The effect of extracorporeal photopheresis alone or in combination therapy on circulating CD4+Foxp3+CD25- T-cells in patients with leukemic cutaneous T-cell lymphoma

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Abstract

Purpose—Extracorporeal photopheresis (ECP) alone or in combination therapy is effective for treatment of leukemic cutaneous T-cell lymphoma (L-CTCL), but its mechanism(s) of action remain unclear. This study was designed to investigate the effect of ECP on regulatory T-cell and CD8$^+$ T-cells in L-CTCL patients.

Experimental Design—Peripheral blood from 18 L-CTCL patients at baseline, Day 2, 1-month, 3-month, and 6-month post-ECP therapy were analyzed by flow cytometry for CD4$^+$CD25$^{+/high}$, CD4$^+$Foxp3$^+$CD25$^{+/-}$, CD3$^+$CD8$^+$, CD3$^+$CD8$^+$CD69$^+$, and CD3$^+$CD8$^+$IFN-γ^+ T-cells. Clinical responses were assessed and correlated with changes in these T-cell subsets.

Results—Twelve of 18 patients achieved clinical responses. The average baseline number of CD4$^+$CD25$^{+/high}$ T-cells of PBMCs in L-CTCL patients was normal (2.2%), but increased at 6-month post-therapy (4.3%, p<0.01). The average baseline number of CD4$^+$Foxp3$^+$ T-cells out of CD4$^+$ T-cells in 9 evaluable patients was high (66.8±13.7%), mostly CD25 negative. The levels of CD4$^+$Foxp3$^+$ T cells in responders were higher (n=6, 93.1±5.7%) than non-responders (n=3, 14.2±16.0%, p<0.01), and they declined in parallel with malignant T-cells. The numbers of CD3$^+$CD8$^+$CD69$^+$ and CD3$^+$CD8$^+$ IFN-γ^+ T-cells increased at 3-month post-therapy in 5 of 6 patients studied.

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Conclusions—ECP alone or in combination therapy might be effective in L-CTCL patients whose malignant T-cells have a $CD4^+Foxp3^+CD25^-$ phenotype.

Introduction

Cutaneous T-cell Lymphomas (CTCL) are extra-nodal non-Hodgkin T-cell lymphomas that first present as cutaneous lesions with clonal expansion of malignant helper T-cells (1). The leukemic variants (L-CTCL) include Sézary Syndrome (SS) and a portion of mycosis fungoides (MF) harboring blood $CD4^+CD26^-$ or/and $CD4^+CD7^-$ malignant T-cells with diffuse erythroderma. The degree of blood involvement in patients with L-CTCL is associated with worse overall survival (2-4). To date, L-CTCL/SS are rarely curable except long-term complete response after allogeneic stem cell transplantation(5, 6).

Extracorporeal photopheresis (ECP) is used commonly as frontline therapy for L-CTCL, with which leukocytes are exposed *ex vivo* to 8-methoxypsoralen (8-MOP) and UVA radiation, and then reinfused into the patient circulation. The overall response rate of ECP in CTCL patients is between 54% and 74% with a 14%-33.3% complete response rate (7-9). It is well-tolerated with minimal side effects and increased overall survival (9-11). To achieve more complete responses, biological response modifiers (BRM), especially interferons and retinoids, are often administered together with ECP and is known as combined immunomodulatory therapy. However, many question about how the therapy works remain unclear.

Regulatory T-cells (T_{reg} cells) are "professional" regulatory/suppressor T-cells critical for maintenance of immune homeostasis and prevention of autoimmunity (12). T_{reg} cells are characterized by constitutive expression of the transcription factor forkhead box P3 (Foxp3) essential for T_{reg} cell development and suppressive activity. The expression of CD25, the α-chain of IL-2 receptor, is also a feature of T_{reg} cells, but its expression is less specific, because CD25 is also expresses by conventional activated T-cells. However, T_{reg} cells express higher levels of CD25 compared to conventional T-cells (12). Therefore, the expression of Foxp3 and the high level of CD25 are widely-used as phenotypic markers for T_{reg} cells.

Interestingly, malignant T-cells in L-CTCL, especially in SS, share many features with T_{reg} cells. SS cells derive from $CD4^+$ helper T-cells, and a portion of them are positive for CD25(13), are anergic to activation stimuli, and are also immunosuppressive (14). Berger *et al* reported that after being co-cultured with dendritic cells loaded with apoptotic tumor cells *ex vivo*, $CD4^+$CTCL cells adopted a T_{reg} cell phenotype expressing CD25 and Foxp3 and secreting IL-10 and TGF-β (15). They, therefore, proposed that CTCL might be a malignancy of T_{reg} cells. Heid *et al* also found that a subset of SS patients had malignant $CD4^+Foxp3^+CD25^-$ T-cells with regulatory function (16). However, discordant findings have simultaneously been reported, especially in MF patients (17-20). How T_{reg} cells are

modulated during therapy with ECP has not been established. Addressing the controversy of T_{reg} cells in CTCL and understanding the effects of ECP on T_{reg} cells may be helpful to develop more effective and less immunosuppressive therapies.

Although the immune tolerance mediated by T_{reg} cells may explain the effects of ECP in graft-versus-host disease (GVHD), the anti-tumor immunity mediated by CD8^{+} cytotoxic T lymphocytes may underlie the efficacy of ECP in L-CTCL[21]. Higher numbers of blood CD8^{+} T-cells are associated with better clinical response to ECP[22]. Clinical improvement after ECP in CTCL patients is associated with a shift from a Th2 phenotype to a IL-12/Th1 phenotype [23]. We recently reported that in patients with L-CTCL, ECP augments blood myeloid dendritic cells (mDC), a subset of DCs producing IL-12 that polarizes naïve T-cells toward a Th1 phenotype (24).

This translational pilot study was designed to further investigate the effect of ECP treatment on T_{reg} cells and CD8^{+} T-cell function. By flow cytometry, we analyzed CD4^{+}CD25[+/high], CD4^{+}Foxp3^{+}CD25^{+/-}, CD3^{+}CD8^{+}, CD3^{+}CD8^{+}CD69^{+}, and CD3^{+}CD8^{+}IFN-γ^{+}T-cell subsets in peripheral blood from L-CTCL patients at baseline, Day 2, and 1, 3, and 6 months during ECP therapy. Clinical responses over six months of therapy were correlated with changes in these T-cell subsets.

Materials and Methods

Study Design and patients

Patients with L-CTCL starting ECP treatment during 04/2007 -11/2010 signed informed consents to enroll in this study. The study was approved by the University of Texas MD Anderson Cancer Center Institutional Review Board, and conducted according to the Declaration of Helsinki Principles. The revised diagnosis criteria for staging of MF and SS by ISCL/EORTC was used (25). All patients were treated with the UVAR XTS photopheresis system (Therakos, Inc. Raritan, NJ) over 2 consecutive days every 2-4 weeks per cycle. Fresh peripheral blood was collected at baseline (BL) and after ECP on Day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-7 months (6-7M). Peripheral blood samples from normal donors (ND) were obtained from the Department of Transfusion Medicine at our institution. Peripheral mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation.

Clinical response analysis

Evaluation of skin involvement by modified severity-weighted assessment tool (mSWAT) was performed (MD) at baseline (BL) and after treatment at 1 month, 3-4 months, and 6-7 months. In this study, we defined circulating malignant T-cells as CD3^{+}CD4^{+}CD26^{-} and/or CD7^{-} T-cells by flow cytometry (26), and assessed them before and at 1 month, 3-4 months, and 6-7 months over a treatment course (10, 27-30). Patients with complete responses (CR) had complete disease disappearance in skin or blood. Patients with partial responses (PR) had greater than 50% improvement in skin or blood involvement. Minor response (MR) patients had 25%-50% improvement of skin or blood. Patients with CR, PR and MR were considered as responders. Patients with stable disease (SD) whose changes in skin or blood

were within 25%, and patients with progressive disease (PD) whose changes in skin or blood were 25% worse from baseline, were grouped as non-responders.

Flow cytometry analysis of regulatory T-cell subsets

Immunofluorescence surface staining for CD3$^+$CD4$^+$CD25$^{+/high}$ T-cells—The standard three-color flow cytometry was used to analyze CD3$^+$CD4$^+$CD25$^{+/high}$ T-cells with fresh PBMCs. Antibodies used were APC/Cy7 anti-human CD3 (BioLegend, San Diego, CA), FITC anti-human CD4, and PE anti-human CD25 (BD Biosciences). APC/Cy7-, FITC- or PE-conjugated IgG1κ, IgG2α were isotype controls.

Immunofluorescence intracellular staining for Foxp3$^+$ T-cells—Frozen PBMCs from all time points for each patient were examined by flow cytometry at the same time. PBMCs were stained with CD3, CD4, and CD25 surface markers first. After a fixation and permeabilization step (Fixation/Permeabilization Solution, BD Bioscience), PE anti-human Foxp3 antibody (clone PCH101, eBioscience, Inc. San Diego, CA) was then added. Counting beads (Spherotech, Inc. Lake Forest, IL) were added afterward. Samples were analyzed on a Gallios flow cytometer using Kaluza software (Beckman Coulter, Inc. Brea CA), and absolute numbers were calculated (31).

Flow cytometry analysis of CD8$^+$ T-cell subsets

Cell activation—Frozen PBMCs from all time points for each patient were incubated with the leukocyte activation cocktail (BD Biosciences) at 2μl/10^6 cells for 4 hours at 37°C before staining.

Immunofluorescence intracellular staining for CD3$^+$CD8$^+$IFNγ$^+$ T-cells—FITC anti-human CD3 (BD Biosciences) and APC anti-human CD8- (Biolegend) were added into activated cells. After a fixation and permeabilization step, cells were then incubated with PerCyPCy 5.5 anti-human CD69 (Biolegend), followed with PE anti-human IFN-γ (R&D Systems). Isotype controls were also run in parallel.

Quantitative Real-Time PCR—Total RNA extraction from PBMCs, 1st strand cDNA synthesis, quantitative RT-PCR for Foxp3 (Hs00203958), and fold change calculation were done as previously described (32-34).

Statistical analysis

Differences in each parameter [mean (SD) and median (range)] among groups at baseline were examined by Kruskal-Wallis tests. Linear mixed models were used to assess the changes from baseline as a function of time point and clinical response (PR or MR vs. PD or SD). P-values <0.05 were considered as significantly different. A correlation was determined by Pearson product-moment correlation coefficient R 0.5-1.0 based on the Cohen scale. Statistical analyses were done in SPSS Statistics 17.0 and Microsoft Office Excel 2007.

Results

Response of L-CTCL patients to ECP alone or in combination therapy

Eighteen L-CTCL patients completed the 6-month treatment course and were evaluable for clinical response in skin and blood (35). Their demographics were summarized in Table 1. All patients had generalized erythroderma except Patient #17 who had high numbers of circulating tumor cells but no visible skin lesions as previously reported (33). All patients had 50% circulating malignant T-cells exception Patient #4 (Table 2). The circulating tumor cells in 17 patients had a $CD4^+CD26^-$ phenotype except for Patient #12 who had a $CD4^+CD7^-$ phenotype. All L-CTCL patients had previously received skin directed therapies, but none had received radiation, chemotherapy, or immunosuppressive agents. Six of 18 L-CTCL patients were treated with ECP only. Twelve patients who had no improvement on ECP alone at 3 months had addition of biological response modifiers, bexarotene or/and IFN-α.

After 6 months of treatment, there was an average 54.1 % reduction of circulating malignant T-cells and a 34.6 % reduction in mSWAT. Twelve of 18 patients (66.7%) achieved an overall response (Figure 1, Table 1): 11 had responses in skin and 3 had responses in blood. Of interest, four of six L-CTCL patients who received ECP alone and 8 of 12 who received ECP combination immunotherapy achieved the same overall clinical response rate of 66.7%. There were no significant differences in baseline mSWAT scores between responders and non-responders, but marginally higher percentages of $CD4^+CD26^-$ or $CD7^-$ malignant T-cells were present in the responders compared to non-responders (Table 2).

$CD4^+CD25^{+/high}$ T-cell subsets in L-CTCL patients increased after ECP

The first analysis by flow cytometry was to measure $CD4^+CD25^{+/high}$ T-cells in peripheral blood from 18 L-CTCL patients at baseline. The average numbers of $CD4^+CD25^{+/high}$ T-cells in PBMCs were 2.2±3.8% for all 18 L-CTCL patients, and 0.8±0.6% for 15 patients if excluding three outliers (Patient# 8, #12, and #15), which were comparable to those in healthy donors (0.6±0.5%, n=8, Figure 2A, B).

After treatment, the average numbers of $CD4^+CD25^{+/high}$ T-cells increased, and average percentages of $CD4^+CD25^{High}$ T-cells in PBMCs of 18 patients were 2.2% at baseline, 2.0% at 1 month, 3.9% at 3-4 months, and 4.3% at 6-7 months post-ECP (p<0.01, Figure 2A,C, Table S1). The average absolute numbers of $CD4^+CD25^{High}$ T-cells were 221.1 cells/μl at baseline, 174.2 cells/μl at 1 month, 236.6 cells/μl at 3-4months, and 281.3 cells/μl at 6-7months post-ECP, respectively (Figure 2D, Table S1). In the 12 responders, the numbers of $CD4^+CD25^{+/high}$ T-cells increased from 1.3% or 114.6 cells/μl at baseline to 4.5% or 224.33 cells/μl at 6-7 months post-ECP. However, in the 6 non-responders, the numbers of $CD4^+CD25^{+/high}$ T-cells were unchanged from 3.9% or 416.4 cells/μl at baseline to 3.9% or 395.4 cells/μl at 6 months post-ECP (Table S1).

$CD4^+Foxp3^+$ T-cells in L-CTCL patients decreased after ECP

Malignant T-cells in L-CTCL have been reported to have a regulatory T-cell phenotype ($CD4^+Foxp3^+$) with either CD25 positive or CD25 negative (15, 16). In this study, we found

increased Foxp3 mRNA levels in PBMCs of L-CTCL patients (n=17, 13.4±8.5 fold) compared to normal donors (n=5, 0.9±0.2 fold). Two patients (Patient#6 and Patient #17) had extremely high levels (80.3 and 126.8 fold). Patient #17, who had 1.19% CD4$^+$CD25$^{+/high}$ T-cells in PBMCs, was previously reported as an invisible leukemic cutaneous T-cell lymphoma with a regulatory T-cell clone (33).

Next, we examined the intracellular expression of Foxp3 in CD4$^+$ T-cells by flow cytometry. Due to limited cell numbers, we were only able to examine CD4$^+$Foxp3$^+$ T-cells in 9 L-CTCL patients. There were higher numbers of CD4$^+$Foxp3$^+$ T-cells out of total CD4$^+$ T-cells in L-CTCL patients (n=9, 66.8±13.7%, Figure 3A) compared to normal donors (n=5, 5.8±2.2 %, t-test, p<0.05). About two thirds of CD4$^+$Foxp3$^+$ T-cells were CD25 negative (48.5±30.4%, Figure 3B). Interestingly, of 9 L-CTCL patients tested, 6 responders had much higher percentages of CD4$^+$Foxp3$^+$ T-cells (93.1±5.7%) which were similar to the levels of CD4$^+$CD26$^-$ malignant T-cells (82.3%) and TCRvβ clonal T-cells (84.5%). In contrast, 3 non-responders had only low numbers of CD4$^+$Foxp3$^+$ T-cells (14.2±16.0%, p<0.01) although of high levels of CD4$^+$CD26$^-$ malignant T-cells (75.7%) and TCRvβ clonal T-cells (95.0%) (Figure 3C, Table 2). CD4$^+$Foxp3$^+$ T-cells were predominantly CD25 negative in both responders (67.5±16.2 %) and non-responders (10.6±12.1%) compared to CD25 positive portions in responders (24.9±17.7 %) and non-responders (1.9±2.2 %), respectively.

After ECP, the average absolute numbers of malignant T-cells in 18 patients decreased from 5903.9 cells cells/μl at baseline to 4326.5 cells/μl at 1 month, 3070.3 cells/μl at 3-4 months, and 2711.8 cells/μl at 6-7 months post-ECP. Meanwhile, the average numbers of CD4$^+$Foxp3$^+$ T-cells were 3640.7 cells/μl at baseline (n=9), 595.1 cells/μl at 1 month (n=5), 2010.9 cells/μl at 3-4 months (n=3), and 314.9 cells /μl at 6-7 months (n=5) post treatment (Figure 3D, Table S1).

Activated and functional CD3$^+$CD8$^+$ T-cells in L-CTCL patients increased after ECP

Compared to CD3$^+$CD8$^+$ T-cells in normal donors (596.6±412.8 cells/μl, n=3, Figure 4A), the average numbers in L-CTCL patients were lower (174.6±124.1 cells/μl, n=6, p<0.01, Figure 4B). Using CD69, a human transmembrane C-Type lectin protein as an activation marker, we found that activated CD8$^+$CD69$^+$ T-cells were much lower in L-CTCL patients (6.3±6.5 cells/μl, n=6, Figure 4B) than in normal donors (288.5 ± 185.6 cells /μl, n=3, p<0.01, Figure 4A). In addition, functional CD8$^+$ T-cells were highly suppressed as indicated by their low expression of intracellular IFN-γ after activation. The CD3$^+$CD8$^+$IFN-γ$^+$ T-cells in L-CTCL patients were rare (4.0 ±6.3 cells/μl, n=6, Figure 4B) versus normal donors (160.1±111.3 cells /μl, n=3, p<0.01, Figure 4A).

As shown in Table 2, responders had only slightly higher baseline CD8$^+$ T-cell numbers (186.5±139.4 cells/μl, n=11) versus non-responders (152.8±82.9 cells/μl, n=6). Responders had higher baseline activated CD8$^+$CD69$^+$ T-cells (11.5±4.8 cells/μl, n=3) than non-responders (1.2±0.8 cells/μl, p<0.05, n=3). Functional CD3$^+$CD8$^+$INF-γ$^+$ T-cells were also higher in responders (7.7±7.7 cells/μl, n=3) than in non-responders at baseline (0.3±0.3 cells/μl, n=3, p<0.01).

After treatment, the absolute numbers of CD8^{+} T-cells decreased in most patients (Table S1). In contrast, the percentages of CD8^{+} T-cells were unchanged or slightly increased during ECP (Table S1, Figure 4B-E). Interestingly, of 6 L-CTCL patients tested, activated CD3^{+}CD8^{+}CD69^{+} T-cells were increased in 5 patients until 3-4 months after ECP but their numbers were not sustained at 6 months. On average, CD3^{+}CD8^{+}CD69^{+} numbers increased from 6.3 ± 2.7 cells/μl at baseline to 73.4 ± 65.2 cells/μl at 1 month, and to 111.3 ± 111.3 cells /μl at 3 months post-ECP (Figure 4C-E). Moreover, functional CD8^{+} T-cells also increased in 5 of 6 patients over ECP therapy. On average, CD3^{+}CD8^{+} IFN-γ^+ T-cell numbers increased from 4.0 ± 2.6 cells/μl at baseline to 9.6 ± 5.1 cells/μl at 1 month, and 8.1 ± 8.0 cells/μl at 3-4 months after therapy.

Discussion

In this study, 12 of 18 L-CTCL patients (66.7%) achieved overall clinical responses to ECP alone or in combination therapy. Interestingly, the decrease in the numbers of CD4^{+}Foxp3^{+} T-cells mirrors the blood improvement over 6 month treatment in some of L-CTCL patients. Indeed, 6 of 9 patients who had high numbers of circulating CD4^{+}Foxp3^{+}CD25^{-} cells were responders compared to patients who had low CD4^{+}Foxp3^{+} T-cells at baseline and did not respond. Meanwhile, we also observed an increase in activated and functional CD8^{+} T-cells during the first three months of therapy. To our knowledge, there is no previous study on the clinical response to ECP or ECP combination therapy in L-CTCL patients stratified by levels of blood CD4^{+}Foxp3^{+} T-cells.

There are previous studies supporting the idea that malignant T-cells in L-CTCL patients are malignant T_{reg} cells (15, 16). In this study, we showed that average Foxp3 mRNA expression was high in PBMCs from L-CTCL patients, which was consistent with our previous study (33, 34). CD4^{+}Foxp3^{+}T-cell populations were high at baseline in 6 of 9 L-CTCL patients studied, which were similar to the numbers of CD4^{+}CD26^{-} malignant T-cells and clonal T-cells in the peripheral blood. Over 6 months of ECP therapy, the numbers of circulating CD4^{+}CD26^{-} malignant T-cells declined, as did the numbers of CD4^{+}Foxp3^{+} cells. Our results suggest that the majority of circulating malignant T-cells in some L-CTCL patients might have a CD4^{+}Foxp3^{+} phenotype which appears to be more sensitive to ECP therapy. The future study with a bigger sample size needs to test this hypothesis. Recently, a distinct epigenetic modification in a region of the Foxp3 locus called the T_{reg}-cell-specific demethylated region (TSDR) was found to be fully demethylated in T_{reg} cells, and it was also found in SS patients with high numbers of Foxp3^{+} malignant T-cells (16).

Human T_{reg} cells are functionally and phenotypically diverse. They can display molecular and functional heterogeneity, can sometimes be naïve and effector- or memory-like cells, and can produce effector cytokines (36-38). CD4^{+}Foxp3^{+}T_{reg} cells might also become unstable. Under certain physiological states and inflammatory conditions, they might adopt a phenotype that is more characteristic of effector CD4^{+}T-cells (36, 39). CD25 expression on T_{reg} cells can be down-regulated in a lymphopenic environment (36, 40). In this study, we found that the heterogeneity of T_{reg} cells with respect to expression of CD25 was observed in peripheral blood of L-CTCL patients. Two thirds of L-CTCL patients had high numbers of CD4^{+}Foxp3^{+} T-cells. At baseline, two thirds of CD4^{+}Foxp3^{+} T-cells were CD25

negative, and one-third were CD25 positive. Although there were high numbers of $CD4^+Foxp3^+CD25^-$ T-cells, the numbers of $CD4^+Foxp3^+CD25^+$ T cells and $CD4^+CD25^{+/high}$ T-cells were relatively normal in most of L-CTCL patients. It is possible that malignant T-cells in these L-CTCL patients may be T_{reg} cells, but multiple phenotypes may co-exist. $CD4^+Foxp3^+CD25^-$ T-cells may be present in the setting of chronic inflammatory environments in CTCL. In fact, two separate T_{reg} makers in our study, Foxp3 and CD25, showed discordant changes in ECP responders: decreasing in $CD4^+Foxp3^+$ T-cells but increasing in $CD4^+CD25^{+/high}$ T-cells. Further study to understand the discrepancy and diversity of T_{reg} cells in CTCL may help us uncover the mystery of ECP therapy in CTCL.

Studies have also shown that activation-induced Foxp3 expression in activated T-cells could be one component of homeostasis initiated by these cells to exert negative feedback during an immune response (12). Long *et al* found that low-dose antigen stimulation can promote Foxp3 expression in human $CD4^+$T-cells (41). Chronic antigen or superantigen stimulation, perhaps from staphylococci or viruses, has been suggested to initiate T-cell proliferation and accumulation in CTCL lesions (42-44). As an example, human T-lymphotropic virus type 1 (HTLV-1), first isolated from an MF patient, is associated with the development of MF lesions (45). In support of this concept, PBMCs from $HTLV-1^+$ patients have been found to have high levels of Foxp3 expression. $CD4^+CD25^-$ T-cells from normal healthy donors were shown to acquire both CD25 and Foxp3 expression after being infected with HTLV-1(46). Krejsgaard *et al* even proposed a scheme describing the plasticity model where the phenotype of malignant T-cells in CTCL is modulated by the local microenvironments in skin lesions and blood(47).

Decreased numbers of $CD8^+$ T-cells are thought to contribute to the impaired cell immunity seen in L-CTCL (22, 48, 49). As expected, we observed a low number of $CD3^+CD8^+$ T-cells in the blood of L-CTCL patients. Depressed activity and function of $CD3^+CD8^+$ T-cells were indicated by low CD69 expression and IFN-γ secretion. Activated $CD8^+CD69^+$ T-cells and IFN-γ secreting $CD8^+$T-cells were increased at 1 or 3 months after ECP treatment in L-CTCL patients. The increase in $CD8^+$T-cells was more profound in L-CTCL patients who responded to ECP than in unresponsive patients. The enhanced activity and function of $CD8^+$ T-cells after ECP could be partially attributed to the removal of suppression from malignant T-cells and/or by dendritic cells stimulation as we have previously reported (35). We conclude that ECP alone or in combination therapy exerts multiple effects on immune cells in L-CTCL patients: 1) reduce immunosuppressive malignant T-cells; 2) augment immunostimulatory dendritic cells; and 3) enhance the activity and function of $CD3^+CD8^+$ T-cells.

The limitation of this study is that two thirds of patients were given ECP therapy combined with biological response modifiers, thus it is not known whether effects on T-cell subsets in these patients are from ECP or from other biological modifiers. The small sample size is another weakness of this study. We only examined 9 patients for $CD4^+Foxp3^+$ T-cells in our cohort, so our results have low statistical power and may be due to the chance, and must be confirmed in a larger study.

Nevertheless, our results from this study allow us to propose a new working hypothesis that ECP therapy alone or in combination therapy might be more effective in in L-CTCL patients whose malignant T-cells have a CD4$^+$Foxp3$^+$CD25$^-$ phenotype. Analysis of CD4$^+$Foxp3$^+$ T-cell subsets might be useful to better tailor ECP therapy for CTCL patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary statement

Extracorporeal photopheresis (ECP) is effective for treatment of leukemic cutaneous T-cell lymphoma (L-CTCL), but its mechanism(s) of action remain unclear. Regulatory T-cells (T_{reg}) express Foxp3 and CD25. Although malignant T-cells of CTCL gain the T_{reg} cell phenotype *in vitro*; it is unclear whether this also occurs *in vivo*. Our results indicate that ECP therapy alone or in combination therapy might be more effective in patients with high baseline percentages of CD4$^+$Foxp3$^+$ T-cells, and this T-cell subset declines in parallel with malignant T cells during treatment. Analysis of CD4$^+$Foxp3$^+$ T-cell subsets might be useful to better tailor ECP therapy for CTCL patients.
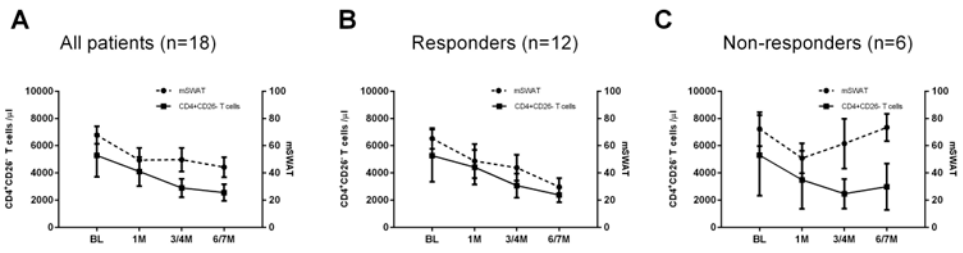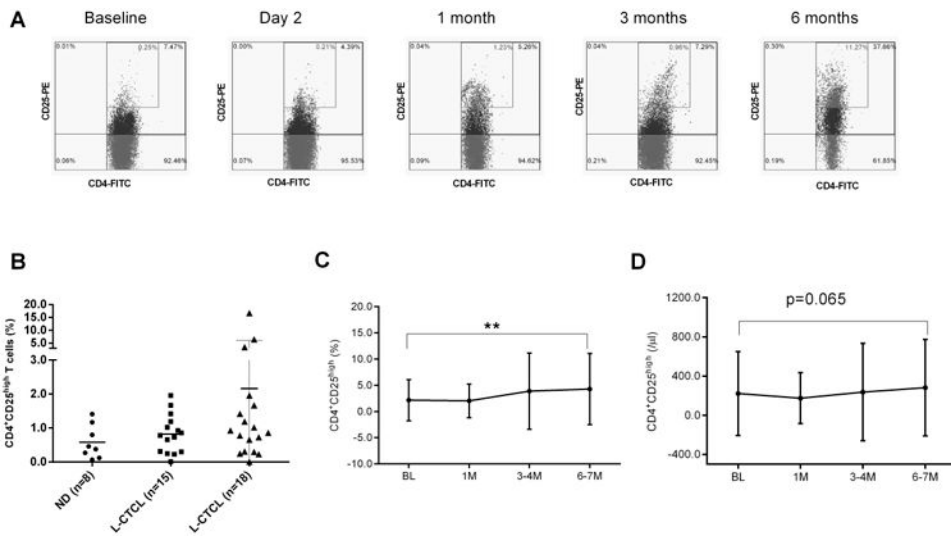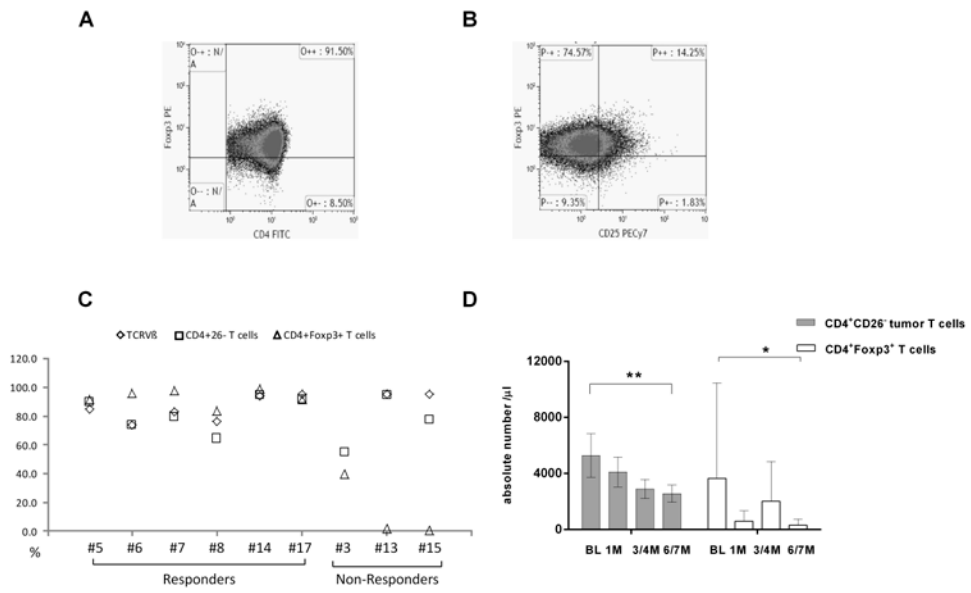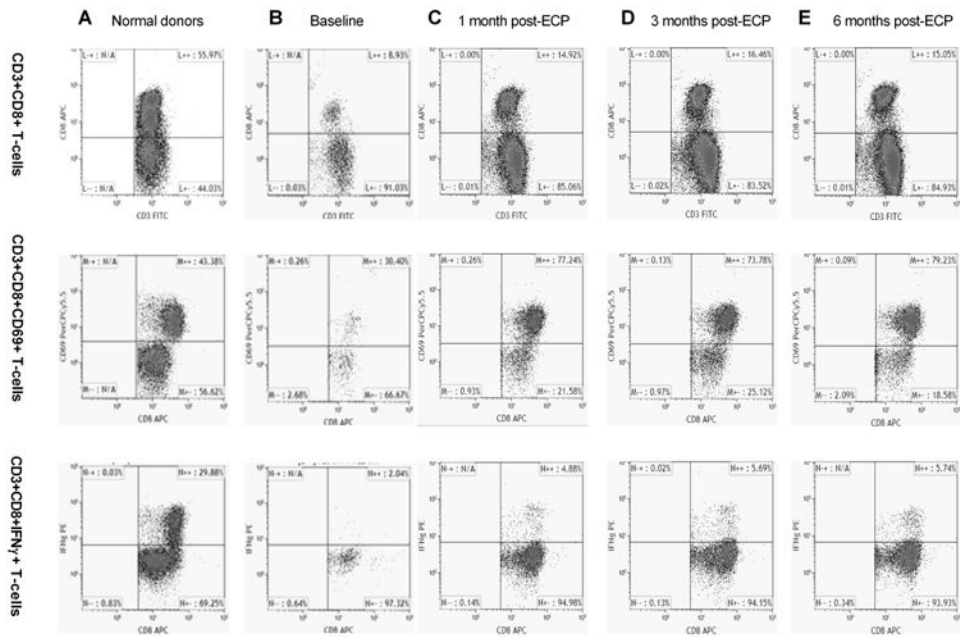
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Clinical characteristics and responses of L-CTCL patients

Characteristics	Patients (n=18)
Age - Years old	
Median	67.0
Range	54.0 – 79.0
Gender - no. of patients (%)	
Female	5 (27.8%)
Male	13 (72.2%)
Stage - no. of patients (%)	
III	2 (11.1%)
IV	16 (88.9%)
Modified skin-weighted assessment tool (mSWAT)	
5.0	1(5.5%)
39.0– 80.0	10 (55.6%)
80.0	7 (38.9%)
Blood involvement *	
B1: low blood tumor burden	4 (22.2%)
B2: high blood tumor burden	14 (77.8%)
TCR vβ - no. of patients (%)	
Positive	16 (88.9%)
Negative	2 (11.1%)
ECP - no. of cycles**	
Median	9.5
Range	6 - 13
Combination immunotherapy - no. of patients (%)	
ECP alone	6 (33.3%)
+ Bexarotene	5 (27.8%)
+ IFN-α	2 (11.1%)
+ Bexarotene and IFN-α	5 (27.8%)
Response - no. of patients (%)	
Partial response (PR)	8 (44.4%)
Minor response (MR)	4 (22.2%)
Stable disease (SD)	3 (16.7%)
Progressive disease (PD)	3 (16.7%)

[*]: circulating $CD4^+CD26^-$ or $CD4^+CD7^-$ T-cells;

[**]: excluding Patient#8 who had L-CTCL plus GVHD

Table 2

Flow cytometry analysis of T-cell subsets in L-CTCL patients at baseline

Patient #	TCR vβ (%)	Circulating CD4+CD26 malignant T-cells %	/µl	CD4+CD25+/high T-cells (%)	CD4+Foxp3+ T-cells (%) ** Total	CD25-	CD25+	CD8+ T-cells (/µl) Total	CD69+	IFN-γ+	Clinical response
1	70.0	64.2	1751.0	0.3	-	-	-	364.0	8.3	0.6	MR
2	91.0	82.2	413.0	0.31	-	-	-	3.0	-	-	PR
5	85.0	89.9	3928.0	0.25	91.5	74.6	14.3	426.0	-	-	PR
6	73.7	73.7	2218.0	1.02	95.5	67.8	28.3	322.0	-	-	PR
7	83.0	79.1	1671.0	1.67	97.7	38.7	59.0	175.0	-	-	PR
8	76.0	64.4	227.0	6.51	83.4	61.4	19.9	13.0	-	-	MR
10	93.0	85.9	2752.0	0.66	-	-	-	80.0	-	-	PR
11	97.1	87.5	11581.0	1.96	-	-	-	106.0	17.1	15.8	PR
14	94.0	94.7	22751.0	0.78	99.1	83.8	15.0	240.0	-	-	PR
16	n/a	91.9	4300.0	0.86	-	-	-	----	-	-	PR
17	95.0	91.7	5139.0	1.19	91.3	78.5	12.7	184.0	-	-	MR
18	97.0	91.7	5500.0	0.01	-	-	-	138.0	9.2	6.6	MR
N=12	86.8±9.97‡	83.1±10.6	5185.9±6311.3	1.3±1.7	93.1±5.7	67.5±16.2	24.9±17.7	186.5±139.4	11.5±4.8	7.7±7.7	
3	n/a	55.5	525.0	0.7	39.8	29.9	5.5	192	-	-	PD
4	54.0	20.6	69.0	1.4	-	-	-	55	-	-	SD
9	97.0	93.8	17981.0	0.9	-	-	-	192	-	-	SD
12*	94.0	56.0	1284.6	3.4	-	-	-	179	0.2	0.2	PD
13	95.0	94.5	99977.0	0.2	2.1	1.5	0.3	252	1.8	0.7	PD

Patient #	TCR vβ (%)	Circulating CD4+CD26- malignant T-cells		CD4+CD25+/high T-cells (%)	CD4+Foxp3+ T-cells (%) **			CD8+ T-cells (/µl)			Clinical response
		%	/µl		Total	CD25-	CD25+	Total	CD69+	IFN-γ+	
15	95.0	77.1	2600.0	16.7	0.6	0.5	0.1	47	1.5	0.1	SD
N=6	**87.0±18.5**	**66.3±28.2**	**5406.1±7158.1**	**3.91±6.4**	**14.2±16.0**	**10.6±12.1**	**1.9±2.2**	**152.8±82.9**	**1.2±0.8**	**0.3±0.3**	
p value	*NS*	*0.0811*	*NS*	*NS*	*0.0001*	*0.0017*	*0.0679*	*NS*	*0.0217*	*0.0035*	

n/a: not available; PR: partial response, MR: minimal response, SD: stable disease, PD: progressive disease;

*
: Patient#12 with CD4+CD7- phenotype;

**
: % of CD4+ T-cells;

†: Mean ±SD