The Immunological Effects of Extracorporeal Photopheresis Unraveled: Induction of Tolerogenic Dendritic Cells In Vitro and Regulatory T Cells In Vivo

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Extracorporeal photopheresis (ECP) may represent an alternative to immunosuppression, as a means of reducing rejection after thoracic organ transplantation. The mechanism by which ECP exerts its protective effects has, until now, remained elusive. We analyzed peripheral blood mononuclear cells of four children with chronic heart and lung transplant rejection, who received ECP in addition to conventional immunosuppressive treatment. The effects of ECP were evaluated at each cycle, comparing blood samples from the same patient collected before and after treatment. In vitro, peripheral blood mononuclear cells treated with ECP undergo apoptosis and are phagocytosed by immature dendritic cells, which, in turn, acquire a tolerogenic phenotype. The frequency of T cells, with a regulatory phenotype and strong suppressive activity, was significantly increased in the blood of ECP-treated patients. The immunomodulatory effects of ECP may be explained by its ability to increase the frequency of regulatory T cells with inhibitory action on transplant immune rejection.

Keywords: Regulatory T cells, Photopheresis, Transplant rejection, Apoptosis.

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Extracorporeal photopheresis (ECP) has been proposed as an alternative therapy for immune-mediated disease, including transplant rejection (*1*). The treatment consists of exposing peripheral blood mononuclear cells (PBMC) to ultraviolet A (UVA) light, in the presence of 8-methoxypsoralen, a DNA-intercalating agent. The cells thus treated are reinjected into the same patient.

ECP was first used successfully for the treatment of cutaneous T-cell lymphoma (*2*). In heart transplantation, ECP prevents chronic rejection (*3*, *4*), reduces acute rejection episodes (*5*), and is associated with an increased number of apoptotic cells infiltrating the transplanted heart (*4*). In patients with graft-versus-host-disease, ECP reduces alloreactive T-cell responses (*6*, *7*); a partial success has also been reported in type 1 diabetes (*8*) but ECP had no beneficial effect in multiple sclerosis (*9*).

The mechanisms by which ECP exerts its protective effects are still poorly understood (*1*, *10*). It has been suggested that ECP-treated lymphocytes undergo apoptosis, but monocytes are more resistant (*1*, *3*). A modulation of dendritic cell (DC) subpopulations and a shift of cytokine profile from Th1 to Th2 have also been observed (*7*).

A T-cell regulatory (Treg) population, actively involved in transplant tolerance, has been described (*11*, *13*). Treg cells are $CD4^+$ and constitutively express the IL-2 α chain receptor ($CD25^+$) without activation markers (e.g. CD69, CD40L). Treg cells do not expand after T-cell receptor stimulation and express the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (*11*). The aim of our work was to study the effect of ECP on the immune system of heart- and lung-transplanted children.

Ten transplanted patients—five with heart and five with lung transplants—were enrolled in the study. Four patients—two with lung and two with heart transplants—were treated with ECP in addition to conventional immunosuppression. The remaining six patients received immunosuppressive therapy alone (Table 1).

The diagnosis of chronic rejection was based on the detection of coronary artery vasculopathy or *bronchiolitis obliterans* after heart or lung transplantation, respectively (*14*, *15*). Drug toxicity was the indication for the additional treatment with ECP. ECP was performed twice a week, 2 weeks per month, for a total of 6 months using the UVARR apparatus (Therakos, West Chester, PA). The protocol was approved by our institution's ethics committee and patients gave their informed consent. Five healthy individuals provided control PBMC. All four patients were analyzed for the first time when ECP treatment was already started (2–4 months from the start) and, since then, they were bled before and after each subsequent ECP cycle (at least three different time points).

To investigate the effects of ECP on PBMC, we compared the viability of leukocytes collected before and after ECP. We measured the frequency of apoptotic cells by annexin V binding in both ECP-treated and untreated cells, immediately and at 24 and 48 hours of culture. The results (Fig. 1A and B) showed that 60% of $CD3^+$ T cells and $CD14^+$ monocytes already underwent apoptosis after 48 hours in culture, only if they had been treated with ECP.

Thus, the treatment basically consists of reinjecting

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TABLE 1. Clinical characteristics and pharmacologic therapy of ECP treated patients

Patient	Transplant	Weight (kg)	Age (yrs)	Time from transplantation	CsA (blood trough level, ng/ml)	MMF (mg/day)	Creatinine (mg/dl)	Clinical conditions after ECP
1	Lung	29	10	5 months	300		0,8	Progressive deterioration of lung function stopped
2	Lung	50	29*	7 years	120		1	Progression stopped and immunosuppression reduced
3	Heart	50	17	10 years	180	1500	1,7	Intravascular ultrasound showed reduction coronary intimal thickening
4	Heart	55	20	9 years	100	1750	1,5	Progression stopped, but patient died of endocardiac thrombosis

* The patient was transplanted twice: the first transplantation was at 17 years of age and the second at 22 years.
ECP, extracorporeal photopheresis; CsA, cyclosporin; MMF, mycophenolate mofetil.

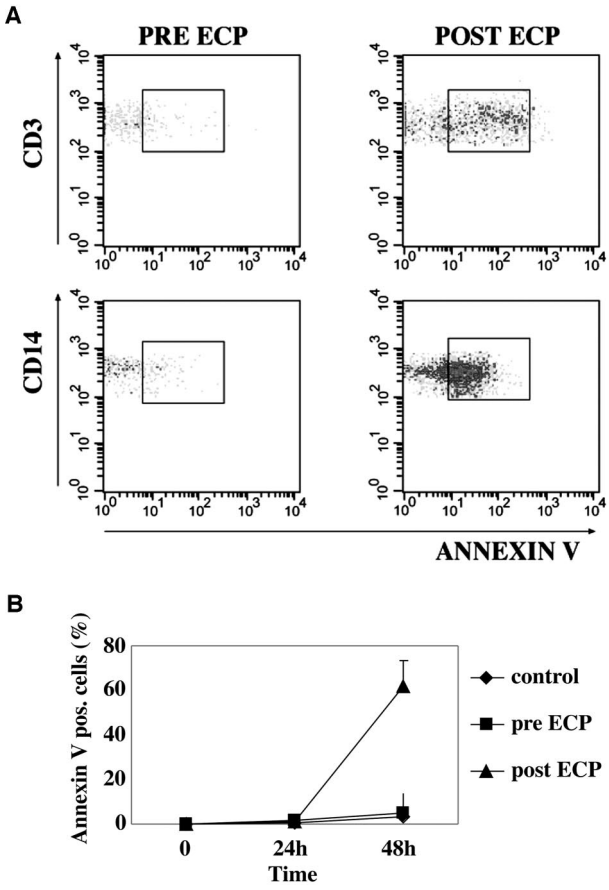

FIGURE 1. Apoptosis is induced by ECP treatment. (A) Density plots show the annexin V staining on $CD3^{pos}$ T cells and $CD14^{pos}$ monocytes. Cells collected pre- and postphotopheresis were analyzed after 48 hours in culture. (B) The percentage of annexin V positive T cells at different time points is shown. Each point represents the mean of five experiments on the peripheral blood of three patients.

cells that are on the verge of apoptosis. It was previously claimed that apoptosis induced by ECP preferentially targeted activated cells, but that monocytes were spared by the killing process and selectively survived (*1*, *3*). Our data do not support this conclusion, because T, B, and NK cells and monocytes were equally susceptible to ECP-induced apoptosis in our study. In addition, activated cells were not detected in the blood of patients either before or after the ECP (data not shown).

Physiologically, apoptotic cells, resulting from the normal cellular turnover, are removed by DCs. We questioned whether DCs would also recognize and phagocytose ECP-induced apoptotic cells. Patients' PBMC, collected before or after ECP, were labeled with the cell-tracking reagent CMFDA and cultured together with unlabelled immature DCs. FACS analysis was performed after 24 hours. DCs could be distinguished from PBMC based on their larger size and different shape. CMFDA fluorescence was detected in the DC population incubated with ECP-treated PBMC, but not in the population cultured with ECP-untreated PBMC (Fig. 2A). This suggests that DCs had recognized and phagocytosed cells undergoing apoptosis after ECP.

In order to confirm this finding, we labeled patient's cells, collected either before or after ECP, with the vital DNA dye Hoechst 3342 and cultured them on a glass coverslip together with immature DCs. After 24 hours, ECP-treated PBMC had been phagocytosed and their Hoechst-stained nuclei could be visualized in the cytoplasm of DCs. No phagocytosis of untreated PBMC was observed (Fig. 2 B).

Immature DCs become mature upon exposure to microbial products, whereas they acquire a tolerogenic phenotype in contact with apoptotic cells (*13*, *16*, *17*). Mature DCs are characterized by the upregulation of major histocompatibility complex (MHC) class II (HLA DR), costimulatory molecules CD80, CD86, CD83, and secretion of TNF-α. In contrast, tolerogenic DCs only upregulate MHC class II molecules (*13*, *16*, *17*).

We compared the phenotype of immature DCs (Fig. 2C, *thin line*) to that of DCs induced to mature with LPS (Fig. 2C, *thick line*, right panels) or incubated with PBMC collected before (Fig. 2C, "pre," *thick line*, middle panel) and after ECP (Fig. 2C, "post," *thick line*, right panel). We found that DCs cocultured with cells collected before ECP remained phenotypically immature, but DCs, which had phagocytosed ECP-treated PBMC, acquired a tolerogenic phenotype (*13*, *16*) (Fig. 2C).

It has been demonstrated that apoptotic cells in the blood collect in the spleen. Here, they are captured by resi-

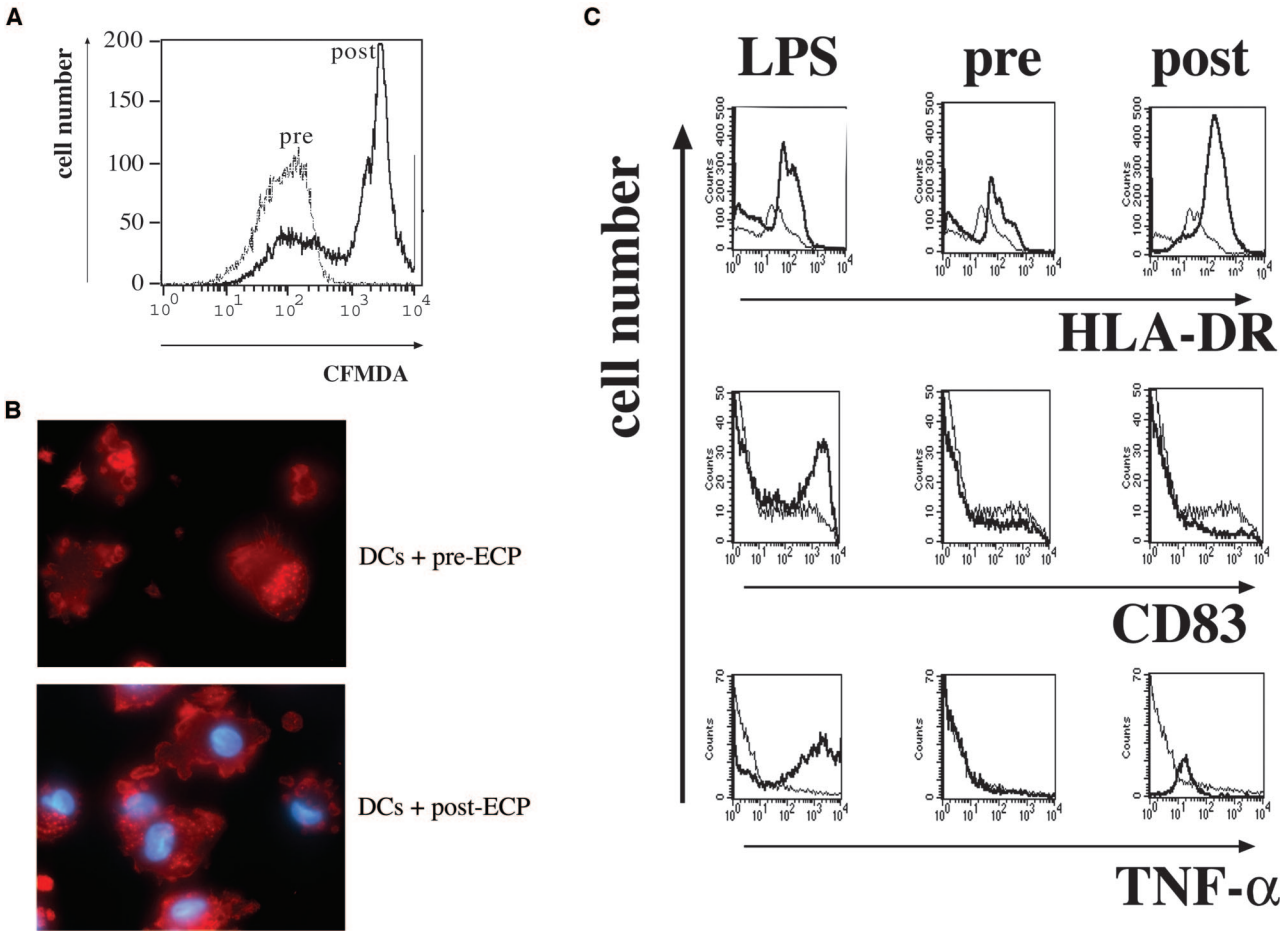

FIGURE 2. Dendritic cells phagocytose PBMC treated with ECP and acquire a tolerogenic phenotype. (A) The histograms show the green fluorescence of DCs, identified as large, granular cells and selected by electronic gating. DCs remained unlabeled if cocultured with CMFDA-labeled patient cells obtained before ECP (pre, *thin line*) and acquired green fluorescence after incubation with ECP-treated cells (post, *thick line*). (B) Pre-ECP: the phalloidin staining (*red*) on DCs incubated with cells collected before ECP. Pre-ECP cells cannot be detected because they are not adherent and were washed off during the staining procedure. Post-ECP: Hoechst-stained nuclei of ECP treated cells (*blue*) were found in the cytoplasm of DCs (phalloidin, *red*) after 24 hours of coculture (post-ECP). (C) The expression of HLA-DR, CD83, and TNF-α was compared in DCs induced to mature with LPS (*left*), DCs cocultured with cells collected pre-ECP (*middle*) or post-ECP (*right*). In each histogram the staining of immature DCs, for the indicated marker is shown by the thin line.

dent immature DCs (*13*, *16*), which in turn develop into tolerogenic DCs (*13*, *16*). It has also been shown that induction of tolerogenic DCs results in increased levels of circulated Treg cells (*13*, *16*). Similar mechanisms may be triggered by the intravenous injection of ECP-treated cells, which, as we have demonstrated, will die of apoptosis.

In the peripheral blood of ECP-treated patients, there was no major change in the frequency and activation status of T, B, and NK cells and monocytes (not shown).

In all four patients, the only significant change was an increased frequency of Treg cells ($CD25^+$ and $CD69^-$), compared to healthy controls (14% vs. 7%. $P < 0.05$) (Fig. 3B). In contrast, Treg cells were significantly diminished in transplanted children treated with conventional immunosuppressive therapy (4% vs. 7% healthy controls, $P < 0{,}05$; vs. 14% ECP-patients, $P < 0.001$) (Fig. 3A). The increased frequency of Treg cells was a constant finding in all ECP-treated patients.

In order to prove that the population identified as Treg cells had a suppressive function, we compared the proliferation induced by CD3 and CD28 M-ab in either the total $CD4^+$ T cells or in $CD4^+$ T cells depleted of Treg. A 30–60% increase of proliferation was observed by removing Treg cells (Fig. 3C). These, upon T-cell receptor crosslinking, upregulated CTLA-4 surface expression (Fig. 3D). Hence, $CD4^+CD25^+$ T cells of patients treated with ECP are functionally regulatory T cells.

Compared to conventional immunosuppressive drugs, the real advantage of ECP is that T- and B-cell responses to novel or recall antigens are conserved in ECP patients (*18*). Thus ECP permits a balance between tolerance to the transplant and the physiological defense against invading pathogens. This balance is of vital importance for organ-transplanted patients not only because it is difficult to defeat infections in individuals who are immunosuppressed, but also because infections often trigger transplant rejection (*15*, *18*).

The idea that regulatory T cells could be the explana-

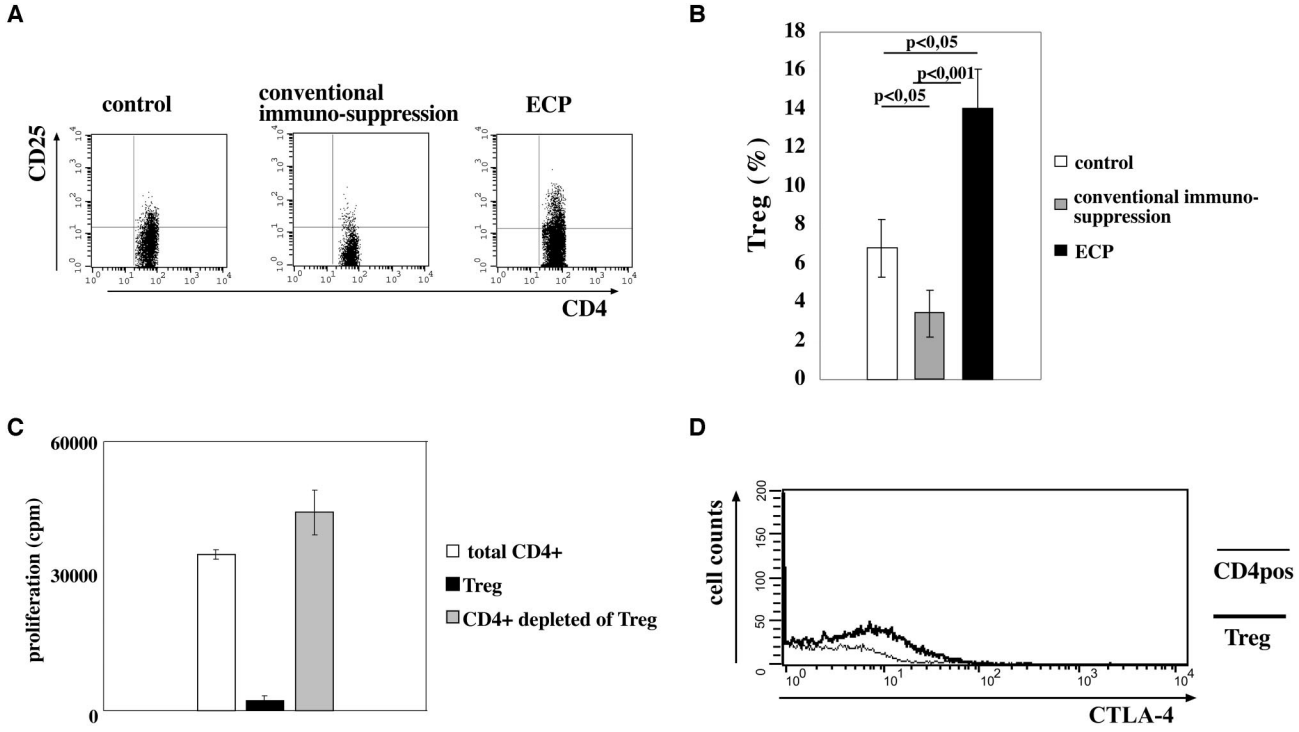

FIGURE 3. Treg cells are increased in the blood of patients treated with ECP and have a suppressive function. (A) Cells were stained with antibodies to CD4, CD3, CD69 and CD25. Dot plot shows CD25 expression of $CD4^+$ $CD3^+$ T cells in a representative control (*left*) and in patients treated either with conventional immunosuppression (CIS, *middle*) or ECP+CIS (*right*). $CD69^+$ activated cells were excluded from analysis by electronic gating. (B) The bars show the frequency of $CD4^+$ $CD25^+$ Treg cells in five normal individuals (*white bar*) and in transplanted patients treated either with CIS (six patients, *gray bar*), or with additional ECP (four patients, *black bar*). Student's *t* test was used for statistical analysis. $P < 0.05$ was considered significant. (C) Depletion of $CD4^+CD25^+$ Treg cells increases T-cell proliferation in ECP-treated patients. The white bar shows the proliferation of $CD4^+$ T cells, the grey bar the proliferation of Treg depleted $CD4^+$ cells. Treg cells do not proliferate upon stimulation (*black bar*). A representative result of three independent experiments is shown. (D) CTLA-4 surface expression of sorted $CD4^+$ T cells (*thin line*) and Treg cells (*thick line*) from an ECP-treated patient analyzed after stimulation with anti-CD3 and anti-CD28.

tion for the immunomodulatory effects of ECP is not new (*10*), but has never been directly proven in organ-transplanted patients. On the other hand, the idea that tolerogenic DCs could be induced by ECP was not even contemplated. We indeed found that $CD4^+CD25^+$ Treg cells, with immunosuppressive function, were significantly increased in the peripheral blood of ECP-treated patients. Most relevantly, we found that in two patients the levels of Treg cells were still higher than normal up to one year after the last ECP cycle and, only at that time, they started to decrease (data not shown). This finding illustrates the long-lasting effects of ECP, but also suggests that "recall" cycles may be necessary to maintain a constant frequency of Treg. In addition, as Treg cells were reduced in patients receiving conventional immunosuppressive therapy alone, an impairment of donor-specific transplant tolerance could be an important consequence of conventional immunosuppression.

During development, $CD4^+CD25^+$ Treg cells originate in the thymus (*16*). In the adult, an increased level of circulated Treg is induced by tolerogenic DCs (*16*, *17*). Our findings suggest that the generation of tolerogenic DCs and the induction of Treg cells explain the immunomodulatory effects of photopheresis. Recent evidence has indicated that the suppressive function of Treg cells is enhanced in vivo by Tr1 cells, another type of Treg with the ability to cause a systemic suppression mediated by IL-10 (*12*). We are currently exploring whether Tr1 cells may amplify the function of conventional Treg cells in organ-transplanted patients treated with ECP.

Our study was not designed to directly prove or disprove the clinical efficacy of ECP, but the treatment appeared to be beneficial (Table 1). Clearly, the association between levels of Treg and an improved clinical outcome remains to be demonstrated in larger trials.

In summary, the elucidation of the immunological mechanisms trigged by ECP may represent the basis for optimizing current therapeutic protocols in the field of transplantation and for its introduction in the treatment of other immunological disorders. It remains to be seen whether the same immunological phenomena, identified after ECP in organ-transplanted patients, apply to the other conditions in which ECP is or has been used (e.g., cutaneous T-cell lymphoma, graft-versus-host disease, and autoimmune diseases). The unraveling of the basic mechanisms of ECP-

induced immunomodulation opens new hopes for rebalancing immunosuppressive therapy in organ-transplanted patients.

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