94.2 | 21.3 | 72.9 | 77.4 | 75.6 | 8.0 | 67.7 | 89.4 |
| 10 | 74.3 | 32.8 | 41.5 | 55.9 | 52.8 | 7.5 | 45.3 | 85.8 | 41.1 | 15.2 | 25.9 | 63.1 |
| 11 | 70.1 | 26.3 | 43.8 | 62.5 | 85.2 | 6.2 | 79.0 | 92.7 | 57.6 | 1.1 | 56.5 | 98.1 |
| M | 75.1 | 29.9 | 45.3 | 61.1 | 75.9 | 8.7 | 70.8 | 89.1 | 69.1 | 8.8 | 60.3 | 86.5 |
| SD | 11.9 | 10.0 | 6.5 | 9.4 | 16.9 | 5.7 | 16.0 | 6.2 | 16.5 | 4.2 | 16.7 | 9.3 |

PD (%) CFSE: Percentage of division of CFSE⁺ cells = 100 × divided events/(divided events + undivided events); number of events was displayed in FACS analysis.

PD (%) CD71: Percentage of division of CD71⁺ cells = CD4⁺ CD71⁺ or CD8⁺ CD71⁺ T cells; displayed in FACS analysis.

Δ Delta PD (%) = Pre- or Post-exP stimulated PD – Pre- or Post-exP unstimulated PD.

Δ Expr: Δ Expression = Pre-exP Delta PD% – Post-exP-Delta PD%

Inh (%): Percentage of inhibition: 100 – (Post-ECP Delta PD/Pre- ECP Delta PD) × 100.

M, Mean; SD, Standard deviation; exP, experimental photopheresis.

Photopheresis was confirmed by labelling the same samples with CFSE fluorescent dye and FACS analysis (Table 1/Fig. 4c). CD4⁺ T cells showed a ΔExpr of 60-3% with a range of 25-9%–79.4% 4 days after stimulation. Analogously to the CD71 analysis, CD8⁺ T cells presented a slightly reduced ΔExpr of 51-2% (range: 26-6%–68-7%) in the CFSE assay. Direct comparison of results obtained 4 days after stimulation in
the CD71 and CFSE assays showed a strong correlation of \( \Delta \text{Expr} \) in CD4\(^+\) \( (r = 0.8717; \ P = 0.0005) \) and CD8\(^+\) T cells \( (r = 0.9114; \ P < 0.0001) \), indicating that the simple CD71 assay may be suitable to replace the CFSE proliferation assay for quality control in photopheresis (Fig. 4d).

**Comparison of CD71 and apoptosis analysis**

Finally, the utility of CD71 expression analysis in PBMC was directly compared to that of apoptosis quantification, since several laboratories have used apoptosis detection as a quality control method in photopheresis. The photopheresis-induced increase in total apoptosis in CD4\(^+\) and CD8\(^+\) T cells was significant after 16 h of culture \( (P < 0.001 \text{ for CD4}^+ \text{ and } P < 0.01 \text{ for CD8}^+) \text{; data not shown}) \). Comparison of overall induced \( \Delta \text{CD71} \) and \( \Delta \text{Apoptosis} \) in PBMCs after photopheresis indicated the superiority of CD71 analysis to detect the effect of photopheresis in non-patient samples, especially for CD4\(^+\) T cells \( (P < 0.001) \) (Fig. 4e).

**Prospective CD71 analysis in patient photopheresis samples and comparison with the standard CFSE fluorescence proliferation test**

Photopheresis patients frequently take different immunosuppressive drugs targeting T-cell proliferation or activation. In these patients, stimulation-dependent quality control tests sometimes cannot indicate photopheresis-dependent proliferation inhibition [18]. Therefore, applicability of the new CD71 assay to patients was prospectively validated on clinical ECP procedures and directly compared to the 'gold' standard CFSE proliferation assay. All patients received combinations of different immunosuppressive drugs. The resting period between ECP and T-cell stimulation was extended to 26 h due to the observation of a delayed photopheresis effect in preliminary results with patient samples. CD71 expression was analysed 16 h and 3 and 4 days after stimulation (Table 2). In patient samples, a stimulation time of 16 h was not optimal to obtain a clear \( \Delta \text{Expr} \) of CD71 in CD4\(^+\) (mean: 8.4\%, SD: 4.8\%) and CD8\(^+\) T cells (mean: 6.0\%,

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### Table 2: Comparison of absolute and relative inhibition of CD71/CFSE expression in CD4⁺/CD8⁺ T cells of ECP patients

#### CD71 Assay (CD4⁺)

|         | 16h after stimulation | 3 days after stimulation | 4 days after stimulation | 4 days after stimulation |
|---------|------------------------|--------------------------|--------------------------|--------------------------|
|         | Pre-ECP Delta PD (%)   | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    |
| Pat/ECP | Δ Expr (%)             | Inh (%)                  | Δ Expr (%)               | Inh (%)                  | Δ Expr (%)               | Inh (%)                  | Δ Expr (%)               | Inh (%)                  |
| 1a      | 15.8 12.8 3 19.0       | 18.9 17.6 1.3 6.9        | 20.8 12.6 8.2 39.4      | 9.9 4.4 5.5 55.8        |
| 2b      | 14.9 12.5 2.4 16.1     | 21.6 14.9 6.7 31.0       | 20.9 8.9 12 57.4        | 10.6 3.9 6.7 63.6       |
| 3c      | 73.9 62.1 11.8 16.0    | 94.9 64.4 30.5 32.1      | 79.2 50.2 29 36.6       | 22.0 3.7 18.3 83.0      |
| 4c      | 83.6 66.7 16.9 20.2    | 98.3 57.8 40.5 41.2      | 83.3 58.0 25.3 30.4     | 39.3 3.7 35.6 90.6      |
| 5d      | 24.1 16.2 7.9 32.8     | 42 5 37 88.1             | 17.9 3.7 14.2 79.3      | 6.9 4.3 2.6 37.6        |
| 6d      | 28.9 13.7 15.2 52.6    | 67 4.1 62.9 93.9         | 72.3 3.0 69.3 95.9      | 57.9 7.6 50.3 86.8      |
| 7e      | 57 48.7 8.3 14.6       | 86.4 52.1 34.3 39.7      | 59.9 46.3 13.6 22.7     | 7.9 0.1 7.8 99.2        |
| 8e      | 58.4 51.9 6.5 11.1     | 95.8 60.9 34.9 36.4      | 79.2 50.2 29 36.6       | 23.8 0.0 33.8 100.0     |
| 9f      | 75.4 69.9 5.5 7.3      | 90.7 70.7 20 22.1        | 79.9 61.0 18.9 23.7     | 40.9 4.2 36.7 89.7      |
| 10f     | 83.3 76.6 6.7 8.0      | 95.4 75.3 20.1 21.1      | 78 65.3 12.7 16.3       | 61.4 13.9 47.6 77.4     |
| M       | 51.5 43.1 8.4 19.8     | 71.1 42.3 28.8 41.2      | 59.1 35.9 23.2 43.8     | 29.1 4.6 24.5 78.4      |
| 5D      | 28.0 26.5 4.8 13.6     | 31.9 28.4 17.7 28.2      | 27.9 25.6 17.8 26.0     | 20.7 3.9 18.3 20.2      |

#### CD71 Assay (CD8⁺)

|         | 16h after stimulation | 3 days after stimulation | 4 days after stimulation | 4 days after stimulation |
|---------|------------------------|--------------------------|--------------------------|--------------------------|
|         | Pre-ECP Delta PD (%)   | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    | Pre-ECP Delta PD (%)     | Post-ECP Delta PD (%)    |
| Pat/ECP | Δ Expr (%)             | Inh (%)                  | Δ Expr (%)               | Inh (%)                  | Δ Expr (%)               | Inh (%)                  | Δ Expr (%)               | Inh (%)                  |
| 1a      | 4.5 3.7 0.8 17.8       | 13.8 9.4 4.4 31.9        | 3.8 5.5 0.0 0.0         | 10.7 6.7 4.0 37.5       |
| 2b      | 2.7 1.6 0.1 40.7       | 8.0 6.7 1.3 16.3         | 2.8 3.1 0.3 0.0         | 2.8 2.0 0.8 29.9        |
| 3c      | 42.4 34.1 8.3 19.6     | 69.2 41.7 27.5 39.7      | 53.9 41.2 12 23.6       | 15.8 1.4 14.4 90.8      |
| 4c      | 59 49.8 9.2 15.6       | 93 36.5 56.5 60.8        | 57 40.9 16.1 28.2       | 42.4 7.6 34.9 82.2      |
| 5d      | 9.2 7.5 1.7 18.5       | 18.6 1.2 17.4 93.5       | 1.4 1.2 0.2 14.3        | 4.5 2.4 2.1 46.6        |
| 6d      | 7.3 1.6 0.5 78.1       | 49.6 1.5 48.1 97.0       | 44.5 1.0 43.5 97.8      | 43.4 5.5 37.9 87.2      |
| 7e      | 21.8 16.2 5.6 25.7     | 68.0 34.7 33.3 49.0      | 31.6 32.4 0.8 0.0       | 5.8 4.1 1.6 28.3        |
| 8e      | 17.5 13.2 4.3 24.6     | 86.3 38.4 47.9 55.5      | 49.1 21.7 27.4 55.8     | 13.8 0.9 12.9 93.3      |
| 9f      | 46.7 39 7.7 16.5       | 79.6 51.3 28.3 35.6      | 67.8 42.8 25 36.9       | 28.3 0.0 28.3 100.0     |
| 10f     | 63.7 48.5 15.2 23.9    | 92.9 57.9 35 37.7        | 60.9 52.2 8.7 14.3      | 69.5 29.2 40.3 58.0      |
### Table 2 (Continued)

| Pat/ECP | CD71 Assay (CD8<sup>+</sup>) | CFSE Assay (CD8<sup>+</sup>) |
|---------|--------------------------------|--------------------------------|
|         | 16 h after stimulation | 3 days after stimulation | 4 days after stimulation |
|         | Pre-ECP Delta % | Post-ECP Delta % | Δ Expr | Inh % | Pre-ECP Delta % | Post-ECP Delta % | Δ Expr | Inh % | Pre-ECP Delta % | Post-ECP Delta % | Δ Expr | Inh % |
| M       | 75.1 | 25.1 | 60.9 | 28.1 | 75.9 | 27.9 | 30.0 | 51.7 | 37.3 | 24.2 | 13.1 | 27.1 |
| SD      | 4.3 | 9.4 | 1.4 | 19.0 | 3.4 | 21.3 | 18.4 | 26.2 | 25.8 | 20.1 | 15.1 | 30.7 |

PD (%): CFSE: Percentage of division of CFSE<sup>+</sup> cells = 100 x divided events/(divided events + undivided events); number of events was displayed in FACS analysis.

ΔPD (%): CD71: Percentage of division of CD71<sup>+</sup> cells = CD4<sup>+</sup> CD71<sup>+</sup> or CD8<sup>+</sup> CD71<sup>+</sup> T cells; displayed in FACS analysis.

ΔExpr: ΔExpression = Pre-ECP Delta PD% - Post-ECP Delta PD.

Inh (%): Percentage of inhibition: 100 - (Post-ECP Delta PD% / Pre-ECP Delta PD%) x 100.

M, Mean; SD, Standard deviation.

Discussion

Here, we present data on a novel, simple alternative quality control assay for ECP using CD71 expression as a surrogate marker of T-cell proliferation. In addition to CD71, we have investigated K63, CD25, and CD69 as possible quality control markers for extracorporeal photopheresis.

The expression levels were systematically investigated after UV/8-MOP treatment in PBMCs isolated from blood donors and in patient samples. To validate our results, we performed experiments in patient samples. The kinetics of the induction of cell proliferation inhibition is required. Based on the data, we suggest a simple alternative quality control test.

Apoptosis detection represents an alternative to proliferation-dependent quality control tests. Finally, we investigated the suitability of apoptosis quantification in patient samples. The kinetics of the induction of apoptosis in patient samples were assessed in CD8<sup>+</sup> T cells and 64% of CD8<sup>+</sup> T cells showed a marked and non-significant increase in total apoptosis 24 h after ECP (Fig. 5a).

From 42 h after ECP, CD8<sup>+</sup> T cells showed a small and non-significant increase in total apoptosis (Fig. 5a). The percentage of CD8<sup>+</sup> T cells that showed a significant increase in total apoptosis after 24 h, 42 h, 4 days, and 5 days after ECP was determined (Table 3). The expression levels were systematically investigated after UV/8-MOP treatment in PBMCs isolated from blood donors and patient samples. The kinetics of the induction of cell proliferation inhibition is required. Based on the data, we suggest a simple alternative quality control test.
results, CFSE and apoptosis assays, representing accepted quality control methods [6,18,21], were also performed and directly compared. CD71 was found to be a highly reliable, rapid and simple test marker to display UVA/8-MOP-related effects. As early as 16 h after stimulation, sufficient T-cell activation and suppression after experimental photopheresis was detectable. The ability of CD71 to display T-cell proliferation has been documented in several previous studies [24,25,27,28]. In a direct comparison between Ki-67 and CD71, Motamedi et al. demonstrated that both markers display a comparable expression pattern after stimulation, and thus, the simpler CD71 measurement can replace the intracellular Ki-67 measurement beyond a certain stimulation time [24]. The slower expression of Ki-67 displayed in our results might be due to the de novo synthesis of the protein in the first cell cycle after stimulation [29]. The novel CD71 assay was subsequently validated in samples from blood donors. Overall, strong and uniform CD71 expression could be triggered in each sample after CD3/28/IL-2 stimulation, which was dramatically reduced in the UVA/8-MOP-treated samples. The CFSE assay was found to be a reliable test system, in agreement with previously reported results [20]. We observed a strong inhibitory effect in all tested samples of healthy donors similar to the results of Szczepiorkowski et al. [30]. In the context of ECP quality control, CD71 was examined for the first time in this study; therefore, no comparative values are available in the literature. Possible applications of the fast and simple CD71 assay are conceivable for the routine quality control of ECP procedures, as well as with respect to the effective validation of novel ECP equipment, such as tubing systems and apheresis machines. A major advantage of the CD71 assay is the rapid availability of distinctive test results within 24 h.

In patient specimens, T-cell activation was <30% in 3 (CD4+) and 4 (CD8+) out of 6 patients 16 h after stimulation, probably because these patients had received immunosuppressive therapy, which inhibited T-cell activation and proliferation. Accordingly, the proportion of proliferating cells determined via CFSE assay was also low, at 29.1% (CD4+) and 23.7% (CD8+). The partially reduced proliferation behaviour of patient PBMCs in photopheresis is in agreement with the studies of Faivre et al.[6] and Evrard et al.[18]. All patient ECP procedures investigated in our study were under concurrent potent dual or triple immunosuppressive regimens including tacrolimus (a calcineurin inhibitor), mycophenolate mofetil, prednisolone and everolimus (a mammalian target of rapamycin (mTOR) inhibitor). Despite the lower proliferation levels, the CD71- and CFSE-assay display an inhibitory ECP effect in almost all tested samples. Our results are comparable to the study of Faivre et al., who recommends a minimum inhibition rate of the CFSE assay of approximately 70% [6]. In patient samples, however, the CD71 assay is not faster than the CFSE assay due to incubation times, but it is easier to perform. Direct apoptosis detection might represent an alternative quality control method, since we found a marked photopheresis effect after 4 days in all patient samples. Furthermore, since PBMCs can remain unstimulated and only Annexin-V and SYTOX staining is performed, this assay is very simple to perform. However, on the other hand, apoptosis detection in cultured blood samples definitively requires strict negative controls, since
| Table 3 | Kinetics of total and late apoptosis in unstimulated T cells in patient samples |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Total apoptosis (CD4<sup>+</sup>) | Total apoptosis (CD8<sup>+</sup>) | Late apoptosis (CD4<sup>+</sup>) |
| Experiment/Patient | Pre- ECP | Post- ECP | Δ Apoptosis | Pre- ECP | Post- ECP | Δ Apoptosis | Pre- ECP | Post- ECP | Δ Apoptosis | Pre- ECP | Post- ECP | Δ Apoptosis | Pre- ECP | Post- ECP | Δ Apoptosis |
| 24 h after ECP | 42 h after ECP | d4 after ECP | d5 after ECP | 24 h after ECP | 42 h after ECP | d4 after ECP | d5 after ECP | 24 h after ECP | 42 h after ECP | d4 after ECP | d5 after ECP | 24 h after ECP | 42 h after ECP | d4 after ECP | d5 after ECP |
| 1a | 5.9 | 8.6 | 2.7 | 7.5 | 11.8 | 4.3 | 7.9 | 35.4 | 27.5 | 13.6 | 85.3 | 71.7 | 16.2 | 56.2 | 40.3 |
| 2b | 4 | 19.7 | 15.7 | 10.3 | 21 | 10.7 | 17.3 | 37.8 | 20.5 | 16.2 | 56.2 | 40.3 | 8.6 | 63.5 | 54.9 |
| 3c | 2.1 | 6.8 | 4.7 | 7.1 | 11.1 | 4 | 5.8 | 40.7 | 34.9 | 6 | 44.7 | 38.7 | 11.2 | 52.6 | 41.4 |
| 4c | 4.8 | 11 | 6.2 | 8.6 | 9.1 | 0.5 | 6 | 44.7 | 38.7 | 11.2 | 52.6 | 41.4 | 8.6 | 63.5 | 54.9 |
| 5d | 12.4 | 11.1 | -1.3 | 8.1 | 27.6 | 19.5 | 8.9 | 89.8 | 80.9 | 15.5 | 98.2 | 82.7 | 10.5 | 92.6 | 82.1 |
| 6d | 4.1 | 12.6 | 8.5 | 7.5 | 22.2 | 14.7 | 16.1 | 94.5 | 78.4 | 10.5 | 92.6 | 82.1 | 3 | 60.1 | 56.8 |
| 7c | 5 | 7 | 2 | 11.1 | 20.5 | 9.4 | 18.4 | 54.3 | 35.9 | 30.6 | 81.3 | 50.7 | 25.6 | 75.9 | 49.4 |
| 8c | 6.6 | 11.4 | 4.8 | 8.3 | 22.6 | 14.3 | 12 | 57 | 45 | 26.5 | 75.9 | 49.4 | 3.2 | 49.6 | 46.4 |
| 9f | 1.7 | 3.3 | 1.6 | 3.4 | 10.4 | 7 | 3.3 | 60.1 | 56.8 | 3.2 | 49.6 | 46.4 | 3.6 | 86.2 | 82.4 |
| 10f | 2.2 | 7.1 | 4.9 | 3.1 | 8.2 | 5.1 | 4 | 64 | 60 | 3.6 | 86.2 | 82.4 | 14 | 74.1 | 60.2 |
| M | 4.9 | 9.9 | 5.0 | 7.5 | 16.5 | 9.0 | 10.0 | 57.8 | 47.9 | 14.0 | 74.1 | 60.2 | 8.9 | 17.4 | 17.6 |
| SD | 3.1 | 4.5 | 1.4 | 2.6 | 7.0 | 5.9 | 5.6 | 20.5 | 15.6 | 8.9 | 17.4 | 17.6 | 3.1 | 4.5 | 1.4 |

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significant apoptosis will occur in all samples over time. In summary, our study revealed that CD71 detection represents a simple and reliable quality control assay for extracorporeal photopheresis in normal PBMC samples devoid of T-cell suppressive drugs.

Conflict of interest

HH was member of the scientific advisory board of macropharma until 2017. The other authors have disclosed no conflicts of interest.

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