Biomarker profile predicts clinical efficacy of extracorporeal photopheresis in steroid-resistant acute and chronic graft-vs-host disease after allogenic hematopoietic stem cell transplant

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Abstract
We conducted a multicenter interventional study to assess the efficacy of Therakos ECP to treat steroid-resistant graft-vs-host disease (SRes-GVHD) after allogeneic HSCT and to identify biomarkers of GVHD response. A total of 62 patients were treated for acute SRes-GVHD (n = 37) or chronic SRes-GVHD (n = 25). Median time to best response was 35 days (range, 28-85) and 90 days (range, 27-240) in acute and chronic SRes-GVHD, respectively. Overall, 27 patients (72.9%) with SRes-aGVHD responded to treatment (40.5% CR and 32.4% PR). The response rate was significantly higher in grade I-II than in grade III-IV aGVHD (100% vs 50.0%, respectively, \( P \)-value = .001). In chronic SRes-GVHD, 22 patients (88%) achieved a clinical response (24.0% CR and 64% PR). Response was higher in moderate than in severe SRes-cGVHD (100% vs 75%, \( P = .096 \)). In both acute and chronic SRes-GVHD patients, the percentage of peripheral blood CD3⁺CD4⁺ was higher and CD3⁺CD8⁺ lower in responding than nonresponding patients. Acute SRes-GVHD responding patients presented a higher number of Treg cells (CD4⁺CD25⁺CD127low/−) at day 0 (\( P = .028 \)) than nonresponding patients, differences that were maintained over the observation period. Phenotypic analysis of T-cell maturation showed a trend toward reduction in TCD8 naïve cells, along with an increased percentage of TCD8 Mem Efect T cells after starting ECP in responding patients. None of the studied serum cytokines displayed statistically significant changes in either acute or chronic SRes-GVHD. ECP is an effective treatment for patients with SRes-GVHD. Biomarkers could help guide decision-making on ECP treatment initiation and duration.
1 | INTRODUCTION

Steroid-resistant GVHD (SRes-GVHD) is the leading cause of early and late allogenic hematopoietic stem cell transplant (allo-HSCT)-related mortality and morbidity, due not only to immune-mediated organ damage caused by GVHD itself, but also the side effects associated with immunosuppressive drugs, especially steroids and calcineurin inhibitors (CNI) used as first- or subsequent-line treatment of this complication. Systemic immunosuppression increases the risk of infection, disease relapse, and organ toxicity after HSCT.

There is no standard treatment for SRes-GVHD. Available therapies such as mycophenolate mofetil (MMF), daclizumab, or anti-T lymphocyte globulin (ATG) have shown limited clinical activity, with response rates ranging from 20% to 40% and poor long-term survival. More recently introduced agents such as ruxolitinib, mesenchymal cells, and ibrutinib have shown promising results in either acute or chronic SRes-GVHD, but phase III trial results are still pending.

Extracorporeal photopheresis (ECP) is an immunomodulatory treatment that has demonstrated beneficial results in retrospective and prospective studies in patients with steroid-resistant acute (SRes-aGVHD) or chronic GVHD (SRes-cGVHD). One method that has been proposed is to induce alloreactive T-cell depletion and immunotolerance by generating regulatory T-cells (Tregs) and tolerogenic dendritic cells (DCs). Owing to their pathogenic involvement, blood levels of certain T cell or NK subsets have been described as cellular biomarkers of GVHD or response to ECP. However, conflicting data have been reported on the effect of ECP on Treg levels in patients with SRes-cGVHD. Discrepant results may stem from differences in severity of GVHD and immunosuppressive treatments used before or combined with ECP across series.

Considerable research has been conducted to identify early biomarkers for risk stratification and to predict GVHD response to ECP in order to inform treatment initiation and duration. However, published results are mostly derived from single institutions, and still await validation in prospective studies or randomized clinical trials to bring them from bench to bedside. Some studies have shown the significant impact of a quality response (ie, complete response) in terms of improved overall or disease-free survival. It therefore seems pertinent to investigate whether biomarkers could help early identification of SRes-GVHD patients most likely to respond to ECP treatment.

In the current study, we evaluated the clinical efficacy of Therakos ECP to treat SRes-GVHD following standard clinical indications predefined by the participating centers (Spanish Group for Hematopoietic Transplantation and Cell Therapy, GETH). While often treated as equivalent to refractory, in our study we stratified SRes-GVHD into true steroid-refractory (SR-GVHD), steroid-dependent (SD-GVHD), or steroid-intolerant (SI-GVHD). Our second aim was to identify biomarkers predicting clinical response to ECP through analysis of different peripheral blood immune populations, including T, B, and NK cells, before episodes of clinical GVHD and the kinetic profile of plasma cytokines related to GVHD.

2 | MATERIALS AND METHODS

2.1 | Study design

A prospective, multicenter, interventional study in line with routine clinical practice was conducted in five Spanish centers from the GETH.

2.2 | Patients

Sixty-two consecutive adult patients with acute or chronic SRes-GVHD after allo-HSCT were included in the study between September 2012 and October 2016. All patients were treated with the Therakos Cellex ECP system, which combines cell collection, photoactivation, and reinfusion technologies in an integrated system, following the manufacturer’s recommendations. The study protocol was written in accordance with the European Union Directive on Good Clinical Practice and the current version of the Declaration of Helsinki. Each participating center obtained study approval from their respective local Research Ethics Committees. All patients gave informed consent to participate in the study. The study was registered in Clinical Trials (NCT03124056).

Patients were eligible to participate in the study if they had:
• Active grade II-IV aGVHD in the following clinical situations: (a) SR-aGVHD, defined as GVHD progression in any organ within 3-5 days of standard first-line treatment with a CNI and high dose methylprednisolone (2 mg/Kg/day) or failure to improve within 7 days or incomplete response after more than 28 days; (b) SD-aGVHD, defined as a GVHD flare-up or evidence of progression in the same or newly affected organ after reduction of full dose methylprednisolone previously administered for at least 2 weeks; (c) SI-aGVHD including avascular necrosis, severe myopathy, uncontrolled diabetes mellitus, systemic viral or fungal infections, or other significant side effects as defined by the attending physician.

• Moderate or severe cGVHD diagnosed ≤3 years from allo-HSCT, that was steroid-resistant (SR-cGVHD), defined as lack of response or disease progression after administration of at least 1 mg/kg of prednisone or equivalent, either alone or combined with CNI) or SD-cGVHD (requiring more than 10 mg prednisone or equivalent to control GVHD manifestations) or SI-cGVHD (including avascular necrosis, severe myopathy, uncontrolled diabetes mellitus, systemic viral or fungal infections), as evaluated by the attending physician.

• White blood cell (WBC) count >1000/mm³, platelets >25 000/mm³, and a Karnofsky Performance status score >50%.

Exclusion criteria were intolerance to methoxsalen, heparin, or citrate products, active gastrointestinal bleeding, previous treatment with ECP, pregnancy, or lactation.

2.3 | Treatment and study assessments

The initial treatment schedule was based on GVHD type (acute or chronic) and was later adapted according to clinical response.

In SRes-aGVHD, ECP was initiated with three treatments during the first week, followed by two treatments per week from weeks 2 through 12. In each session, 1500 mL of blood was processed using the Therakos Cellex photopheresis system following the standard instructions provided by Therakos Inc (Therakos Inc. THERAKOS CELLEX Photopheresis System: Operator’s Manual. In. Vol Rev. 5.0-14 604 152 014). In case of disease progression after 2 weeks of treatment or lack of response after 6-8 weeks, treatment was withheld and alternative therapies were considered by the physician. In the event of partial (PR) or complete response (CR), treatment frequency was reduced according to the following schedule: two treatments/week from week 2 through week 12; one treatment every 2 weeks from week 13 until total discontinuation of steroid treatment.

In SRes-cGVHD, ECP was initiated using three treatments during the first week, followed by two treatments every 2 weeks from weeks 2 to 12. In patients with no prior progression, the first evaluation was made at week 12, except for sclerodermic SRes-cGVHD, in which case evaluation was made after 28 weeks. The same blood volume was processed and the procedure detailed above for aGVHD was followed in each session. In case of clear progression after 1 month of treatment or lack of response at the first evaluation at week 12, treatment was withheld and alternative therapies were considered by the physician. If the patient achieved a partial (PR) or complete response (CR) in week 12, two treatments were applied every month for three additional months after suppression of steroid treatment. Tapering during treatment with ECP was guided by GVHD manifestations, at the discretion of the treating physician.

At the start of ECP all patients were receiving immunosuppressive (IS) therapy with prednisone and a CNI. All other IS therapy except MMF was discontinued before starting ECP. Tapering of immunosuppressors during ECP treatment was guided by GVHD manifestations at the discretion of the attending physician.

Diagnostics and grading of patients with aGVHD were based on modified Glucksberg criteria, while in SRes-cGVHD patients followed NIH Consensus Group 2005 criteria.

Acute GVHD response criteria:

If no previous progression had occurred, evaluation was scheduled after day 28 after ECP onset without additional systemic immunosuppressants. Complete response was defined as resolution of all target organ symptoms. Partial response was defined as an improvement at any organ stage by at least one stage without increasing in any other target organ stage. All other treatment outcomes were classified as nonresponse/progression. Patients who died before response assessments were considered nonresponders.

For chronic GVHD response assessments the NIH Consensus Group 2006 criteria were used. Response assessment was carried out by the same trained physician in each center to minimize criteria variability.

2.4 | Sample extraction

Aiming to identify early biomarkers of response to ECP, we obtained samples pre-ECP and on days +7, +14, and +21 in aGVHD, and pre-ECP and on days +15, +30, +45, and +60 in SRes-cGVHD. Sample collection was as
follows: 2 × 4.5 mL whole blood were collected in blood collection tubes with sodium heparin anticoagulant, were processed on the day of collection at each participant center to obtain mononuclear cells (MNC) following the same protocol, and were stored frozen until transportation to the centralized processing laboratory at the Hospital Clínico Universitario of Valencia, along with patients’ clinical data.

2.4.1 Analyses of lymphocyte subsets and dendritic cells

The samples were defrosted at 37°C on the day of cytometric analysis. Subsequently, one or two washes were carried out with phosphate-buffer saline (PBS) and the sample was resuspended in it. From the total sample, 100 μL was pipetted into each tube with the appropriate combination of antibodies according to the study populations, using manufacturer recommended amounts of antibody (BD Biosciences, San José, California). They were incubated in the dark for 15 minutes. Cells were then washed, resuspended in 200 μL of 1% paraformaldehyde in PBS, and run within 2 h with a FACSCanto II (BD Biosciences, San José, California) using FACSDiva software vs 1.8 (BD Biosciences, San José, California), then analyzed with Infinicyt vs 1.8 (Cytognos). Lymphocytes were selected by SSC-A vs FSC-A gating and dendritic cells were selected by gating SSC-A vs FSC-A and CD123+ vs HLA-DR. The following monoclonal antibodies and combinations were used in the procedure (BD Biosciences):

1. CD3-FITC, CD16-PE, CD19-PerCP, CD4 PE-Cy7, CD56-APC, CD8-APC-Cy7: T, B, NK lymphocytes.
2. CD4-FITC, CD123-PE, CD25 PE-Cy7, CD127-APC, HLA-DR Pacific Blue: Treg lymphocytes and dendritic cells.
3. CD45 RA-FITC, CCR7-PE, CD4-PerCP, CD62L PE-Cy7, CD3-APC, CD8-APC-Cy7: lymphocyte subpopulations.

Cytokine measurement in plasma was assessed using peripheral blood obtained from patients, centrifuged immediately and stored at −80°C. Cytokine determinations were performed by normal or high sensitivity ELISA (R&D Systems, Minneapolis, Minnesota). The following parameters were used in the analysis:

1. BAFF: Quantikine Human BAFF/BLyS/TNFSF13B ELISA (assay range: 62.5-4000 pg/mL)
2. IFN-gamma: Quantikine Human IFN-gamma ELISA (assay range: 15.6-1000 pg/mL)
3. IL-10: Quantikine Human IL-10 ELISA (assay range: 0.8-50 pg/mL)
4. TNF-alfa: Quantikine Human TNF-alfa ELISA (assay range: 15.6-1000 pg/mL)
5. IL-6: Quantikine Human IL-6 ELISA (assay range: 0.2-10 pg/mL pg/mL)

The control group consisted of 10 healthy blood donors.

2.5 Statistical analysis

Analyses were conducted in R version 4.0.0 (A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: at https://www.R-project.org/). For categorical variables, frequencies of groups were compared using chi-square test. For each cell population, changes across time were compared with pre-ECP levels using Wilcoxon signed rank test for two paired samples. In each cell population, numbers were compared between groups (patients vs healthy donors; responding patients vs nonresponding patients) using the Mann-Whitney test. Two-sided exact P-values were calculated whenever possible and P-values ≤.05 were considered statistically significant. Overall survival (OS) was defined as time from ECP onset to time of death from any cause. The probability of OS was estimated using the Kaplan-Meier method. The OS curves for different groups of patients were compared using the log-rank test.

In the analysis of each cell population type, only patients with valid values at the beginning of the protocol (day 0) were considered and presented in the results section as figures. All results are expressed as percentages of the lymphocyte count. Changes over time in the mean level (with the SE of the mean) for each cell population are also presented in the results section as figures, adding the number of valid values for each time point, the P-value associated with the comparison with the pre-ECP level (day 0) (in black) and the P-value associated with the comparison with healthy blood donor levels in the control group (in gray).

3 RESULTS

A total of 62 adult patients were included in the study. A complete description of the series characteristics can be found in Table 1. Most patients included in the study had SR-GVHD (n = 49, 79%), in both acute (n = 30, 81.1%) and chronic GVHD (n = 19, 76%) groups, while only 21% were SD-GVHD (11.3%) or SI-GVHD patients (9.7%).
### TABLE 1  Characteristics of the study population

|                              | Total              | Acute GVHD group | Chronic GVHD group |
|------------------------------|--------------------|------------------|--------------------|
| N (%)                        | 62 (100.0%)        | 37 (59.7%)       | 25 (40.3%)         |
| Sex, M/F (%)                 | 42 (67.7%)/20 (32.3%) | 26 (70.3%)/11 (29.7%) | 16 (64.0%)/9 (36.0%) |
| Age (years), median (range)  | 55 (18-71)         | 53 (20-71)       | 55 (18-71)         |
| Diagnosis (%)                |                    |                  |                    |
| Acute leukemia               | 28 (45.2%)         | 16 (43.2%)       | 12 (48.0%)         |
| Lymphoproliferative malignancies<sup>a</sup> | 22 (35.5%)      | 12 (32.4%)       | 10 (40.0%)         |
| Myelodysplastic syndrome (MDS) | 9 (14.5%)         | 7 (18.9%)        | 2 (8.0%)           |
| Others                       | 3 (4.8%)           | 2 (5.4%)         | 1 (4.0%)           |
| DRI<sup>b</sup>              |                    |                  |                    |
| High                         | 42 (67.7%)         | 25 (67.6%)       | 17 (68.0%)         |
| Intermediate                 | 18 (29.0%)         | 11 (29.7%)       | 7 (28.0%)          |
| Low                          | 2 (3.2%)           | 1 (2.7%)         | 1 (4.0%)           |
| Type of transplant           |                    |                  |                    |
| Related                      | 32 (51.6%)         | 17 (45.9%)       | 15 (60.0%)         |
| Unrelated                    | 30 (48.4%)         | 20 (54.1%)       | 10 (40.0%)         |
| HLA (A, B, C, DR) match      |                    |                  |                    |
| Yes                          | 45 (72.6%)         | 23 (62.2%)       | 22 (88.0%)         |
| No                           | 17 (27.4%)         | 14 (37.8%)       | 3 (12.0%)          |
| Haploidentical               | 2 (3.2%)           | 2 (5.4%)         | 0 (0.0%)           |
| Type of conditioning         |                    |                  |                    |
| Myeloablative                | 18 (29.0%)         | 11 (29.7%)       | 7 (28.0%)          |
| Reduced intensity conditioning| 44 (71.0%)        | 26 (70.3%)       | 18 (72.0%)         |
| GVHD prophylaxis             |                    |                  |                    |
| Tacrolimus plus sirolimus    | 29 (46.8%)         | 19 (51.4%)       | 10 (40.0%)         |
| Cyclosporine plus methotrexate| 21 (33.9%)        | 10 (27.0%)       | 11 (44.0%)         |
| Others                       | 12 (19.4%)         | 8 (21.6%)        | 4 (16.0%)          |
| Previous GVHD treatment lines|                     |                  |                    |
| 1                            | 37 (59.7%)         | 27 (73.0%)       | 10 (40.0%)         |
| 2                            | 14 (22.6%)         | 8 (21.6%)        | 6 (24.0%)          |
| >2                           | 11 (17.7%)         | 2 (5.4%)         | 9 (36.0%)          |
| Leucocytes (/mm³) at ECP initiation, median (range) | 5850 (800-18200) | 5410 (800-14700) | 6860 (1300-18200) |
| Corticosteroid status at study entry |          |                  |                    |
| SR-GVHD                      | 49 (79.0%)         | 30 (81.1%)       | 19 (76.0%)         |
| SD-GVHD                      | 7 (11.3%)          | 4 (10.8%)        | 3 (12.0%)          |
| SI-GVHD                      | 6 (9.7%)           | 3 (8.1%)         | 3 (12.0%)          |
| Time from GVHD diagnosis to ECP (days), median (range) | 67 (4-1038) | 26 (4-250) | 268 (60-1038) |

<sup>a</sup>Non-Hodgkin lymphoma (NHL), Hodgkin lymphoma (HL), Multiple Myeloma (MM), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL); SR-GVHD, steroid-refractory GVHD; SD-GVHD, steroid-dependent GVHD; SI-GVHD, steroid-intolerant GVHD.

<sup>b</sup>DRI: Disease Risk Index. Low: Acute myeloid leukemia (AML) with favorable cytogenetics, CLL, CML, indolent B-cell NHL; Intermediate: AML with intermediate cytogenetics, Myelodysplastic syndrome (MDS) with intermediate cytogenetics, myeloproliferative neoplasms, MM, HL, Diffuse large B cell lymphoma/transformed indolent B-NHL, Mantle cell lymphoma (MCL), T-cell lymphoma nodal; High: AML with adverse cytogenetics, MDS with adverse cytogenetics, T-cell lymphoma extranodal.
3.1 SRes-aGVHD patients

3.1.1 Clinical efficacy

In the time elapsed between diagnosis of SRes-GVHD and initiation of ECP, some patients had received a second (n = 8, with MMF, n = 7 or ruxolitinib, n = 1), third (ATG, n = 1), or fourth (mesenchymal stromal cell, n = 1) line of treatment. Median time from diagnosis of SRes-aGVHD to ECP initiation was 31 days (range, 4-158 days). The median time to evaluation was 35 (range, 9-85 days), 31 (range, 9-64 days), 33 (range, 28-85 days), and 36 (range, 28-45 days) days for all patients, and progression, PR and CR groups, respectively. Median time to response in responding patients was 35 days (28-85).

Table 2 shows the organ affected and clinical grade at study entry of the 37 patients treated according to the SRes-aGVHD protocol. A total of 20 of the 37 SRes-aGVHD patients had severe (grade III-IV) aGVHD on inclusion in the study. Two patients classified grade I at study entry had been grade II with partial response to steroids, but were deemed candidates because of associated infection (SI-aGVHD).

The median ECP treatment time was 3.0 months (range, 0.3-23.2 months). No significant ECP-related side effects were observed in any patient. Overall, 15 patients (40.5%) achieved CR, 12 (32.4%) had PR, and 10 (27.0%) had aGVHD progression (Prog). Response rate (complete or partial) was significantly higher in the milder forms of aGVHD (grade I-II) than in the most severe forms (grade III-IV) (100% vs 50.0%, respectively, P-value = .001) (Table 3).

Median follow-up of the series from treatment initiation was 12.8 months. The median time to additional therapy in the PR group (n = 12) was 45 days (36-128). A total of 17 patients (45.9%) died during study follow-up, with an estimated median OS of 27 months (Figure 1A). Four deaths were related to underlying hematological disease, while the rest were attributed to aGVHD, in most cases (85%) directly linked to a fatal infectious episode. Patients who achieved CR had longer survival (median of over 47 months) than those who achieved partial or no response (median of 12.3 months) (P = .031) (Figure 1B).Patients with SD-aGVHD or SI-aGVHD (n = 7) had longer survival than patients with SR-aGVHD (n = 30), but the difference did not reach statistical significance (P-value = .090) (Figure 1Sa). Better survival was also

| Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|--------|--------|--------|--------|--------|
| Overall | 0 (0.0%) | 2 (5.4%) | 15 (40.5%) | 9 (24.3%) | 11 (29.7%) |
| Skin    | 9 (24.3%) | 3 (8.1%) | 5 (13.5%) | 16 (43.2%) | 4 (10.8%) |
| Digestive | 7 (18.9%) | 16 (43.2%) | 5 (13.5%) | 3 (8.1%) | 6 (16.2%) |
| Liver   | 26 (70.3%) | 3 (8.1%) | 5 (13.5%) | 2 (5.4%) | 1 (2.7%) |

TABLE 2 Overall and organ grade in SRes-aGVHD patients at study entry

| Total | Grade I and II | Grade III and IV | Total | Stage I and II | Stage III and IV |
|-------|----------------|------------------|-------|----------------|------------------|
| Overall | 37 (100.0%) | 17 (100.0%) | 20 (100.0%) | 28 (100.0%) | 8 (100.0%) | 20 (100.0%) |
| Response | 27 (73.0%) | 17 (100.0%) | 10 (50.0%) | 20 (71.4%) | 3 (37.5%) | 17 (85.0%) |
| Complete | 15 (40.5%) | 10 (58.8%) | 5 (25.0%) | 9 (32.1%) | 2 (25.0%) | 7 (35.0%) |
| Partial | 12 (32.4%) | 7 (41.2%) | 5 (25.0%) | 11 (39.3%) | 1 (12.5%) | 10 (50.0%) |
| NR/progression | 10 (27.0%) | 0 (0.0%) | 10 (50.0%) | 8 (28.6%) | 5 (62.5%) | 3 (15.0%) |

TABLE 3 Response to treatment by aGVHD overall grade and stage of the affected organ at study entry

| Total | Stage I and II | Stage III and IV | Total | Stage I and II | Stage III and IV |
|-------|----------------|------------------|-------|----------------|------------------|
| Overall | 30 (100.0%) | 21 (100.0%) | 9 (100.0%) | 11 (100.0%) | 8 (100.0%) | 3 (100.0%) |
| Response | 20 (66.7%) | 15 (71.4%) | 5 (55.6%) | 6 (54.5%) | 5 (62.5%) | 1 (33.3%) |
| Complete | 12 (40.0%) | 9 (42.9%) | 3 (33.3%) | 3 (27.3%) | 3 (37.5%) | 0 (0.0%) |
| Partial | 8 (26.7%) | 6 (28.6%) | 2 (22.2%) | 3 (27.3%) | 2 (25.0%) | 1 (33.3%) |
| NR/Progression | 10 (33.3%) | 6 (28.6%) | 4 (44.4%) | 5 (45.5%) | 3 (37.5%) | 2 (66.7%) |

Abbreviation: NR, nonresponse.
observed in patients with a lower grade of aGVHD at study entry, with differences approaching statistical significance ($P = .077$) (Figure 1Sb).

### 3.1.2 | Biomarker identification

#### Analysis of evolution of T-cell populations and serum cytokines in SRes-aGVHD patients

Figures 2, 2S, 3S, and 4S show the changes over time in different T and NK cell subsets in all SRes-aGVHD patients.

The percentage of CD3$^+$CD4$^+$ cells saw a statistically significant increase on day +7 compared with day 0 ($P = .018$), maintaining this level until day +21 ($P = .037$). Throughout this period, the values were statistically lower than those of healthy donors (dashed line) ($P < .001$ for all analyzed time points) (Figure 2A).

The %CD3$^+$CD8$^+$ cells decreased gradually through the same period with a statistically significant decrease on day +21 ($P = .001$) compared with day 0. Throughout this period, values up to day +21 were significantly higher than those of healthy donors (dashed line) until day 15 ($P = .001$) (Figure 2B). The CD3$^+$CD4$^+$/CD3$^+$CD8$^+$ ratio showed a general increase through the observed period, reaching statistical significance on day +21 ($P = .003$) compared to the initial value. Despite this, throughout the entire period proportions were lower than those of healthy donors (dashed line) ($P < .001$) (Figure 2C).

The percentage of Treg cells (CD4$^+$CD25$^+$CD127$^{low/−}$) decreased gradually until day +21. All values were statistically lower than those of healthy donors (dashed line) from day 7 ($P = .004$) (Figure 2D).

Analysis of the NK cells subsets showed no statistically significant differences with respect to basal levels (Figure 2S). Phenotypic analysis of T-cell maturation showed a trend for reduction of TCD4naive and TCD8naive cells, stable levels for TCD4MemCentral and TCD8MemCentral cells along with increased percentage of TCD4 MemEfect and TCD8MemEfect T cells, suggesting a maturation change in T-cell profile (Figure 3S).

None of the studied serum cytokines showed statistically significant changes during the first month of treatment (Figure 4S).

#### Analysis of evolution of cell populations and serum cytokines comparing ECP response groups in SRes-aGVHD patients

Figures 3, 5S, 6S, and 7S show changes in mean level (with the SE of the mean) in the two ECP response patient groups (CR + PR vs no response [NR]) for each cell population and time point analyzed. The small sample size of nonresponding patients must be taken into account in all comparisons. In all analyses, we included patients with a valid cell population value at day 0.

For %CD3$^+$CD4$^+$, the values of the patients who achieved a response (CR + PR) were higher than nonresponders during the whole follow-up, with the greatest difference observed on day +7 ($P = .047$) (Figure 3A). The median percentage of CD3$^+$CD4$^+$ cells increased significantly in patients with a response on day +7 ($P = .029$) and day +14 ($P = .043$) compared with pre-ECP level (day 0).

For %CD3$^+$CD8$^+$ cells, values in responding patients were lower than in nonresponding ones and decreased...
continuously during the whole follow-up, with a statistically significant difference on day \(+21\) \((P = .001)\) (Figure 3B).

For the \(CD3^+CD4^+/CD3^+CD8^+\) ratio, the values of responding patients \((CR + PR)\) were higher than nonresponding patients during the whole follow-up, the
greatest difference being observed on day +7 (P = .008) (Figure 3C). Responding patients saw a continuous rise, with significant differences on day +21 (P = .005) compared to pre-ECP level (day 0), while in nonresponding patients, growth began as late as day +21.

Responding patients presented higher numbers of Treg cells (CD4+CD25+CD127low/−) at day 0 (P = .028), and the differences were maintained during the observation period in these patients (Figure 3D).

Analysis of the NK cell subsets showed no statistically significant differences from basal levels (Figure 5S). Phenotypic analysis of T-cell maturation revealed a trend toward reduction of TCD4naive and significant reduction of TCD8naive cells, along with increased percentages of TCD4MemEffect and TCD8MemEffect T cells through days +14 to +21 from starting ECP, in both responding patient groups (CR + PR vs NR), suggesting treatment-induced maturation changes in T-cell profile (Figure 6S).

None of the studied serum cytokines (TNF-alfa, IL-10, IFN-gamma, IL-6, and BAFF) showed statistically significant changes between responding and nonresponding patients during the first month of treatment (Figure 7S) though the low number of samples analyzed precluded meaningful conclusions.

### 3.2 SRes-cGVHD patients

At a median of 647 days (250-994) after diagnosis of chronic GVHD, all patients (n = 25) had received systemic corticosteroids alone (n = 15) or in combination with a CNI (n = 10). Among patients included in the chronic SRes-GVHD group of our study, six patients had received a second line (cyclosporin, n = 3; MMF, n = 2; mesenchymal stromal cell, n = 1), five patients a third line (sirolimus, n = 3; ruxolitinib, n = 2), and four patients a fourth line of treatment (sirolimus, n = 2; rituximab, n = 1; irbritinib, n = 1). Prior to enrollment in our study, CNI and MMF were the only agents not discontinued.

Among the 25 patients treated according to the SRes-cGVHD protocol, at study entry 1 patient (4%) had mild (previously graded moderate which become intolerant to steroids), 12 patients (48%) had moderate, and 12 patients (48%) had severe SRes-cGVHD, respectively. Regarding the affected organs, 24 (96.0%) had mucosal/cutaneous, 10 (40.0%) scleroderma, 9 (36.0%) genital, 4 (16.0%) hepatic, 3 (12.0%) fasciitis, 2 (8.0%) pulmonary, and 1 (4.0%) intestinal involvement.

Median ECP treatment duration was 7.8 months (range, 0.3–16.2 months). No treatment-derived side effects were observed in patients except for mild pain during venous insertion of the apheresis catheter in seven patients (11%). No patient had apheresis catheter-related hemorrhage or thrombosis. Overall, 6 patients (24.0%) achieved CR, 16 (64.0%) had PR, and 3 (12.0%) had disease progression at the end of the treatment. The percentage of patients with response (complete or partial) was higher in the moderate SRes-cGVHD group than the severe one (100% vs 75%, P = .096) (Table 4).

Median time to best response was 90 days (range, 27-240 days) for patients who achieved PR and 92 days (range, 90-173 days) for those reaching CR.

Median follow-up of the series was 14.3 months. Four patients (16%) died during follow-up, two, due to infectious events and two to progression of the underlying disease, with an estimated median OS of over 47 months (Figure 4A). Patients achieving CR had longer survival than those who had PR or NR (light gray) (Figure 4B). During follow-up, 3 of the 19 patients with resistance (15.8%) and 1 of the 6 patients with dependence or contraindication (16.7%) died. None of the 13 patients in the mild/moderate group died, compared to the 4 deaths observed among the 12 patients of the severe group (33.3%).

| Table 4 | Response to treatment by overall cGVHD grade and affected area |
|---------|---------------------------------------------------------------|
| Total   | Response Complete Partial Progression | 6 (24.0%) | 16 (64%) | 3 (12%) |
| Mild–Moderate | 13 (100.0%) | 13 (100%) | 3 (23.1%) | 10 (76.9%) | 0 (0.0%) |
| Severe  | 12 (100.0%) | 9 (75%) | 3 (25%) | 6 (50%) | 3 (25%) |
| Mucosal/cutaneous | 24 (100.0%) | 21 (87.5%) | 5 (20.8%) | 16 (66.7%) | 3 (12.5%) |
| Scleroderma | 10 (100.0%) | 10 (100%) | 1 (10%) | 9 (90%) | 0 (0.0%) |
| Genital | 9 (100.0%) | 6 (66.7%) | 2 (22.2%) | 4 (44.4%) | 3 (33.3%) |
| Hepatic | 4 (100.0%) | 4 (100%) | 1 (25%) | 3 (75%) | 0 (0.0%) |
| Fasciitis | 3 (100.0%) | 3 (100%) | 0 (0.0%) | 3 (100%) | 0 (0.0%) |
| Pulmonary | 2 (100.0%) | 2 (100%) | 1 (50%) | 1 (50%) | 0 (0.0%) |
| Intestinal | 1 (100.0%) | 1 (50%) | 1 (50%) | 0 (0.0%) | 1 (50%) |
3.2.1 | Analysis of T-cell subsets and serum cytokines in SRes-cGVHD patients

Figures 5, 9S, 10S, and 11S show the evolution over time of different T and NK cells subsets in all SRes-cGVHD patients.

Figure 5 shows changes over time for each T-cell subset in SRes-cGVHD patients.

A decrease in the CD3⁺CD4⁺ cell percentage was observed compared to the initial value, but without reaching statistically significance. The values were lower than in healthy donors (dashed line) throughout this period, with statistically significant differences at each time point (P < .001) (Figure 5A).

%CD3⁺CD8⁺ levels remained very stable, with a modest increase at day +30. The values were above
healthy donors (dashed line) throughout the period, with statistically significant differences only on day +30 ($P = .035$) (Figure 5B).

A fall in the CD3+CD4+/CD3+CD8+ ratio could be observed at day +30 compared to the initial value, but the differences were not statistically significant. Throughout this period, values were lower than in healthy donors (dashed line) with statistically significant differences over the entire time (Figure 5C).

A decrease in the Treg cell (CD4+CD25+CD127low/−) percentage was observed with respect to the initial value, with statistically significant differences on day +45 ($P = .003$). Throughout this period, values were lower than in healthy donors (dashed line) with statistically significant differences throughout the whole period (Figure 5D).

Analysis of the NK cell subsets did not show statistically significant differences compared with basal levels (Figure 9S). Phenotypic analysis of T-cell maturation found no statistically significant differences with respect to basal levels either (Figure 10S).

None of the studied serum cytokines levels showed statistically significant changes during follow-up (Figure 11S). BAFF serum levels and the BAFF/CD19 ratio show a progressive increase, peaking on day +45 (Figures 11Sa and 11Sb).

### 3.2.2 Analysis of T-cell subset and serum cytokine evolution comparing ECP response groups in SRes-cGVHD patients

Figures 12S, 13S, 14S, and 15S show the changes in the mean level (with the SE of the mean) in the two ECP response patient groups (CR and PR vs NR) for each cell population and time point analyzed.

During the first 45 days responding patients (CR + PR) showed higher CD3+CD4+ percentages than non-responders, with a trend toward statistical significance compared to pre-ECP level (day 0) on day +30 ($P = .095$) (Figure 12Sa). Regarding %CD3+CD8+, responding patients (CR + PR) displayed stable, lower percentages than nonresponding patients throughout the whole period (Figure 12Sb). %CD3+CD4+/%CD3+CD8+ values in responding patients were above those of nonresponding ones (Figure 12Sc). Basal Treg cell (CD4+CD25+CD127low/−) cells did not show significant differences between responders and nonresponders, with a continuous decline in both groups, statistically significant different on day +45 compared to pre-ECP level (day 0) ($P = .003$) (Figure 12Sd).

Phenotypic analysis of T-cell maturation showed a trend toward reduction in TCD8naive cells at day +15, along with an increased percentage of TCD8MemEfect T cells across days +15 to +45 after starting ECP in both responding patient groups (CR + PR vs NR), suggesting treatment-induced maturation change in T-cell profile (Figure 14S).

None of the studied serum cytokines (TNF-alfa, IL-10, IFN-gamma, IL-6, BAFF, and BAFF/CD19) displayed statistically significant changes between responding and nonresponding patients during the first 45 days of treatment (Figure 15S). Again, the low number of samples analyzed precluded meaningful comparison.

### 4 DISCUSSION

The present study shows that the Therakos ECP is clinically effective in treatment of both acute and chronic SRes-GVHD patients and provides detailed data on the evolution of the most commonly recognized cell and cytokine biomarkers which may mediate the effect of ECP.

Overall, 73% of patients with SRes-aGVHD and 88% of those with SRes-cGVHD responded to ECP treatment. It should be underlined, however, that at least as regards SR-aGVHD, partial responses were usually transient and did not translate into survival improvement. In this setting, tapering and discontinuation of corticosteroids and other immunosuppressors, which confers significant survival advantage, is usually only permitted in CR. However, given the good safety profile of ECP, it might serve as a bridge therapy or as part of a combined therapy, as is being evaluated by other authors. Of note, SRes-aGVHD grade at ECP initiation was significantly associated with efficacy, with a 59% CR rate being observed in patients with grade I and II as compared to 25% in patients with grade III and IV. These results seem inferior to those found in other studies, such as the one conducted by Greinix et al. In that study including 59 patients, the largest series of patients with aGVHD receiving ECP treatment, 86%, 55%, and 30% of patients with grade II, grade III, and grade IV aGVHD achieved CR, respectively. By affected organ, complete response in SRes-aGVHD was higher for cutaneous involvement (82%), and somewhat lower for liver and gut involvement (61%) in the same phase II study. Differences in response rates can mainly be explained by variations in the studied population characteristics, as 27% of the aGVHD group patients and 60% of the cGVHD group patients had received two or more lines of treatment prior to ECP. In addition, the proportion of true steroid-refractory GVHD (SR-GVHD) at study entry was much higher in our study, in both acute and chronic GVHD, than in other studies which had lower or even much
lower percentages. In general, prognosis with true steroid-resistant GVHD$^{8,23}$ (progression of GVHD symptoms and manifestations while patients are receiving full dose corticosteroid treatment) is worse than prognosis in steroid-dependent patients (GVHD flare-up during or after tapering of steroid treatment or steroid intolerance).$^{24}$

Turning to SRes-cGVHD, in a randomized, standard therapy-controlled trial Flowers et al reported that ECP treatment of SRes-cGVHD with cutaneous manifestations was associated with improvements in total skin score and quality of life, reduction in corticosteroid use, and a parallel improvement in extracutaneous manifestations at week 12 was greater in the group than in the control arm (14.5% vs 8.5%) although the difference was not statistically significant ($P\ value = .48$).$^6$ In our series, 24% of patients with SRes-cGVHD achieved CR, a similar outcome to the study conducted by Apisarnthanarax et al where 22% reached CR.$^{25}$

Likewise, differences in the biomarkers study results could derive from variations in the treatment protocol or time points selected to evaluate treatment response, given that our study focused on the first weeks of treatment in order to identify early predictors of response while in Greinix’s study response assessment was extended to the first 3 months of treatment.

The search for biomarkers for diagnosis, risk stratification, and prediction of response of GVHD to ECP has attracted substantial research interest.

In our series, clinical response was associated with a trend toward normalization in the CD4$^+$/CD8$^+$ ratio owing to rising levels of T CD3$^+$/CD4$^+$ cells along with decreasing levels of TCD3$^+$/CD8$^+$ cells early after ECP photochemotherapy initiation in patients with acute GVHD. A reduction of TCD4naive cells and TCD4MemCentral cells along with increased TCD8 effector memory T-cell percentage was also observed, suggesting a maturation T-cell profile change. These figures are comparable to those reported in previous studies.$^{26-29}$

With respect to the Treg cells subset, our data showed different results for SRes-aGVHD and SRes-cGVHD. Interestingly, in our series basal Treg levels in patients with SRes-aGVHD were statistically higher in the responding than the nonresponding group. These levels were maintained thereafter, but without progressive increase observed. In contrast, our results in patients with SRes-cGVHD are more in keeping with those reported by Gandelman et al$^7$ but are strikingly different from other published data.$^{30-33}$ As in our series, Gandelman et al found that the percentage of CD4$^+$ cells that were Tregs at study completion did not differ between patients who responded or not to ECP.

The reason for this discrepancy with other studies is unclear. It is probably not due to differences in either clinical or demographic characteristics between patients in our series. Neither can GVHD type account for this, since rising Treg levels have been described in both acute and chronic GVHD. Performing immunophenotypic rather than functional characterization of Tregs would not appear to be the reason as other studies did the same. One potential explanation could be that we limited the observation period to the first weeks of treatment both in SRes-aGVHD and SRes-cGVHD in line with our search for early predictive biomarkers of response. Both higher basal levels and ECP-induced rising levels are likely to be predictive of response; this should be confirmed in future prospective analyses.

Unlike other studies, in our study no cytokine serum levels show statistically significant differences between responding and nonresponding patients. This may be related to the low number of patients included, as well as the fact that some patients were being treated with corticosteroids, which are known to reduce serum levels of several cytokines.$^{34}$ Consistent with previous studies showing rising serum BAFF levels in cGVHD patients,$^{35}$ our cohort of SRes-cGVHD patients showed a progressive increase in serum levels of BAFF and in the BAFF/CD19$^+$ ratio$^{34}$ (Supporting Information) but the low sample size and high response rate to ECP precluded meaningful comparison between responding and nonresponding patients.

Lastly, we could not confirm previously published observations that an increase$^{17}$ or decrease in levels of some NK cell subsets were predictive of ECP response (Supporting Information).

Despite providing new insight into the relationship between certain biomarkers and ECP, this study is not without limitations, such as the small sample size included in the study and the low number of nonresponding patients, which hampers comparisons between responding and nonresponding groups.

In conclusion, we have shown that ECP is an effective treatment for patients with acute and chronic steroid-resistant GVHD. Higher response rates were observed in SRes-aGVHD patients with higher Treg levels at baseline, early normalization of TCD4$^+$ and TCD8$^+$ cell percentages in both SRes-aGVHD and SRes-cGVHD and a trend toward a more mature phenotypic CD4$^+$ and CD8$^+$ T-cell profile during the first 3 weeks of ECP treatment in SRes-aGVHD. These findings help pinpoint a patient subgroup in whom continuing ECP treatment could be a reasonable strategy, provided that our results are supported by other prospective studies. On the other hand, switching to an alternative drug or early addition of a second drug could improve outcomes in other patients with SRes-GVHD after allo-HSCT.
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CONFLICT OF INTEREST
Carlos Solano reports personal fees from Incyte, Therakos, Terumo BCT, and grants from Astellas and Pfizer outside the submitted work. Other authors declare no potential conflicts of interests.

AUTHOR CONTRIBUTIONS
Paula Amat and Andreu Martínez, performed T-cell and cytokines assays, and collected the data. Lucía López-Corral, Rosa Goterris, Ariadna Pérez, Olga López, Inmaculada Heras, Cristina Arbona, María Cruz Viguria, Juan Carlos Hernández-Boluda, and Carlos Solano attended the patients. Paula Amat, Francisco Martínez-Ruiz, Lucía López-Corral, and Carlos Solano analyzed the data and wrote the manuscript. All reviewed the data and participated in interpretation.

ETHICS STATEMENT
The study protocol was written in accordance with the European Union Directive on Good Clinical Practice and the current version of the Declaration of Helsinki. Each participating center obtained study approval from their respective local Research Ethics Committees.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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