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Title: Regulatory T-cells in chronic graft-versus-host disease following extracorporeal photopheresis: correlation with skin and global organ responses, and ability to taper steroids

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Helen Denney participated in senior authorship of the paper, the performance of the research, and in data analysis. Robert Whittle participated in research design and in performance of the research. Peter Taylor participated in the writing of the paper and assessment of the clinical response. Statistical support was provided by Jennifer Lai and Richard Jacques of the School of Health and Related Research (ScHARR), at the University of Sheffield. The authors declare no conflict of interest.

**Disclosure**

The authors declare no conflict of interest.

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Abbreviations

**APC**, antigen-presenting cells

**BC**, Beckman Coulter

**cGvHD**, chronic graft-versus-host disease

**CI**, 95% confidence interval

**ECD**, energy-coupled dye

**ECP**, extracorporeal photopheresis

**FITC**, fluorescein isothiocyanate

**FOXP3**, forkhead box protein-3

**HSCT**, haematopoietic stem cell transplant

**IL**, interleukin

**OR**, odds ratio

**PB**, peripheral blood

**PE**, phycoerythrin

**RBC**, red blood cells

**TGF- \( \beta \)**, transforming growth factor-beta

**T-reg**, regulatory T cell, defined by the phenotypic expression profile \( \text{CD4}+\text{CD25}+\text{FOXP3}+\text{CD127}^{\text{dim/-}} \)

**WBC**, white blood cells
Abstract

Background

Induction of immune tolerance by an increase in regulatory T-cells (T-regs) following extracorporeal photopheresis (ECP) is thought to contribute to how ECP exerts its therapeutic effect in patients with chronic graft-versus-host disease (cGvHD). We investigated whether percentages and absolute counts of T-regs changed post-ECP, and examined correlation with response.

Methods

Absolute counts and % of CD4+ T cells and T-regs (CD4+CD25+FOXP3+CD127dim/-) were evaluated using flow cytometry in 32 patients with cGvHD treated by ECP for a minimum of 3 months (m), and up to 12m. CD4+ or T-regs at baseline to 12m post-ECP were compared to changes in skin disease scores or global organ involvement, or the ability to taper steroids, at 14, 28, and 56wk.

Results

T-reg % increased significantly above any overall changes in CD4+ % at 6, 9, and 12m post-ECP. There was no statistically significant association between T-regs and skin or steroid response, whereas a larger increase in CD4+ count from baseline to 1-3m corresponded to increased odds of being able to reduce steroid dose by ≥50% at 14wk. Skin and global organ responders at 28wk had higher median T-reg counts 3m post-ECP than non-responders, as did steroid responders at 56wk who were 12m post-ECP.
Conclusions
T-reg counts and % varied greatly amongst cGvHD patients, and the increase post-ECP was not significant until 6m. No clear correlation was found between T-reg counts and clinical improvement, suggesting that increases in T-reg numbers &/or proportions are not driving the mechanism leading to a response following ECP.
INTRODUCTION

Chronic graft-versus-host disease (cGvHD) is an immunological disorder that remains a major cause of morbidity and mortality following allogeneic haematopoietic stem cell transplantation (HSCT). It involves alloreactivity of donor T cells to host histocompatibility antigens, which can affect many sites, such as the skin, mucous membranes, liver and lungs. Corticosteroids and immunosuppressive drugs are common treatments for cGvHD, but the resulting unpredictable and variable response rates, toxicities and profound immunodeficiency, can cause deaths from infection. Extracorporeal photopheresis (ECP) is used as a safe and efficacious treatment for some immune-mediated diseases such as steroid-refractory cGvHD, following on from its development in 1987 for use in treating cutaneous T-cell lymphoma. Clinical improvement in cGvHD patients treated with ECP enables tapering or discontinuation of immunosuppressive agents and corticosteroids. As T- and B-cell responses to novel and recall antigens are not destroyed by ECP, it brings a reduced risk of infection compared to the use of immunosuppressive drugs\(^1\).

ECP is an apheresis therapy that involves centrifugal separation and collection of white blood cells (WBC) from blood, with the return of red blood cells (RBC) and plasma to the patient. The photosensitising agent, 8-methoxypsoralen (8-MOP) is administered to the collected WBC, followed by circulation through an irradiation chamber, where exposure to ultraviolet-A radiation occurs. The photoactivated 8-MOP causes cross-linking of DNA in cell nuclei, inducing apoptosis of the treated WBC, which are then reinfused to the patient. Apoptotic cells express specific membrane proteins which are recognised by receptors on antigen-presenting cells (APC) in the circulation. The full effects of ECP on the immune system remain poorly
understood, but it is thought that returning apoptosing WBC to the patient induces immune tolerance by modulating APC, switching their activity in favour of anti-inflammatory cytokine production, whilst decreasing production of pro-inflammatory cytokines, and inducing regulatory T cells (T-regs).

T-regs are a subpopulation of T cells which can derive from the thymus (natural T-reg), or be induced in peripheral lymphoid organs (induced T-reg). T-regs can suppress effector T cell responses and the activity of other immune cells, such as dendritic, and B cells. The balance between effector T cell and T-reg populations is critical in achieving proper control of the quality and extent of adaptive immune responses, for establishing self-tolerance, and intolerance to non-self-antigens. T-regs are CD4+ cells that express high levels of CD25 (the interleukin (IL)-2 receptor α-chain) and the transcription factor, forkhead box protein-3 (FOXP3). FOXP3 is a key regulator of T-reg development and function. Non-regulatory T cells can also express the intracellular marker FOXP3, so a more stringent definition of T-regs incorporates an additional marker of high specificity. T-regs downregulate expression of the IL-7 receptor α-chain (CD127) compared to conventional T cells, so a CD127dim/- marker is also often used for T-reg characterisation.

The ability of T-regs to suppress aberrant immune responses, regulate T cell homeostasis, and to maintain self-tolerance, prompted interest in their impact on cGvHD, and how ECP might influence their numbers. Evidence that T-regs play a key role in GvHD was demonstrated in a mouse model where removal of the CD4+CD25+ T cells present in the stem cell graft accelerated GvHD, and adding freshly isolated CD4+CD25+ T cells at the time of HSCT delayed, or even prevented,
this disease. Administration of cultured and activated CD4+CD25+ T-regs was shown to inhibit GvHD lethality in mice. Striking results in murine GvHD, coupled with the ready availability of donor T-regs, has led to GvHD prevention emerging as the first clinical application for human T-reg. Evidence for treating existing GvHD with T-regs remains sparse, however, but an anecdotal report suggested amelioration of cGvHD in one patient following transfer of in vitro expanded donor T-regs.

The potential impact of T-regs in ECP was observed when established experimental GvHD was reversed in mice by increasing donor T-regs as a consequence of transferring splenocytes from ECP-treated animals. T-regs whose development is promoted during ECP are antigen-specific, corresponding to the clones of cytotoxic T-cells that were collected in the apoptotic WBC product after predominating in the circulation during an active immune response. Using a hypersensitivity model in mice, Maeda and colleagues showed that antigen-specific T-regs were induced by apoptotic cells generated from ECP, and that transferring these T-regs to other syngeneic mice created the same antigen-specific immune suppression.

Despite frequent suggestions that T-regs play an integral part in the mode of action of ECP when used to treat cGvHD patients, reported levels of T-regs in this disease remain inconclusive. Compared to HSCT recipients without cGvHD, both an increase and a decrease, in T-regs have been documented in cGvHD. A study of 29 HSCT patients reported that a numerical deficit of T-regs following HSCT was associated with acute, but not chronic, GvHD, whereas a different study
reported elevated T-reg numbers and percentages in both acute and chronic GvHD\(^{17}\).

There is a paucity of studies reporting the effects of ECP on T-reg numbers and proportions in cGvHD patients. We performed a study of 32 patients, using flow cytometry to determine changes in CD4+ T-cell and T-reg numbers and proportions after a minimum of 3 months (m) ECP treatment, and examined how these cell measures correlated with skin and global organ responses, and the ability to taper steroids. Here, we report that T-reg numbers increase statistically significantly post-ECP above any overall change in CD4+ T-cells, and that these T-reg increases are not a universal feature of response to ECP. This is the largest study to date that comprehensively examines the effect of ECP on T-reg numbers and their association with clinical improvement in cGvHD patients.

**MATERIALS AND METHODS**

**Study Population**

Adult patients (\(n=32\), 50% male, aged 19-62, median=46.5 years) diagnosed with cGvHD provided written consent and were included in the study, subject to the inclusion and exclusion criteria detailed in Table 1. At ECP start, 78% of patients had skin involvement, and 91% were on steroids. Patient characteristics are detailed in Table 1. ECP was performed using the THERAKOS™ CELLEX\(^{®}\) or UVAR XTS\(^{®}\) Photopheresis Systems at the Photopheresis Unit, Rotherham NHS Foundation Trust. Patients received 2 treatments over 2 consecutive days (1 cycle) every fortnight for 14wk, then 1 cycle every 4wk, although some variation did occur (see Table 1). The study period was 56wk: all 32 patients were treated for a minimum of
3m; 28 for ≥6m; 26 for ≥9m; and 26 patients were treated for ≥12m. Reasons for stopping ECP in the 6 patients that received ECP for <12m include death from infections \( (n=4) \), an unscheduled stop \( (n=1) \) and cGvHD improvement (maximal response) \( (n=1) \). Peripheral blood (PB) samples were taken every month immediately before each treatment cycle commenced. PB was also collected from 25 age-matched healthy volunteers, who gave informed consent, to enable comparison with patient CD4+ T-cell counts and %. The study was approved by York Research Ethics Committee, and internally by the R&D Department.

**Measures of Response to ECP**

Clinical response scores were obtained at 14, 28, and 56wk post-ECP, where possible. **SKIN**: The modified Rodnan scoring system was used to evaluate skin disease, where a high score equates to more severe skin disease and the % reduction in skin score from baseline was used to calculate response. Patients with a 0-24% reduction in skin score were classed as non-responders, whereas responders experienced a 25-100% reduction from pre-ECP values. **STEROIDS**: The ability to reduce steroid dose (mg/kg) by ≥50%, whilst disease activity was controlled or improved by ECP, was considered a response. Patients whose steroids were tapered only 0-49%, or received an increased dose, were considered non-responders. **GLOBAL ORGAN**: Global organ response was derived based on changes in the level of involvement of the main organ affected (skin, gut, liver, eyes, mucous membranes, or lung), using the response definition detailed above for skin, and the 2005 National Institutes of Health (NIH) cGvHD Consensus Response Criteria\(^{18} \) for all other organs. For our analyses, either a partial or complete response, as detailed by the NIH response definitions, were considered to be a
global organ response. Non-responders were defined according to the criteria for organ progression.

**Flow Cytometry of CD4+ Cells and T-regs**

Fresh anti-coagulated PB samples were used for enumeration of CD4+ T-cells. CD4+ T-cells were identified following incubation of 100µl PB at 4 °C for 30 min with anti-human antibodies as follows: CD4 conjugated with phycoerythrin-cyanine 7 (PE-Cy7); PE-Texas Red (energy-coupled dye, ECD)-conjugated CD45; and CD3-PE anti-human antibodies. All antibodies were from Beckman Coulter (BC) (Buckinghamshire, UK), except CD4-PE-Cy7 (BD Biosciences, Oxford, UK). RBC were lysed with IOTest® 3 Lysing Solution (BC) after antibody staining of WBC.

For enumeration of T-regs, intracellular labelling was required. Mononuclear cells were first separated from fresh PB samples using LymphoPrep™ density gradient medium (Axis-Shield PoC AS, Norway), according to the manufacturer’s recommendation, and washed 2 times with RPMI-1640 medium (Sigma-Aldrich, Dorset, UK). Cells were labelled for cell surface markers by incubating 100µl cell preparation at 4 °C for 30 min with anti-human antibodies: CD127 conjugated with fluorescein isothiocyanate (FITC) (BD Pharmingen™, Oxford, UK); CD25-PE-Cy5 (BC); CD4-PE-Cy7; and CD45-ECD. Labelled cells were subsequently washed with cold 0.9% NaCl solution, fixed and permeabilised using a kit (Miltenyi, Surrey, UK) according to the manufacturer’s protocol, and intracellular staining performed using an anti-human FOXP3-PE antibody (Miltenyi). Cells were resuspended in 500µl 0.9%NaCl solution prior to analysis.
T-regs were identified by the phenotypic expression profile CD4+CD25+FOXP3+CD127dim/-.. The frequencies (%) of lymphocytes that were CD3+CD4+, and the % of CD3+CD4+ T-cells that were T-regs were evaluated using five-colour flow cytometry on an FC-500 Flow Cytometer (BC), following gating of lymphocytes according to CD45 expression and side-scatter. Absolute counts (cells/µl) of CD3+CD4+ T cells were calculated by multiplying their % by the lymphocyte count, obtained by automated differential using an Advia® 2120i Haematology System (Siemens, Germany). T-reg counts were derived by multiplying their % by the count of CD3+CD4+ T-cells.

**Statistical Analysis**

All analyses were performed using SPSS (PASW) 20 software. Multiple analyses (see SDC, Materials and Methods) were performed to address the following questions:

- **Are there statistically significant differences in:**
  1. T-regs/CD4 of prior acute and non-prior acute GvHD patients?
  2. CD4 measures of healthy controls and cGvHD patients?
  3. T-regs/CD4 with duration of ECP treatment?
  4. T-regs/CD4 of responders and non-responders?

- **Are there associations between:**
  5. The number or % of T-regs/CD4 and clinical response?
  6. The change of T-reg/CD4 measures from pre-ECP to post-ECP levels, and clinical response?
  7. T-reg/CD4 measures and skin or steroid scores?
RESULTS

Response rates of the study group

To establish whether our patient cohort gave results that were representative of previously established response rates, we examined the skin, steroid, and global organ response at the last measured time point (≤12m) for each patient. Four (12.5%) patients had died by 8m of commencing ECP. Of the 26 patients that had skin involvement, 65.4% undergoing ECP achieved at least a 25% improvement in skin scores. Similarly, 60% of the 30 patients on steroids demonstrated a response to ECP, where a ≥50% dose reduction was achieved. Global organ responders accounted for 71.9% of the 32 patients in our cohort. Our response rates were comparable to the study of 32 cGvHD patients by Apisarnthanarax et al., who found 64% achieved a ≥50% steroid dose reduction, and to a study by Couriel et al. of 71 patients with severe cGvHD, where overall improvement was seen in 61%, with 59% of patients achieving a skin response.

Prior acute GvHD

No statistically significant differences were found in T-reg or CD4+ measures between patients that had acute GvHD prior to their cGvHD, and those that did not.

Differences in CD4+ measures between cGvHD patients and healthy controls

Patients with cGvHD had statistically significantly lower CD4+ % and counts at baseline (P<0.001) and at the last measured time point post-ECP (P<0.001), than the healthy control group.
Changes in the proportion of T-reg/CD4+ cells with ECP treatment duration

After accounting for overall changes in CD4+ measures, there was a statistically significant increase in T-reg measures over time ($P=0.01$). Compared to pre-ECP measures, there were statistically significant increases of T-reg proportions at 6m ($P=0.003$), 9m ($P=0.008$), and 12m ($P=0.02$). Median T-reg count increased 3.7-fold at 9m from the pre-ECP value. No statistically significant change in CD4+ % ($P=0.111$) or count ($P=0.165$) was found over time (Figure 1).

Associations between T-reg/CD4+ measures and clinical response

No statistically significant associations were found between T-reg/CD4+ counts or % and a skin disease score reduction of ≥25%, or the ability to taper steroids by ≥50%.

Differences in the distribution of T-reg/CD4 between responders and non-responders

Skin response

At 28wk, patients demonstrating a skin response had statistically significantly different distributions of T-reg counts at 3m post-ECP ($P=0.025$), with a higher median, than non-responders (7.7 vs 3.1 cells/µl) (Figure 2). Skin responders at 56wk had lower medians than non-responders ($P\leq 0.044$), of: CD4+% at 0-9m post-ECP (SDC, Figure 1); and CD4+ counts at 1 & 9m (SDC, Figure 2).

Steroid response

Patients demonstrating a steroid response at 56wk had higher median counts of T-regs ($P=0.009$) (Figure 2) and CD4+ ($P=0.027$) (SDC, Figure 2) at 12m post-ECP.
**Global organ response**

At 28wk, patients demonstrating a reduction in global organ involvement had statistically significantly different distributions, with higher medians, of T-reg counts at 3m post-ECP than non-responders ($P=0.023$) (Figure 2), but similar T-reg % (SDC, Figure 3). At 56wk, responders had lower median: CD4+ % at 0-2 and 6m ($P \leq 0.044$) (SDC, Figure 1); and CD4+ counts at 1m ($P=0.024$) (SDC, Figure 2).

**Associations between response and the post-ECP changes at 1-12m in T-reg/CD4+ measures from pre-ECP values**

**Skin response**

No association was found between post-ECP changes in T-reg or CD4+ measures and an improvement in skin disease of $\geq 25\%$.

**Steroid response**

No association was found between post-ECP changes in T-reg measures and the ability to taper steroids. For the CD4+ analyses, after controlling for pre-ECP CD4+ count and platelet count, a 1 cell/$\mu$l increase in the difference between pre-ECP and 1, 2, and 3m CD4+ counts increased the odds of a steroid response at 14wk (1m odds ratio (OR) 1.0067, confidence interval (CI): 1.0002-1.0132, $P=0.043$; 2m OR 1.0100, CI: 1.0002-1.0198, $P=0.045$; 3m OR 1.009, CI: 1.001-1.016, $P=0.027$).

Similar results were found for 3m CD4+ counts with cyclosporine as a covariate (OR 1.008, CI: 1.001-1.016, $P=0.028$).
**Associations between T-reg/CD4+ measures and skin scores**

Moderate positive associations were observed between pre-ECP T-reg counts and baseline skin scores (higher T-reg counts associated with greater skin disease) \( (P=0.025) \) (SDC, Figure 4a), or 14wk \( (P=0.034) \). Moderate positive associations were also seen between skin scores at 56wk and CD4+ % at pre-ECP \( (P=0.042) \), 1m \( (P=0.019) \), 2m \( (P=0.036) \), 3m \( (P=0.008) \), 6m \( (P=0.006) \) and 9m \( (P=0.008) \), and CD4+ counts at 1m \( (P=0.047) \) and 9m \( (P=0.023) \).

**Associations between T-reg/CD4+ measures and steroid dose**

Lower T-reg counts at 12m were associated with higher steroid dose at 56wk and, therefore, greater disease activity (negative association, \( P=0.009 \))(SDC, Figure 5a). A higher pre-ECP T-reg %, however, was positively associated with greater baseline steroid dose \( (P=0.016) \)(SDC, Figure 5b), whereas lower pre-ECP CD4+ counts were associated with greater baseline steroid dose \( (P=0.003) \). Similarly, lower 3m CD4+ counts were associated with higher 14wk steroid dose (negative association, \( P=0.033 \)) (SDC, Figure 5c).

**DISCUSSION**

Our study of 32 cGvHD patients treated with ECP revealed a statistically significant increase in T-reg from baseline at 6m post-ECP and beyond, which is at variance with some previous reports. Rao and colleagues, for example, did not find a statistically significant difference in T-reg in 11 cGvHD patients for their study duration of 0-6m post-ECP\(^2\). Our results also conflict with the study of 27 cGvHD patients by Di Biaso and colleagues, who reported a statistically significant increase in T-reg at the sixth ECP cycle (approximately 3m post-ECP), but not thereafter.
This study also found that only cGvHD patients who responded to ECP demonstrated an increase in circulating T-reg numbers\textsuperscript{22}, whereas we found no clear pattern, with some non-responders showing an increase in numbers and/or proportion of T-regs, and some responders whose T-reg counts decreased or remained static.

We conducted a high number of tests that led to the issue of multiple testing. Caution should be applied when interpreting results as the more tests performed, the more likely it is to obtain a false positive result.

Interestingly, no statistically significant increase in median CD4+ T-cell count or % was found, even after 12m of ECP (Figure 1e-h). Both pre- and post-ECP CD4+ counts were statistically significantly lower in cGvHD patients compared to healthy controls. Our study indicates that ECP did not normalise CD4+ T-cell numbers, which conflicts with the findings of Bladon & Taylor\textsuperscript{23}, who found an enhancement of CD4+ T-cell numbers after 3m of ECP in their study of 8 cGvHD patients.

The functionality of T-regs was not tested in our study, and it is important to consider that the T-regs we measured may be dysfunctional or possess an elevated suppressive quality, thereby affecting their potential to influence immune responses in a greater or lesser capacity. Indeed, ECP has been shown to augment immunosuppressive T-reg function by triggering CD39-mediated adenosine production by T-reg, which can reduce T-cell proliferation\textsuperscript{24}. T-regs can, therefore, become more suppressive after ECP, but augmentation of adenosine production is only one of a number of mechanisms that operate during T-reg-mediated
suppression. The variety of impairment that may occur in cGvHD of other suppressive mechanisms, such as: the release of IL-10 &/or transforming growth factor-β (TGF-β); consumption of IL-2; and the expression of inhibitory molecules\textsuperscript{16}, may account somewhat for our heterogeneous observations that patients with low or high T-reg counts or % demonstrated a clinical response.

Our analysis of T-reg numbers and proportion in cGvHD patients has shown a high degree of variability. A heterogeneous study population complicates the interpretation of results, with T-reg reconstitution and clinical response being influenced by patient characteristics, stem cell source, T cell depletion strategy of the graft, infections, underlying disease, extent of tissue injury, and immunosuppressive medications\textsuperscript{25}. Changes in T-reg frequencies can be either drug-induced, or disease-related, and it is important to remember that drug use varies as ECP progresses. Intravenous immunoglobulin expands T-regs\textsuperscript{26}, calcineurin inhibitors used to treat GvHD decrease T-reg numbers, whereas sirolimus use produces an increase, and corticosteroids are known to enhance expression of FOXP3 and improve the survival and function of T-regs\textsuperscript{27}. The cytokine milieu in which T-regs operate can also affect their function. For example, tumour necrosis factor (TNF) downmodulates the function of CD4+CD25+ T-regs\textsuperscript{28}, and TGF-β induces FOXP3 expression\textsuperscript{29}.

In an attempt to resolve the debate as to the importance of T-regs in clinical response to ECP, we not only looked at associations of response with T-reg counts and %, but also investigated if it was the \textit{change} in T-reg measures post-ECP that might be pivotal. Furthermore, associations between T-reg measures and skin and steroid scores were examined. From all these analyses, the conclusion of no clear
associations between T-reg measures and clinical improvement is drawn. Consistent with our findings, Nord et al. found in her study of 16 cGvHD patients, who were treated with ECP for a minimum of 3m, that increases in the proportion of T-reggs were not always accompanied by clinical benefit. Any significant impact of T-reggs on the clinical improvement of patients with cGvHD following ECP may be qualitative rather than quantitative, rendering observations based solely on the quantity of T-reggs of limited use. Furthermore, if there is no deficit of T-reggs in cGvHD, as several studies have suggested, it is unlikely that simply boosting peripheral blood (PB) T-reg numbers will be sufficient to generate complete amelioration. A better understanding of the diversity of T-reg subsets, plasticity, and function in the context of ECP therapy and cGvHD is required, with a greater focus on their role in target organs. A greater force than PB T-reggs alone is likely to be needed to suppress the chronic inflammatory process at work in cGvHD, involving cellular and humoral responses, cytokines, and chemokine expression in target tissues.

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REFERENCES


Table 1: Patient Characteristics

cGvHD Patient Characteristics  \(n=32\)

**Inclusion criteria**

- ≥18 years old (for this study).
- Patients must require second-line/third-line salvage therapy because of either steroid-refractory or steroid-dependent cGvHD. Steroid-refractory is defined as minimal, or no, response to prednisolone 1mg/kg or equivalent after a minimum of 4 wks of treatment; steroid-dependent is defined as the inability to reduce steroids to < 10 mg or equivalent daily without flare of cGVHD. Alternatively, patients must be unable to tolerate standard first-line corticosteroid therapy.
- Patients must have cGvHD primarily affecting at least one of the following organs: skin; mucosal membranes (mouth and/or eye disease); liver.
- Patients should have cGVHD confirmed with compatible histology from an affected organ biopsy, wherever possible.
- Patients with cGvHD following full-intensity conditioning transplants, reduced-intensity conditioning transplants or donor lymphocyte infusions are all eligible.

**Exclusion criteria**

- Patients exhibiting a known sensitivity to psoralen compounds.
- Patients with comorbidities that may result in photosensitivity.
- Patients with aphakia.
- < 40 kg in weight (defined by machine characteristics).
- Pregnancy.
- History of heparin-induced thrombocytopenia.
- Uncontrolled infection.
- Absolute neutrophil count < 1.0 x10^9/L.
- Platelet count < 20 x10^9/L despite platelet transfusion support.
- Diarrhoea > 1000 mL daily.
- Other cause thought likely to cause inability to tolerate apheresis procedure.

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<td>Reduced intensity conditioning, % (no.)</td>
<td>53</td>
</tr>
<tr>
<td>NIH Score at ECP Onset</td>
<td></td>
</tr>
<tr>
<td>Mild, % (no.)</td>
<td>12.5</td>
</tr>
<tr>
<td>Moderate, % (no.)</td>
<td>50</td>
</tr>
<tr>
<td>Severe, % (no.)</td>
<td>37.5</td>
</tr>
<tr>
<td>Organ target at GvHD onset % (no.)</td>
<td></td>
</tr>
<tr>
<td>Skin only</td>
<td>25</td>
</tr>
<tr>
<td>Gut only</td>
<td>3.125</td>
</tr>
<tr>
<td>Liver only</td>
<td>6.25</td>
</tr>
<tr>
<td>Eyes only</td>
<td>3.125</td>
</tr>
<tr>
<td>Multiple:</td>
<td></td>
</tr>
<tr>
<td>Skin + 1 of respiratory, joints, gut, liver, mucous membranes</td>
<td>25</td>
</tr>
<tr>
<td>Skin + 2 of respiratory, joints, gut, liver, mucous membranes</td>
<td>18.75</td>
</tr>
<tr>
<td>Skin + 3 (eyes, gut, liver)</td>
<td>3.125</td>
</tr>
<tr>
<td>Skin + 4 of respiratory, joints, gut, liver, mucous membranes, eyes</td>
<td>6.25</td>
</tr>
<tr>
<td>Gut + eyes</td>
<td>3.125</td>
</tr>
<tr>
<td>Gut + 2 (joints, eyes)</td>
<td>3.125</td>
</tr>
<tr>
<td>Gut + 3 (joints, liver, respiratory)</td>
<td>3.125</td>
</tr>
<tr>
<td>Prior acute GvHD</td>
<td></td>
</tr>
<tr>
<td>Yes % (no.)</td>
<td>69</td>
</tr>
<tr>
<td>No  % (no.)</td>
<td>31</td>
</tr>
<tr>
<td>Time from Transplant to cGvHD</td>
<td></td>
</tr>
<tr>
<td>Months: Median (Range)</td>
<td>3.7 (1.2-98.5)</td>
</tr>
<tr>
<td>Time from Transplant to ECP Start</td>
<td>19.8 (3.2-133.8)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Time from cGvHD Diagnosis to ECP Start</td>
<td>11.8 (0.8-91)</td>
</tr>
<tr>
<td>Platelet Count at ECP Start</td>
<td>240.5 (21-614)</td>
</tr>
<tr>
<td>Bilirubin Score at ECP Start</td>
<td>12 (2-282)</td>
</tr>
<tr>
<td>Drugs received at ECP Start</td>
<td></td>
</tr>
<tr>
<td>Intravenous Immunoglobulin, % (no.)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>Prednisolone, % (no.)</td>
<td>90.6 (29)</td>
</tr>
<tr>
<td>Cyclosporine, % (no.)</td>
<td>56.3 (18)</td>
</tr>
<tr>
<td>Tacrolimus, % (no.)</td>
<td>12.5 (4)</td>
</tr>
<tr>
<td>Mycophenolate mofetil (MMF), % (no.)</td>
<td>40.6 (13)</td>
</tr>
<tr>
<td>Azathioprine (AZA), % (no.)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Thalidomide, % (no.)</td>
<td>3.1 (1)</td>
</tr>
<tr>
<td>Total Number of Immunosuppressive Agents Per Patient at ECP Start: Median (Range)</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>Number of ECP Treatments Per Patient Included in the 56wk Assessment Period</td>
<td></td>
</tr>
<tr>
<td>2 treatments per ECP cycle = 16 treatments after fortnightly ECP from start to the 14wk assessment point; 24 treatments to the 28wk assessment (ECP cycles are every 4wk after 14wk); and ~38 treatments to the 56wk assessment.</td>
<td></td>
</tr>
<tr>
<td>Actual Treatments Received: Median (Range)</td>
<td>35.5 (10-49)</td>
</tr>
<tr>
<td>Expected Treatments: Median (Range)</td>
<td>38   (16-38)</td>
</tr>
</tbody>
</table>
Figure 1 - Absolute counts of: (a) T-regs; and (e) CD4+ cells, and proportions of: (b) CD4+ cells that are T-regs; and (f) lymphocytes that are CD4+ cells, at pre-ECP, and 1, 2, 3, 6, 9, and 12 months post-ECP. Fold-change from pre-ECP to 1-12m post-ECP of (c) T-reg counts, (d) T-reg %, (g) CD4+ counts and (h) CD4+ %. Coloured lines in (a), (b), (e), and (f) represent values for individual patients, and the bold black lines in (a-h) show the medians. A statistically significant increase from pre-ECP levels of the proportion of T-regs (after accounting for any changes in CD4+ count) was seen at 6, 9 and 12 m post-ECP (b&d). Pre- and post-ECP CD4+ counts (e) and proportions (f) did not differ significantly.

Figure 2 - Comparison of T-reg cells/µl at 0-12m post-ECP in patients with and without a response in: (a) skin; (b) steroids; and (c) global organ, as measured at 14, 28, and 56 weeks. Plots show medians in 25-75th percentile boxes, or median only if n≤3, with 5-95th percentile range whiskers. The number (n) of patients in each group at each time point is given below the boxes. Outliers are shown as circles. Statistically significant (P <0.05) differences between responders (grey) and non-responders (white) are labelled with their Mann-Whitney P values. Tests were not done if n<4 in any group.
Figure 1: Changes in T-reg and CD4+ Absolute Counts and Proportions at 0-12m Post-ECP

- **a**: T-reg Absolute Cell Counts at 0-12m Post-ECP
- **b**: Proportion of CD4+ cells that are T-regs at 0-12m post-ECP
- **c**: Fold-Change From Pre-ECP Values of T-reg Absolute Counts at 1-12m Post-ECP
- **d**: Fold-Change From Pre-ECP Values of the Proportion of CD4+ Cells That Are T-regs at 1-12m Post-ECP
- **e**: CD4+ Absolute Cell Counts at 0-12m Post-ECP
- **f**: Proportion of Lymphocytes That Are CD4+ at 0-12m Post-ECP
- **g**: Fold-Change From Pre-ECP Values of CD4+ Absolute Counts at 1-12m Post-ECP
- **h**: Fold-Change From Pre-ECP Values of the Proportion of Lymphocytes That Are CD4+ at 1-12m Post-ECP
Figure 2: T-Reg Cell Counts in Skin, Steroid and Global Organ Responders and Non-Responders at 0-12m Post-ECP

a

i T-REG SKIN 14WK

ii T-REG SKIN 28WK

iii T-REG SKIN 56WK

Treatment Duration (months)

b

i T-REG STEROID 14WK

ii T-REG STEROID 28WK

iii T-REG STEROID 56WK

Treatment Duration (months)

c

i T-REG GLOBAL 14WK

ii T-REG GLOBAL 28WK

iii T-REG GLOBAL 56WK

Treatment Duration (months)
Supplementary Online Material
SDC, Materials and Methods

Statistical Analysis

Two-tailed significance was held at 5%. For the logistic regression, statistical significance was also conditional on 95% confidence intervals not including 1. There is no general consensus on what procedure to adopt for multiple comparisons. Despite the large number of multiple tests carried out for this study, Bonferroni corrections were not used to aid the interpretation of the results. As recommended by Altman, we report unadjusted \( P \)-values and confidence limits with a suitable cautionary note on interpretation. The following 7 questions were addressed:

1. **Are there statistically significant differences in T-regs/CD4 between prior acute and non-prior acute GvHD patients?**
   Mann-Whitney U-Tests were used to compare the distribution of T-reg or CD4+ measures between patients that had prior acute GvHD, with those that had not.

2. **Are there statistically significant differences in CD4 measures between healthy controls and cGvHD patients?**
   An independent-samples t-test was conducted to compare patient CD4+ measures pre-ECP or at the last time point available post-ECP, with healthy control CD4+ measures. The data were log-transformed for this analysis, as it was not normally distributed.
3. **Are there statistically significant changes in T-regs/CD4 with duration of ECP treatment?**

   A longitudinal model was constructed to test whether there were any statistically significant changes in CD4+ cells or T-regs over time, accounting for changes in CD4+ by using the proportion of T-regs over CD4+. Cell proportions were log-transformed and comparisons made between the proportion measured at each time point, and the pre-ECP proportion.

4. **Is the number or % of T-regs/CD4 associated with clinical response?**

   A logistic regression was used to assess whether the absolute count or % of T-regs or CD4+ cells pre-ECP and at 1-12m post-ECP were associated with skin response or the ability to taper steroids at 14, 28, and 56wk. Covariates included were either platelet count or treatment with cyclosporine, as these were found to influence response. The pre-ECP CD4+ or T-reg measure was also included in models if it was a statistically significant predictor when tested as the main independent variable. Baseline skin score or steroid dose was added as an additional covariate for analysis with skin or steroid response as the outcome. These analyses considered only the response status of the patient at the specific time point in question.

5. **Are there statistically significant differences in T-regs/CD4 between responders and non-responders?**

   To investigate differences in the distribution of T-reg/CD4+ measures between responders and non-responders (skin, steroid and global organ) at each time
point, Mann-Whitney U-Tests were used where there were at least 4 observations in any group.

6. **Are there associations in the change of T-reg/CD4 measures from pre-ECP to post-ECP levels, and clinical response?**

Logistic regression was used to investigate associations between the change in T-reg and CD4 measures from pre-ECP to 1-12m post-ECP (calculated by subtracting the baseline), and skin or steroid responses, after accounting for baseline cell measures, and platelet count or cyclosporine use. Baseline skin score or steroid dose was added as an additional covariate for analysis with skin or steroid response as the outcome.

7. **Are there associations between T-reg/CD4 measures and skin or steroid scores?**

Spearman’s correlations were used to test the association between raw T-reg or CD4+ measures and skin or prednisolone scores.

**References**

**SDC, Figure 1:** Comparison of CD4+ proportions at 0-12m post-ECP in patients with and without a response in: (a) skin; (b) steroids; and (c) global organ, as measured at 14, 28, 56 and 112 weeks. Plots show medians in 25-75th percentile boxes, or median only if \( n \leq 3 \), with 5-95th percentile range whiskers. The number (\( n \)) of patients in each group at each time point is given below the boxes. Outliers are shown as circles. Statistically significant (\( P < 0.05 \)) differences between responders (grey) and non-responders (white) are labelled with their Mann-Whitney \( P \) values. Tests were not done if \( n < 4 \) in any group.
SDC, Figure 2: CD4+ Cell Counts in Skin, Steroid and Global Organ Responders and Non-Responders at 0-12m Post-ECP

SDC, Figure 2 - Comparison of CD4+ cells/µl at 0-12m post-ECP in patients with and without a response in: (a) skin; (b) steroids; and (c) global organ, as measured at 14, 28, 56 and 112 weeks. Plots show medians in 25-75th percentile boxes, or median only if $n \leq 3$, with 5-95th percentile range whiskers. The number ($n$) of patients in each group at each time point is given below the boxes. Outliers are shown as circles. Statistically significant ($P < 0.05$) differences between responders (grey) and non-responders (white) are labelled with their Mann-Whitney $P$ values. Tests were not done if $n < 4$ in any group.
SDC, Figure 3: T-Reg Proportions in Skin, Steroid and Global Organ Responders and Non-Responders at 0-12m Post-ECP

SDC, Figure 3 - Comparison of T-reg proportions (%) at 0-12m post-ECP in patients with and without a response in: (a) skin; (b) steroids; and (c) global organ, as measured at 14, 28, 56 and 112 weeks. Plots show medians in 25-75th percentile boxes, or median only if n≤3, with 5-95th percentile range whiskers. The number (n) of patients in each group at each time point is given below the boxes. Outliers are shown as circles. Mann-Whitney tests were not done if n<4 in any group. No statistically significant differences between responders (grey) and non-responders (white) were found.
SDC, Figure 4: Associations between T-reg/CD4+ Absolute Counts/% and Skin Scores Pre-ECP and at 14, 28 & 56wk Post-ECP

SDC, Figure 4 – Scatter plot series of skin scores at pre-ECP and 14, 28 & 56wk post-ECP and corresponding time point values of: (a) T-reg counts (black squares); (b) T-reg % (white squares); (c) CD4+ counts (black triangles); and (d) CD4+ % (white triangles). Spearman’s correlation was used to test the association between T-reg/CD4+ counts or % and skin scores. Statistically significant ($P<0.05$) correlations are labelled with their $P$ values, with greater pre-ECP T-reg counts associated with higher skin scores (worse response) at baseline (a) and 14wk (not shown).
**SDC, Figure 5:** Associations between T-reg/CD4+ Absolute Counts/% and Steroid Dose Pre-ECP and at 3, 6 & 12m Post-ECP

**a**

- **Moderate Positive**
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg cells/ul Day 0
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg cells/ul 3m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg cells/ul 6m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg cells/ul 12m

**b**

- **Moderate Positive**
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg % Day 0
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg % 3m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg % 6m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - T-reg % 12m

**c**

- **Moderate Negative**
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ cells/ul Day 0
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ cells/ul 3m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ cells/ul 6m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ cells/ul 12m

**d**

- **Moderate Negative**
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ % Day 0
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ % 3m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ % 6m
  - Pre-ECP Steroid Dose (mg/kg body weight)
  - CD4+ % 12m

**SDC, Figure 5** – Scatter plot series of steroid dose at pre-ECP and 14, 28 & 56wk post-ECP and corresponding time point values of: (a) T-reg counts (black squares); (b) T-reg % (white squares); (c) CD4+ counts (black triangles); and (d) CD4+ % (white triangles). Spearman’s correlation was used to test the association between T-reg/CD4+ counts or % and steroid dose. Statistically significant ($P<0.05$) correlations are labelled with their $P$ values. A greater steroid dose pre-ECP was associated with a higher pre-ECP T-reg % (b), but lower pre-ECP CD4+ counts (c). A greater steroid dose (worse response) at 56wk, however, was associated with lower T-reg counts at 9-12m post-ECP (a).