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Experimental Extracorporeal Photopheresis Inhibits the Sensitization and Effector Phases of Contact Hypersensitivity via Two Mechanisms: Generation of IL-10 and Induction of Regulatory T Cells

Akira Maeda,*† Agatha Schwarz,* Ann Bullinger,‡ Akimichi Morita,† David Peritt,§ and Thomas Schwarz2*

Extracorporeal photopheresis (ECP) is used to treat immune-mediated diseases including transplant rejection and graft-vs-host-disease. An experimental murine model of ECP utilizing contact hypersensitivity (CHS) revealed that ECP inhibits the sensitization of CHS and induces regulatory T cells (Treg). In this study, we find that ECP inhibits not only the sensitization but also the effector phase of CHS, although Treg only inhibited sensitization. IL-10 was determined to be a critical component of the effector phase inhibition and also a driving force in developing Treg. Thus, we propose that the inhibition of the effector phase of CHS by ECP is a process that does not require Treg but may be mediated via enhanced IL-10 as suggested by the use of IL-10-deficient mice. This suggests that ECP has at least two mechanisms of action, one inhibiting the effector phase of CHS and one generating Treg, which in turn can inhibit CHS sensitization and is responsible for the transferable protection. Together, this may help explain the clinical benefits of ECP in prophylactic, acute, and therapeutic settings. The Journal of Immunology, 2008, 181: 5956–5962.

Although ECP has been in use for more than 20 years, the mode of action by which this therapy works is not fully understood. The beneficial effects of ECP in GvHD, in preventing the rejection of solid organ transplants, and in various autoimmune diseases gave rise to the speculation that ECP was functioning via immunomodulation (12). However, in contrast to conventional immunosuppressive drugs, it appears unlikely that ECP induces a generalized immune suppression since patients undergoing long-term ECP therapy have not reported higher risk of developing infections or malignancies (13) and respond normally to both novel and recall Ags (14). PBMC treated in vitro with ECP undergo apoptosis and are phagocytosed by immature dendritic cells, which, in turn, acquire a tolerogenic phenotype (15). More recently, it has been suggested that ECP may induce Ag-specific immunomodulation via regulatory T cells (Treg) (16), which could explain its efficacy in immune-mediated diseases (17, 18) and lack of toxicities. The frequency of Treg was significantly increased in the blood of ECP-treated patients. In patients having received lung transplants, a correlation of functional stabilization with a slight increase or stabilization of the number of peripheral blood CD4+CD25+ cells was observed (19). In vitro these cells exerted suppressive activity and showed features of Treg.

Treg comprise a heterogeneous group of T lymphocytes, which actively inhibit immune responses (20). They have been recognized to play an important role in the prevention of autoimmunity, GvHD, and transplant rejection (21–23). Clinically, there is great enthusiasm about the potential to develop strategies that can enhance Treg number or activity for therapeutic use in immune-mediated diseases. The best-characterized subtype of Treg are those expressing CD4 and CD25 (24). Solar/UV radiation, in particular the mid-wave range (UVB, 290–320 nm), has been long recognized to exhibit the capacity to induce immunotolerance (25). This appears to be at least in part mediated via Ag-specific Treg (26). Interestingly, i.v. injection of UVB-induced Treg inhibits only the sensitization but not the effector phase of contact hypersensitivity.
Adoptive cell transfer

Splenocytes and lymph node cells were obtained from the first generation recipients that were infused with ECP-treated cells. The cell number was adjusted to 2.5 × 10^6/ml and cells were injected i.v. (200 μl) into naïve syngeneic mice (secondary recipients). Twenty-four hours later, secondary recipients were sensitized against DNFB. Five days later, ear challenge was performed and ear swelling was measured 24 h thereafter.

Experimental photopheresis

Experimental ECP was performed as previously described (29). Briefly, donor mice were sensitized against DNFB. Twenty-four hours later, spleens and regional lymph nodes were removed and single-cell suspensions were prepared. After the washed cells were incubated with 200 ng/ml 8-MOP for 30 min in the dark, they were exposed to UVA (5 J/cm²). For UVA irradiation, a UVA high-power device (Sylvania Sunlamp USX; Gevelingen, Germany) was used with an emission peak at 365 nm (output 40 mW/cm²). After washing in PBS, the cell number was adjusted to 2.5 × 10^6/ml and 200 μl of cells were injected i.v. into naïve syngeneic mice. Five days later, recipients were sensitized by application of 50 μl of 0.5% DNFB. Recipients were challenged by application of 0.3% DNFB on the left ear 5 days after sensitization. After another 24 h, the ear swelling response was measured. To monitor the induction of Treg in the primary recipients, spleen and lymph node cells were obtained after challenge and transferred i.v. into naïve secondary recipients which were sensitized 24 h later.

IL-10 measurement

Dendritic cells (1 × 10^6) isolated from bone marrow of naïve mice were coincubated with ECP-Treg (3 × 10^6) in the absence or presence of 0.1 mM DNBS. After coincubation for 48 h, supernatants were harvested and IL-10 levels were measured using an IL-10 ELISA (R&D Systems).

Statistical analysis

Data were analyzed by Student’s t test. Differences were considered significant at p < 0.05.

Results

ECP-Treg release IL-10 upon Ag-specific stimulation

To investigate whether ECP-Treg produce IL-10, we activated Treg with Ag-loaded APCs. For that purpose, leukocytes were obtained from DNFB-sensitized mice and exposed in vitro to 8-MOP and UVA. Cells were injected i.v. into naive mice that were sensitized against DNFB 5 days after injection (29). Five days later, lymph node and spleen cells were obtained and separated into CD4⁺CD25⁻ and CD4⁺CD25⁺ fractions by magnetic bead separation. Cells were then incubated with bone marrow-derived dendritic cells in the absence or presence of the water-soluble DNFB analog DNBS. Forty-eight hours after incubation, supernatants were harvested and IL-10 concentrations were measured using an IL-10 ELISA.

A significant induction of IL-10 was observed when CD4⁺CD25⁺ ECP-Treg were incubated with dendritic cells and DNBS (Table I). In contrast, in the presence of dendritic cells but absence of DNBS, IL-10 was not induced nor was IL-10 produced by DNBS in the absence of dendritic cells. Stimulation of CD4⁺CD25⁻ cells with dendritic cells and DNBS produced minimal amounts of IL-10. This indicates that Ag-specific stimulation of ECP-Treg by APCs induces the release of IL-10 and that ECP-Treg might exert their immunosuppressive activity at least partly via IL-10.

IL-10 is required for the induction of ECP-Treg

We next were interested to study whether IL-10 is necessary for the induction of ECP-Treg. Experimental ECP was performed as described above using IL-10-deficient mice as donors. Injection of 8-MOP/UVA-exposed leukocytes derived from IL-10-deficient mice into wild-type (WT) mice (IL-10KO→WT) did not cause suppression of sensitization in the primary recipients (Fig. 1A).
WT recipients receiving 8-MOP/UVA-exposed leukocytes derived from WT mice (WT→WT) were suppressed in their sensitization, confirming our previous findings (29). Likewise, no Treg were induced in the primary recipients by injection of 8MOP/UVA-exposed leukocytes derived from IL-10-deficient mice since transfer of T cells into naive secondary WT recipients (WT→WT) did not prevent sensitization against DNFB (Fig. 1B). In contrast, injection of T cells from mice that had received 8-MOP/UVA-exposed leukocytes from WT donors (WT→WT) suppressed sensitization, demonstrating the induction of Treg (Fig. 1B). Together, this indicates that IL-10 derived from 8-MOP/UVA-exposed leukocytes is required for the induction of ECP-Treg in this model system.

To study whether generation of ECP-Treg requires IL-10 in the recipients, the experiment described above was repeated with WT mice as donors and IL-10-deficient mice as primary recipients. IL-10-deficient mice were not suppressed in their sensitization response against DNFU under these conditions (WT→IL-10KO), when compared with DNFB-sensitized IL-10-deficient mice as positive controls (Fig. 1A). In addition, Treg did not develop in the IL-10-deficient recipients since injection of T cells into naive WT secondary recipients did not prevent sensitization (Fig. 1B; WT→IL-10KO). The CHS response in these mice was comparable to that of WT mice which were sensitized and challenged with DNFB as positive controls.

**ECP inhibits the effector phase of CHS**

In the design of experimental ECP previously reported (29) and in this study, 8-MOP/UVA-exposed leukocytes, which are injected into naive recipients, inhibit sensitization. However, such an inhibition does not reflect the normal clinical situation in which patients present after disease has initiated. Therefore, we were interested to study whether experimental ECP is also able to suppress an established immune response. To address this issue, 8-MOP/UVA-exposed leukocytes from DNFB-sensitized donors were injected into recipients, which, in contrast to the previous experiments, were already sensitized against DNFB 4 days before treatment. Ear challenge was performed 24 h after injection. Positive control mice, which were sensitized against DNFB and challenged 5 days thereafter, mounted a pronounced ear swelling response (Fig. 2A). In contrast, mice that were sensitized at the same time but had received 8-MOP/UVA-exposed leukocytes on day 4 revealed a significantly reduced CHS response upon challenge 24 h after injection. This indicates that ECP is able to suppress an established immune response.

To prove whether infusion of 8-MOP/UVA-exposed leukocytes into sensitized mice also induces Treg, T cells were obtained from the recipients and transferred into naive mice. These secondary recipients were sensitized against DNFB 24 h after injection and challenge was performed 5 days later (Fig. 2B). The ear swelling response in the secondary recipients was significantly suppressed, indicating that ECP also induces Treg in previously sensitized individuals.

**ECP-Treg do not inhibit the effector phase in already sensitized mice**

From the experiment demonstrated in Fig. 2, it can be indirectly concluded that ECP-Treg, in contrast to UV-induced Treg (27), may exert the capacity to inhibit not only the sensitization but also the effector phases of CHS. To further analyze this, ECP-Treg were injected i.v. into already sensitized mice. Twenty-four hours later, ear challenge was performed. Surprisingly, ECP-Treg did not inhibit the effector phase of CHS when injected i.v. into already sensitized mice.

### Table I. ECP-induced Treg release IL-10 upon Ag-specific stimulation

| T Cells | Bone Marrow-Derived Dendritic Cells | DNBS | IL-10 f (pg/ml) |
|---------|------------------------------------|------|----------------|
| CD4⁺CD25⁺ | − | − | 16.64 ± 5.30 |
| CD4⁺CD25⁻ | − | + | 13.43 ± 2.27 |
| CD4⁻CD25⁺ | + | − | 17.17 ± 3.03 |
| CD4⁻CD25⁻ | + | + | 40.71 ± 1.51* |
| CD4⁺CD25⁻ | + | + | 24.13 ± 2.27 |

*p < 0.001 vs CD4⁺CD25⁺ cells incubated with bone marrow-derived dendritic cells without DNBS.

**FIGURE 1.** Experimental ECP does not work in IL-10-deficient mice. A, Splenocytes and lymph node cells were obtained from DNFB-sensitized IL-10-deficient (IL-10KO) or WT mice and treated with 8-MOP and UVA. Cells were injected into WT recipients that were sensitized against DNFB 5 days later (IL-10KO→WT). Similar experiments were performed with WT mice as donors and IL-10-deficient mice as recipients (WT→IL-10KO). Five days thereafter, recipient mice were challenged with 0.3% DNFB. Ear swelling was measured 24 h after challenge. WT mice and IL-10KO mice were sensitized and challenged as Pos. Co. or challenged without sensitization as Neg. Co. B, Splenocytes and lymph node cells obtained from primary recipients (A) were injected into naive WT mice (secondary recipients). Mice were sensitized against DNFB 24 h after transfer, and ear challenge was performed 5 days thereafter. WT mice served as positive and negative controls. Ear swelling response is expressed as the difference (cm × 10⁻³; mean ± SD) between the thickness of the challenged ear compared with the thickness of the vehicle-treated ear. *, p < 0.001 vs Pos. Co. (WT); **, p < 0.01 vs Pos. Co.; n.s., nonsignificant vs Pos. Co.
sensitized mice (Fig. 3), suggesting that they behave in a similar fashion as has been described for UVB-induced Treg (27).

Suppression of CHS by ECP is mediated via IL-10

On the one hand, the data shown in Fig. 3 imply that ECP and UVB might induce a similar type of Treg. In contrast, the data were quite surprising since on the first glance they appeared to be in contrast to the findings presented in Fig. 2. This experiment clearly showed that infusion of 8-MOP/UVA-exposed leukocytes suppresses the immune response in already sensitized mice and concurrently induces Treg. Thus, we initially concluded that ECP inhibits the CHS response via induction of Treg. But this would imply that ECP-Treg, in contrast to UVB-induced Treg, exert the capacity to act suppressive not only in naive but also sensitized mice. However, according to the data presented in Fig. 3 this is not the case.

Since IL-10 has been shown to exhibit the capacity to inhibit the effector phase of CHS (31, 32), we analyzed whether 8-MOP/UVA-exposed leukocytes can function as a source of IL-10. Leukocytes obtained from DNFB-sensitized mice were exposed to 8-MOP and UVA and cultured for 24 h. Supernatants were harvested and IL-10 was measured with an ELISA. Significantly increased amounts were detected upon exposure of leukocytes to 8-MOP/UVA (861.3 ± 48.2 pg/ml) in comparison to untreated leukocytes (80.3 ± 6.7 pg/ml).

In addition, serum samples were obtained from mice 48 h after injection of either untreated or 8-MOP/UVA-exposed leukocytes. The serum samples of mice that had received 8-MOP/UVA-exposed leukocytes revealed significantly elevated levels of IL-10 in comparison to mice that had not received an injection or were injected with untreated leukocytes (Fig. 4). These high levels of IL-10 might explain why the effector phase in the recipients is suppressed.

To prove whether the inhibition of the effector phase of CHS upon injection of 8-MOP/UVA-exposed leukocytes is rather mediated via IL-10 than via ECP-Treg, the experiment demonstrated
the suppression in major parts may be mediated via IL-10 (Fig. 6). Thus, in contrast to our previous conclusions (29), we speculate that the inhibition of CHS is a process which does not require Treg but may be mediated via enhanced IL-10. In parallel, Treg are induced as demonstrated by the adoptive transfer experiments and these cells could be important in maintaining or inhibiting induction of immune responses and explain the Ag specificity.

**Discussion**

One reason why the mode of action of ECP may have remained unclear for such a long time was due to the lack of experimental in vivo models. We established a murine model for ECP using CHS as a model immune response (29). In this model, we could show that infusion of leukocytes that were exposed to 8-MOP/UVA in vitro inhibits the sensitization of CHS against the specific hapten in the recipients. We postulated that this suppression might be due to the induction of Treg since the suppression could be adoptively transferred into a second generation of naive recipients and was Ag specific. In the present study, we confirm the induction of Treg by ECP, but also demonstrate that the inhibition of the effector phase of CHS in the primary recipients is independent of Treg yet dependent on the anti-inflammatory cytokine IL-10.

ECP-Treg appear to be similar in phenotype and activity as the more well-described UVB-induced Treg. Both types of Treg are activated in an Ag-specific fashion and express CD4 and CD25 (28). It is known that UVB-induced Treg also express the negative regulatory molecule CTLA-4 (33), bind the lectin dectin-2 (34), utilize the apoptosis-related Fas/Fas ligand system (35), and express the lymph node homing receptor CD62L (28). Whether the same features apply for ECP-Treg remains to be determined.

One surprising result was that the infusion of 8-MOP/UVA-exposed leukocytes suppressed the immune response in already sensitized mice, an activity the UVB-induced Treg cells did not possess, raising the question whether Treg could fully explain the benefit of ECP. We considered IL-10 as a potential candidate since it is known to inhibit the effector phase of CHS (31, 32). It has been previously observed that 8-MOP/UVA induces the release of IL-10 in macrophages (A. Krutsick, K. Campbell, and D. Peritt, unpublished observations). IL-10 is produced following APC engagement of apoptotic cells (36) and elevated serum levels of IL-10 have been detected in GvHD patients following ECP (37). Therefore, we analyzed whether 8-MOP/UVA-exposed leukocytes can function as a source of IL-10. Significantly increased amounts were detected upon exposure of leukocytes to 8-MOP/UVA in comparison to untreated leukocytes. In addition, the infusion of 8-MOP/UVA-exposed leukocytes appeared to induce IL-10 in the recipients, since serum samples obtained from mice 48 h after injection revealed significantly elevated levels of IL-10 in comparison to mice that had not received an injection or were injected with untreated leukocytes. Similar elevations were observed when 8-MOP/UVA-exposed leukocytes were injected into DNFB-sensitized mice. These concentrations were in the ranges which have been described to be immunosuppressive (38). Because of the high levels, it appears unlikely that the IL-10 detected in the serum is...
derived from the injected cells. We surmise that the infusion of the 8-MOP/UVA-exposed cells stimulates host cells to release IL-10. We do not have any evidence which type of cell represents the major source of IL-10. A potential candidate are certainly macrophages since these have been demonstrated to produce rather high amounts of IL-10 when confronted with apoptotic cells (36). However, other host cells, including Tr1 or Th2 cells, cannot be excluded. The exact source of cell can only be identified by using conditional IL-10 knockout mice (39), although it cannot be excluded that several cell types might be involved. Thus, we speculate that the inhibition of the effector phase of CHS is a process which relied primarily on enhanced production of IL-10 which is also supported by the reduction of the suppression upon injection of a neutralizing anti-IL-10 Ab. In parallel, Treg are induced in the spleen with the requirement of IL-10. These Treg could be important for the Ag-specific and long-term protection. It is interesting to speculate whether these findings could explain both the acute and long-lasting clinical benefit described for ECP.

We previously reported on the importance of the CD11c+ cell population as the primary target for ECP (29) as opposed to the T cell population. ECP induces apoptosis of all cells including the CD11c+ population, albeit at a slightly slower kinetic as compared with CD3+ T cells (29). Thus, we postulated that the infusion of dying but not dead APCs is crucial for the induction of Treg. This assumption is based on similar observations in the UVB system where we could demonstrate that UV-damaged Langerhans cells have to be present in the regional lymph nodes to induce Treg (40). Analogous to these observations, one can speculate that APCs carrying the specific hapten are not suddenly killed but damaged by 8-MOP/UVA. Due to the insult by 8-MOP/UVA, these cells cannot present the Ag in a professional fashion or are tolerogenic and thus do not activate T effector cells but Treg. This has been demonstrated in vitro with human ECP-treated APCs (A. Krutsick, K. Campbell, D. Peritt, unpublished observations). The action of these cells appears to be in the spleen as 8-MOP/UVA-exposed leukocytes are retained in the spleen (29) and ECP-Treg did not develop in splenectomized mice (data not shown).

It is obvious that our experimental ECP model has limitations and that the observations cannot be extrapolated completely to human diseases. We view CHS as a tool to study potential mechanisms of action and investigate potential ECP improvements. Since the introduction of ECP 20 years ago, few attempts have been made to optimize the regimen. It is unclear whether an alteration of the frequency of treatment or a change in the cell number would improve the clinical outcome. Our model may represent a tool to perform such types of studies. Preliminary studies have shown that a reduction of the number of 8-MOP/UVA-exposed cells is strongly correlated with a reduction of the effect and suggested a dose-dependent effect that should be further assessed.

A major difference between the clinical application of ECP and our model is the fact that in the experimental model ECP-exposed leukocytes were infused into syngeneic naive mice (29). Since subsequent sensitization was inhibited, it was concluded that the induction of an immune response, in this setting of CHS was prevented. In the clinical situation, ECP is not used to prevent but to treat a disease. Hence, it was obvious to test the effect of experimental ECP in already sensitized mice. Infusion of 8-MOP/UVA-treated leukocytes obtained from DNFB-immune mice caused a significant suppression of the effector phase in recipients that had been sensitized before infusion. This implied that experimental ECP is also effective in sensitized recipients, but this effect does not appear to be mediated via Treg but at least partially via IL-10.

Therefore, we propose that the inhibition of CHS in experimental ECP is due to enhanced IL-10 levels and the induction of Treg. This could explain why both the sensitization and the effector phases are blocked. Whether this is a direct effect of IL-10 or indirectly mediated via the release of other host-derived immunosuppressive cytokines remains to be explored. The Treg activity could also help explain the delayed clinical efficacy, the low toxicity, and the presence of long-term responders. Since Treg appear to be more effective in the prevention rather than in the down-regulation of diseases, the prophylactic administration of ECP may be useful clinically in a broader range of diseases. Such clinical studies in GvHD have been reported (41) and are under way.

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Disclosures

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