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8-Mop/uva Inhibits Maturation Of Extracorporeal Photochemotherapy (ecp) Generated Dendritic Cells

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8-MOP/UVA inhibits maturation of

Extracorporeal Photochemotherapy (ECP) generated Dendritic Cells

A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

Abigail Hart Baird

2011

Abstract

Extracorporeal photochemotherapy (ECP), a cellular therapy involving a light activated drug, is FDA approved for the treatment of Cutaneous T-cell lymphoma (CTCL) and used for reversal of Graft-versus-Host Disease (GHVD) and solid organ transplant rejection. The mechanism by which ECP functions as an immunomodulating treatment is unknown, although work by our lab and others suggests ECP generates functional Dendritic cells (DC) that may be involved in tolerization. Both radiation and chemotherapy have been shown to be involved in generation of tolerizing DC, and so we hypothesized that UVA activated chemotherapy 8-Methoxypsoralen (8MOP) exposure induces a tolerizing phenotype in ECP generated DC. In this study, normal donor peripheral blood mononuclear cells (PBMC) were treated in an *in vitro* model of the ECP apparatus, and collected (no8MOP/UVA sample) or treated with 8MOP (100ng/ml), UVA (2J/cm^2) (8MOP/UVA sample). We measured phenotype and functionality of generated cells by two-color flow cytometry and functional assays. In all populations, we noted a statistically significant increase in percentage of cells staining double positive for DC markers (HLA-DR+/CD83+) 18 hours post treatment. 8MOP/UVA treatment reduced expression of mature DC markers (membrane HLA-DR/CD83) and co-stimulatory molecules (CD80/CD86) when compared to no8MOP/UVA and positive control DC, and failed to stimulate CD4+Tcells in antigen presenting assay. These data suggest 8MOP/UVA exposure during ECP inhibits DC maturation and induces a tolerizing phenotype. These tolerizing DC may play a role in the immunosuppressive effects of ECP in the treatment of GVHD and solid organ transplant rejection.

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Introduction

Solid organ rejection and Graft-versus-host disease (GVHD) are major problems in clinical transplantation. Both acute cellular and chronic solid organ rejection are largely mediated by T cells that use cytolytic molecules to eliminate the allograft, the incidence of which, varies based on procedure and on chronicity. For instance, among heart transplant recipients, acute cellular rejection affects 30–50% of patients in the first year with 80% of heart transplant recipients surviving 10 years. In lung transplant recipients, a chronic rejection syndrome, bronchiolitis obliterans syndrome (BOS), is expected to develop in 50% of recipients by 5 years after transplantation, and 74% by 10 years (1). Less than 40% of lung recipients are alive 10 years after the operation. Graft-versus-Host Disease is a common complication of allogeneic bone marrow transplantation in which T cells introduced from the graft result in host tissue destruction, typically of the liver, skin, mucosa, and gastrointestinal tract. The incidence of acute GVHD is 30-60% depending on gender and age of patient and donor if HLA matched, and up to 80% incidence if donor and recipient are unrelated. The incidence of chronic GVHD is 50%, and overall, GVHD carries approximately a 50% mortality rate (2).

At the present time, clinical transplantation continues to rely on the use of non-specific immunosuppressive protocols in order to prevent and treat rejection and GVHD. These regimens bring with them complications related to the global immunosuppression that they cause, such as infection and malignancies, and to toxicity related to individual drugs. The most commonly prescribed drugs, such as cyclosporine and tacrolimus

(calcineurin phosphatase inhibitors), induce side effects such as nephrotoxicity, neuro-toxicity, and thrombotic microangiopathy, among many other serious complications.

Extracorporeal Photochemotherapy

Extracorporeal photochemotherapy (ECP) is a promising cell mediated alternative immunosuppressive therapy for transplant rejection, GVHD, and other T cell mediated autoimmune diseases, with few to no side effects. During ECP, a patients' leukapheresed blood is exposed *ex vivo* to an UVA activated chemotherapy agent, 8-methoxypsoralen (8-MOP) in a 1mm thick plastic exposure plate for twenty-to-ninety minutes. During this time the 8-MOP intercalates in the DNA of nucleated cells and is cross-linked to adjacent pyrimidine bases by UVA light activation (3). The cross-link formation is a lethal defect and replicating cells are rendered apoptotic. The processed leukocytes are then reinfused to the patient in a closed-loop system. (Figure 1)

ECP was developed in 1985 as a treatment for Cutaneous T cell lymphoma (CTCL), a malignancy of T cells, after it was shown to reduce cutanous disease involvement in 27 out of 37 patients with treatment-resistant CTCL (4). Numerous studies have been conducted since then (see review by Knobler (5)). Initially believed to be a cytotoxic therapy, rapid clinical response in patients who had less than 10% of total leukocytes processed led researchers to suspect an underlying immunologic mechanism. ECP has since become the most widely used cell based immune therapy in the world, having been administered more than one point five million times (6). Approved for the treatment of CTCL by the Food and Drug Administration in 1988, extracorporeal photochemotherapy use has since been expanded to the treatment of multiple T cell mediated diseases with its primary use now in GVHD (60%), followed by CTCL (30%)

and transplant rejection (10%).

As a treatment for CTCL, ECP is administered on two consecutive days every four weeks. Although there is no standard protocol for ECP treatment in transplantation medicine, most studies describe a similar protocol in transplant and GVHD patients in which therapy begins with one to two sessions a week for four weeks and then is tapered depending on the clinical effect. For instance, the median number of ECP sessions per patient in one particular liver transplant study was 20 (7).

General side effects of the treatment in both adults and children are mild, irrespective of disease, and include malaise, low-grade fever, gastrointestinal complaints, as well as hypotension and syncope resulting from volume shifts during leukopheresis (8-10). There have been some reports of side effects associated with catheter access for apheresis, with risk of thrombosis (11). Notably, opportunistic infections are not increased by ECP treatment, suggesting antigen specific immune regulation (12).

Solid Organ Transplant Rejection

The use of combined psoralen and UVA (PUVA) had been implemented in transplant immunosuppression as early as 1985, when PUVA treatment of a kidney transplant in a rat model prevented rejection (13). This finding led researchers to investigate ECP effect in a primate model for heterotrophic heart xenotransplantion and allotransplantion, where animals received either ECP twice a week starting 3 days after transplantation, or cyclosporine and steroids. The ECP group demonstrated absence of hyperacute rejection, increase in graft survival, and suppression of response in mixed lymphocyte reactions, as well as reversal of biopsy-proven rejection episodes in 2 cases,

whereas medical immunosuppression controls suffered from progressive rejection (14,15).

The most compelling data illustrating ECP efficacy in solid organ transplant in humans would not come for many years later, and data having been generate primarily from retrospective studies in heart and lung transplant. In one study involving cardiac transplant patients, the ECP group had a significant reduction in the number of acute rejection episodes, with 39% of patients having no rejection episodes, compared with 19% among those receiving conventional therapy alone. Furthermore, ECP did not increase the incidence of infection, and cytomegalovirus DNA was detected significantly less frequently in the photopheresis group than in the standard therapy group (16). Recurrent cardiac transplant rejection patients also showed reduced morbidity and mortality when ECP was added to conventional medical therapy (17).

The data supporting ECP in lung transplant recipients has come from retrospective trials looking at halting or slowing an already progressed chronic rejection process BOS. The first of such studies demonstrated histological and clinical improvement (improved FEV1) in patients with acute or chronic rejection (8,18,19). In the last 2 years, two larger accounts of ECP in lung transplantation have been published. The first, by Benden, reviewed their long-term experience in 24 patients. All patients experienced clinical stabilization and none developed BOS after completing the ECP protocol. ECP was well tolerated and not associated with adverse events. The most recent report regarding ECP and lung transplant rejection suggests ECP slowed down the rate of decline in lung function in 60 patients with progressive BOS for whom they had data for the 6 months preceding ECP. All studies to date have suggested that patients

with early-stage BOS are more likely to improve or stabilize lung function with ECP (20).

Other notable case reports include ECP used in two face transplantations. ECP therapy, associated with maintenance immunosuppressive therapy and doses of methylprednisolone, succeeded to reverse the rejection process without the development of other side effects or need to intensify the immunosuppressive regimen (21,22). The dosing of ECP in these cases was started 10 months post-transplantation. ECP was performed twice a week for 4 weeks or once a week for 8 weeks and was well tolerated.

Acute and Chronic GVHD

The most convincing data supporting ECP's efficacy in the treatment of acute GVHD in humans comes from a prospective phase II study, including 59 patients with either steroid-refractory or steroid dependent disease (23). After a median of 1.3 months of therapy, which involved 2 consecutive days of treatment for 1-4 week intervals, 82% of patients with cutaneous involvement, 61% with liver involvement, and 61% with gut involvement achieved a complete resolution of GVHD. In this study, ECP allowed faster corticosteroid tapering and discontinuation in responders, reducing steroid associated morbidity. Overall survival at 4 years, based on Kaplan-Meier estimates, was significantly higher for patients who showed a complete response to ECP, compared with those who did not. The effects of ECP in patients with acute GVHD were sustainable and indicated that ECP might be effective as, not only a second line agent, but also a first-line therapy for acute GVHD.

Several studies support ECP efficacy in chronic GVHD, particularly in regards to cutaneous manifestations with data suggesting 20/25 chronic GVHD patients with long-

standing, refractory disease had skin manifestations responsive to treatment (24,25). Up to 80% were able to discontinue or reduce their steroid or immunosuppressive medications (26). In a retrospective study involving 71 patients who had received ECP, the overall response rate was 61% with a CR of 20% (27). Highest responses were achieved in patients with chronic GVHD of the skin, liver, oral mucosa, and eye. In addition, patients responding with ECP had longer survival compared with those failing treatment (28).

Despite the clinical success of ECP in transplant rejection and GVHD, the exact mechanism by which ECP generates immunotolerization remains unclear. ECP holds enormous promise as an immuno-modulating agent, not only as a supplementary treatment in transplant medicine, but as a possible first line therapy. ECP has been shown to be efficacious and most importantly, generates a specific immunotolerizing effect without general immunosuppressive side effects and morbidity seen with medical immunosuppression. The future of ECP in the treatment of GVHD and transplant rejection depends on a clear understanding of the underlying mechanism of this procedure in order to optimize treatment and dosing protocol. In addition, the immunotolerizing effects of ECP have broad clinical application to an array of T cell mediated diseases. ECP is already beginning to be investigated as an immunomodulatory agent in some autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, SLE, pemphigus vulgaris, multiple sclerosis, generalized extensive lichen planus, diabetes, and refractory Crohn's disease (29-32). A clear understanding of ECP's mechanism will allow for expansion of treatment applications and improvement of patient care.

Mechanism

There is significant evidence in animal and human models to suggest that the underlying mechanism of ECP in GVHD and organ transplant rejection lies in its ability to induce tolerance via antigen-specific regulatory T cells (CD4+CD25+FoxP3+ Tregs). Regulatory T cells (Treg) are a subpopulation of $CD4^+$ lymphocytes, comprising 5% to 10% of the peripheral blood pool that maintain immunological self-tolerance in the periphery. Tregs also regulate or suppress other classes of immune response such as allograft rejection, allergy, tumor immunity, and responses to microbes. These cells express the Foxp3 transcription factor and CD25, the high affinity interleukin-2 receptor (IL-2R) (33, 34) and directly suppress T-cell activation, possibly through membrane expression of molecules such as CTLA-4. In addition, Treg lymphocytes may mediate distal antigen nonspecific suppression through the secretion of cytokines such as IL-10 and TGF-1 (35).

In ECP treated heart and lung allograft recipients, Treg blood levels doubled compared to normal controls, and this increased Treg level persisted for more than a year after treatment (36). Similarly, in a study of lung transplant recipients, ECP was shown to slightly increase or stabilize the number of circulating Tregs, and this finding correlated with decreased organ rejection (37). In a mouse model of heart transplantation, George et al. demonstrated a twofold increase in the number of splenic Tregs in ECP-treated animals (38). In addition, ECP increased survival in two strains of mice, and the effect of ECP could be transferred to a non-ECP-treated animal via the infusion of purified Tregs. In a murine model of immune tolerance, Maeda et al demonstrated that transfer activity was lost when cells were depleted of CD4 (+) or CD25 (+) subpopulations (39, 40). More recently, Schmitt et al. showed that ECP stimulates the conversion of adenosine

triphosphate (ATP) to adenosine, a soluble immunosuppressive mediator of T cell activation (41). At this point, it seems that the upregulation of Tregs in the circulation is at least one of the ways ECP exerts its benefit.

How might ECP induce regulatory T cells? The details of how ECP induces Tregs remain unclear, however, several hypotheses have been proposed. The primary theory is that *in vivo* Dendritic Cell (DC), professional antigen presentation cells, are induced to become immunotolerizing DC secondary to processing the apoptotic cells delivered during ECP. To discuss this theory further, a more detailed understanding of DC is required.

In the steady state, most DCs in lymphoid organs arise from a blood precursor, which proliferate in the lymphoid organ, particularly the T cell areas. Another potentially major source of DCs is monocytes (42). DCs are uniquely located beneath the epithelium at several mucosal surfaces (e.g. airway, intestine, and stomach) and these cells insinuate their dendritic processes between epithelial cells to enter the mucosal lumen (43, 44). The most intricate feature of DCs is their capacity to differentiate or mature along many different lines. The general consensus is that maturity level is directly related to immune function. As shown in Figure 2, in the immature state, DCs have a high capacity for endocytosis, low MHC class I and II expression, as well as low expression of T cell co-stimulatory molecules (CD80/CD86). These immature DC are generally believed to be immune-tolerizing. Maturing DCs express high levels of CD80/CD86, and high membrane MHCII, and present antigens to stimulate T cells. Both immature and mature DC express CD11c, cytoplasmic HLA-DR and CD83 at comparable levels, and thus these molecules are considered non-specific marker for DC.

Maturation is driven by many different types of stimuli including microbial ligands for pattern recognition receptors, innate lymphocytes, immune complexes acting on Fc receptors, and additional environmental and endogenous stimuli, much of which has not been identified. Depending on the stimulus as well as environmental factors affecting lymphocytes, DC then determine the type of response, which can be either immunogenic, providing resistance, or tolerogenic, silencing an immune response.

One sphere of immunity that is particularly sensitive to the type of stimulus encountered by DCs is $CD4^+$ helper T cell differentiation. The specific pathway followed by $CD4^+$ T cells, whether it involves Th1, Th2, Th17, Tf, Tr1, or Treg cell differentiation, is significantly governed by DCs (42). Immature, tolerogenic DCs have been shown to induce the differentiation and proliferation of Regulatory T cells (Tregs) (45). Thus, leading to speculation that tolerizing DC generated by in vivo uptake of apoptotic leukocytes, one of the known stimuli for an immature DC phenotype, is responsible for the increase in Tregs measured in the blood of ECP-treated transplant patients.

As mentioned previously, ECP acts by exposure of peripheral blood mononuclear cells to light activated drug in a thin plastic exposure plate, during which time lymphocytes undergo apoptosis. Reintroduced apoptotic bodies are taken up *in vivo* by dormant Dendritic cells in the spleen and liver. Rapid *in vivo* clearance of apoptotic bodies in the absence of danger signals (e.g. double stranded RNA, LPS, etc) has been demonstrated to be one of the identified stimuli that induces a tolerizing DC phenotype and subsequent immunosuppressive effect (46). When resident DCs engulf apoptotic bodies with out other danger signals they become tolerizing DC and exhibit an immature phenotype with low expression of costimulatory molecules, increased anti-inflammatory

cytokine IL10 (47) and induce antigen specific regulatory T cells. Researchers have proposed that surface interactions between apoptotic cells and monocyte-derived DC via complement components C1q and iC3b is responsible for impairing maturation; with associated reduced secretion of IL-6, TNF-α and decreased T cell allo-stimulatory function (48). Interaction with iC3b-opsonized apoptotic cells prevented up-regulation of MHC class-II Ag, CD86, CC chemokine receptor (CCR)2, CCR5, and β2 integrins, but increased expression of CCR7 in human DC (49). Thus, DC that uptake of iC3b-opsonized apoptotic cells in peripheral tissues remain immature/semi-mature, but capable of migrating in response to CCR7 ligands to secondary lymphoid organs and initiate or maintain peripheral tolerance.

There are convincing data from our lab and others suggesting the mechanism of ECP tolerizing action may be more complicated than simply injection of apoptotic lymphocytes. In 2005, Maeda et al, created a murine model of ECP immunotolerance function in contact hypersensitivity. Splenocytes and lymph node cells of mice that were sensitized with dinitrofluorobenzene were exposed to 8-MOP plus UVA in vitro. In an animal model, more than 50% of $CD3^+$ T cells are apoptotic within 24 h after ECP treatment, whereas apoptosis of $CD11c^+$ cells is slightly delayed. Intravenous injection of these cells into naive mice caused inhibition of a hapten immune response, which was lost upon depletion of CD11c (+) cells but not T cells, suggesting that APCs directly generated during treatment play an essential role in ECP-induced tolerance. Mice that received untreated cells or cells exposed to UVA or 8-MOP alone were not affected. Inhibition was cell-mediated and Ag-specific as demonstrated by transfer of tolerance from the primary recipients into naive animals, which could, however, properly responds

to the unrelated hapten oxazolone. Reinfusion of ECP-treated cells without these preapoptotic $CD11c^{+}$cells fails to induce tolerance (39).

Previous work by our lab has shown that monocytes from ECP-treated CTCL patients, thought to be more resistant to apoptosis, are activated to become immature CD11c+, CD83, cytoplasmic HLA-DR+ dendritic cells (DC) during passage through the 1mm plastic ultraviolet exposure plate[6]. This phenomenon was shown to be generalizable to GVHD patients as well as normal human subjects treated with in vitro ECP plate model. Monocytes were analyzed by flow cytometry for the DC markers cytoplasmic HLA-DR, and CD83, before, immediately after and 18 hours post therapy. Overall, after 18 hours incubation, more than a six-fold increase in DC, over baseline, was found, with 30 percent of the processed monocytes developing this phenotype. Importantly, this effect is independent of disease state and these results validated the first in vitro human ECP system that incorporated a plate apparatus as an appropriate model to study the effects of ECP treatment on DC activation. This finding has been validated by microarray analysis (6). A single ECP cycle generates up to $300x10^{6}$ functional DC, a number comparable to that generated for DC vaccines. Based on Maeda's work in a murine model, DC generated on the plate are critical for tolerization. Thus, we hypothesized that the DC generated on the plate during ECP played an integral role in the immunosuppressive activity of the therapy. It is not so simple as apoptotic load, but the act of directly delivering a antigen-specific tolerizing DC therapy that is responsible for ECP's underlying mechanism.

Tolerizing DC

In addition to uptake of apoptotic or self-antigen, research thus far has shown that

UVB irradiation, infection with RSV, treatment with IL10, and treatment with Mitocycin C (MMC), induced DC with tolerizing affects (50,51). In several studies, irradiation of immature monocyte derived DC with 500-3000rads of gamma-rays have been shown to down regulate the expression of maturity markers (costimulatory receptors CD80/CD86 and HLA-DR molecules) on dendritic cells, and may compromise their ability to capture and present antigen, while maintaining their immature DC migratory/phagocytotic/endocytic capacities. The T cell proliferative response in the MLR and the response to tetanus antigen or LPS was reduced when gamma-irradiated primary DC1 were used to either stimulate or present antigen to T cells (52, 53). DCs were resistant to irradiation-induced apoptosis. IL-12 secretion by irradiated DC was decreased in a dose-dependent manner.

Based on what is known regarding the factors that have been shown to prevent DC maturation (MMC, gamma irradiation) we hypothesized that a similar reactant, the UVA-radiation activated chemotherapy agent 8-methoxypsoralen (8MOP/UVA), might be critical in creating a tolerating DC phenotype during ECP. Exposure of leukocytes to light activated drug is an important step in ECP. Because one can control the concentration and degree of photoactivation, it is among the most finely titratable and focusable pharmacologic agents in clinical use. The effect of 8MOP/UVA on monocyte-to-dendritic cell activation and maturation in ECP generated DC, however, has not been studied in a human model to our knowledge.

Hypothesis

We theorized that with normal human cells treated in an ECP exposure plate model +8MOP/UVA would have decreased costimulatory molecules CD80/CD86 and

membrane MHC classII molecules, all required by maturing DC for antigen presentation, compared to cells passaged through the identical apparatus with out 8MOP/UVA. In addition, we hypothesize that DC generated in the presence of 8MOP/UVA will not be able to present antigen to T cells and will have tolerizing effect.

Specific Aims

1. Our primary aim is to investigate the effect of 8MOP/UVA on DC maturation and function during ECP in order to better understand the immunomodulating effects of the treatment for GVHD and solid organ transplant patients.
2. ECP has a paradoxical effect, in that it is capable of eliciting an anti-tumor response in the Tcell malignancy CTCL and an immunomodulating effect in GVHD and solid organ transplant rejection. It is not well understood how this effect is possible. Since we know that dendritic cells have the capacity to be either immunotolerizing or immunogenic, we hypothesize that these cells generated during ECP play a role in the paradoxical mechanism. The second aim of studying the effects of light activitated drug on ECP generated DC is to contribute further to our understanding of the seemingly complex immunology that underlies ECP efficacy, thus generating new hypotheses that explain both anti-tumor and immunosuppressive effects.

Materials and Methods

***All methods, unless otherwise noted, were performed by student**

Normal blood donors

In order to determine the effect of 8MOP/UVA on DC maturation in a human *ex vivo* ECP model, Peripheral blood mononuclear cells (PBMC) were isolated from 200 cc of

normal human donor whole blood by ficoll/hypaque separation. Leukocytes were counted and pretreatment sample allocated and stained for phenotyping. An Extracorporeal chemotherapy (ECP) apparatus model (shown in Figure 1) was designed to allow for control of UVA exposure variable and incorporated a plastic exposure plate, injection port, 2 collection bag sites with clamps, an attachment for normal saline, a UVA light source with temperature gauge and on/off switch, and a peristaltic pump. Plate primed with 3:1 saline: autologous serum (from ficoll/hypaque separation) for one hour. PBMC resuspended in 50-100 cc RPMI were processed at 100 cc/hour for 1.5 hours. Half the isolate was collected from collection bag [no8MOP/UVA, (n=3)]. 8MOP (100 ng/ml) was equilibrated for 15minutes in apparatus before exposure to UVA ($2J/cm^2$) and collected [8MOP/UVA, (n=6)]. Negative control cells (n=3) were untreated PBMCs. Priming, exposure time, 8MOP dose, and UVA dose estimate from original Therakos ECP apparatus. Aliquots were procured immediately after procedure and stained for phenotyping.

Cell Culture

In ECP, it is not possible to examine phenotypic and functional changes in treated monocytes since those cells are immediately reinfused into patients. Therefore, negative control and treated normal donor cells were cultured for 18 hours to study induced monocyte maturation and function in 1 liter Baxter platelet storage bags (37°C, 5% CO_2) in RPMI-1640 medium, 1%P/S, 10%AB serum. Following overnight culture (18hours), cells were harvested and either stained for flow-cytometry or underwent monocyte enrichment for mixed leukocyte reactions and antigen presentation assays. Monocyte enrichment was achieved using Monocyte Isolation Kit II magnetic microbeads

(Miltenyi) Prior to and after 8- MOP/UVA exposure PBMC were depleted of CD4 T cells by using CD4 magnetic microbeads (Miltenyi), yielding 60-80% monocyte enrichment.

Patient samples

In order to test the validity of the in vitro ECP system, normal blood donors were compared to leukocytes from patients with CTCL or GHVD undergoing ECP using the UVAR XTS Photopheresis System (Therakos). Cells were obtained under the guidelines of the Yale Human Investigational Review Board. Informed consent was provided according to the Declaration of Helsinki ***(performed by others).***

Positive control monocyte derived DC (MoDC) were generated from identical normal donor whole blood collected one week prior to plate experiment. Isolated monocytes by MAC-3 magnetic cell sorting to 5×10^6 cells/mL used for protocol Immature DC were generated over 6 to 7 days, first incubated in 6 well plates RPMI 1640 with 2.05 mM l-glutamine, and 15% ABserum, 15% patient serum with 200 ng/ml of GM-CSF/ml and 4 ng/ml of IL-4 /ml. Half media changed Q2-3 days with fresh cytokines. Induction of DC maturation with LPS (10ng/ml).

Immunophenotyping

5 million cells from each sample set aside for two-color flow cytometry analysis to determine DC immunophenotype and maturation status. Monoclonal antibodies specific for monocytes and DC, included: CD14 (LPS receptor, monocytes); CD36 (receptor for apoptotic cells, monocytes); HLA-DR (class II MHC molecule: immature DC-cytoplasm, mature DC-membrane); CD83 (DC marker); and CD80 and CD86 (B7.1 and B7.2 co-stimulatory molecules). Antibodies were obtained from Beckman Coulter and used at their pre-determined optimal dilutions. Background staining was established with

appropriate isotype controls, and immunofluorescence was analyzed using a FC500 flow cytometer (Beckman Coulter). Combined membrane and cytoplasmic staining was performed following manufacturer's instructions for cell fixation and permeabilization (Intraprep kit, Beckman Coulter).***(Calculation of total cell number based on CD83 staining performed by others)**

Induction of Apoptosis

To test whether immature DC phenotype was induced directly or by uptake of apoptotic lymphocytes, apoptotic lymphocytes were incubated with no8MOP/UVA cells at 1:4 ratio for 18 hours at 37C in an identical manner to other overnight cell culture previously described here. Apoptosis was induced by irradiation of autologous magnetic bead isolated CD4+lymphocytes with a calibrated UV lamp at a dose of 2,500 mJ/cm2 at a density of 1 $x10^{6}$ cells/ml in six-well plates in RPMI or X-VIVO15 medium supplemented as described above. we monitored apoptosis serially after UV irradiation by trypan blue staining.

Antigen presentation assay

Volunteer freshly isolated, magnetic bead-enriched, antigen-experienced CD4+ populations (2 x 10^{6c}cells/cc, 50 µl/well) were added to A) Pos control DC, B) no8MOP/UVA generated DC C) 8MOP/UVA DC D) combination of A and C. DC were plated in flat- bottom 96 well plate at (2 x 10^{6}/cc, 50 µl/well) in the presence of tetanus toxoid (5,10 and 15 µg/ml, 100 µl/well) and 100 µl RPMI 1640/15% autologous serum. Control is responder T cells + responder T cells. CD4+ cell purity determined by flow cytometry. After 5 days of culture, the cells receive1 µCi [3H]- thymidine and incubate overnight, are harvested, and counted in a Beta liquid scintillation counter (Perkin-

Elmer). The results are calculated by averaging the mean background counts per minute (CPM) from 5 samples obtained by culturing responders w/out stimulating cells and subtracting the CPM from the average results of the CD4 T cells responding to allogeneic stimulators (54).

One-Way Mixed Leukocyte reaction

The one-way mixed lymphocyte reaction (MLR) protocol is designed to test the proliferation of one individual's lymphocytes (autologous) in response to foreign histocompatiblity antigens on lymphocytes from another individual (allogeneic) that have been irradiated to prevent them from proliferating. Allogeneic responding CD4+ T cells were obtained from normal blood donor CD4-magnetic bead purification (90–95% CD4+ T cells). Aliquots containing 2×10^5 cells/well allogeneic stimulatory cells gamma irradiated (2000 rad), were co-cultured with 2 × 105 cells/well autologous CD4+ T cells or with out stimulator cells in 5replicate wells of round-bottomed 96-well plates with the addition of 100 µl/ well RPMI 1640 containing 15% AB serum and 15% autologous serum. After 5 days' incubation at 37°C, 5% CO2, 1 µCi 3[H]-thymidine was added, The cells harvested and radioisotope uptake measured 16 hours later, using a liquid scintillation counter. The results were calculated by averaging the mean background counts per minute (CPM) obtained by culturing responders w/out stimulating cells and subtracting the CPM from the average results of the CD4 T cells responding to allogeneic stimulators (54).

Results

In order to study the effects of 8MOP/UVA on DC maturation, we needed to construct and validate an appropriate in vitro human model that allowed for control of

this variable. A model based on Therakos ECP apparatus was created that allowed for cells to pass through the plate in the absence or presence of the light with out significant difference in plate exposure time. Temperature was determined to be stable at room temperature throughout in vitro treatment. To validate our experimental model, normal donor PBMCs (n=6) were processed with the ex-vivo apparatus and compared to leukopheresis from CTCL patients (n=3) and GVHD patients (n=3) treated with Therakos ECP from a prior study, using CD83/HLA-DR coexpression as a measure for monocyte-to-DC conversion. As shown in Figure 3a, we noted a statistically significant increase in percentage of cells staining double positive for DC markers (HLA-DR+/CD83+) 18 hours post-ECP treatment in all population. The difference between mean pretreatment (4%) and immediately post-treatment (2%) co-expression of CD83/HLA-DR was not statistically significant. However, 18 hour incubation produced an average of 30%+/- 8% monocyte-to-DC conversion, more than 7-fold increase in the expression of this standard DC marker over background levels, ($p<0.001$). There was no significant difference in CD83/HLA-DR expression between patient and normal blood donor samples treated with experimental model at 18hr incubation time point. The last set of bars demonstrates the mean and standard deviation for all twelve subjects.

The absolute number of ECP-processed monocytes that expressed this DC phenotype after overnight incubation was assessed in cells from 6 patients and 3 normal subjects using CD83 positivity as the criterion. The DC yield, one day after ECP processing of monocytes from normal subjects ($54 - 316 \times 10^6$), compares favorably to that produced after conventional 5-6 day maturation of leukapheresed human mononuclear cells cultured in GM-CSF/IL4.

To determine whether 8MOP/UVA exposure was required for monocyte-to-dendritic cell conversion during extracorporeal photochemotherapy, we processed autologous human leukocytes from normal volunteers (n=3) in the in vitro ECP apparatus in the presence (8MOP (100ng/ml) + 2J/cm2 UVA (8MOP/UVA)) or absence of 8MOP/UVA with equal plate exposure time of 1.5 hours. We assessed monocyte-to-dendritic cell conversion by measuring coexpression of cytoplasmic CD83 and HLA-DR, dendritic cell markers, using two-color flow cytometry. Negative control cells were not exposed to plate and positive control cells were MoDC (cultured with IL4/GM-CSF for 7 days and matured with LPS 10ng/ml). Figure 4 suggests there was no significant difference in percentage of CD11c+CD83+HLA-DR+ cells after 18 hr incubation between positive control DC (47.33% +/-13.69), no8MOP/UVA treatment (36%+/-8.29), and 8MOP/UVA treatment (32.9% +/-6.86). The mean percentage of CD14+/CD11c+ cells positive for CD83+/HLA-DR+ (34.45%+/- 7.01) for all 6 experimental subjects after 18 hr incubation compared to pre-treatment (0.78%+/-0.51; p<0.001). There was no significant change in CD83+/HLA-DR+ coexpression after 18hr incubation in negative control samples, PBMCs not exposed to ECP apparatus (4.83 +/- 2.91 vs 11.47 +/- 4.65, p=0.12).

The costimulatory markers CD80/CD86 are required for antigen presentation and have the level of expression has been associated with dendritic cell maturity; with low levels of expression suggesting immature, tolerizing DC and high expression associated with mature, immunogenic dendritic cells (Figure 2). Therefore, to test our hyptothesis that 8MOP/UVA exposure prevents maturation of DC during ECP, we measured CD80/CD86 levels in the presence and absence of light activated drug before treatment,

immediately after, and 18hours incubation after treatment. Figure 5a represents a sample of a minimum of 3 experiments and suggests 8MOP/UVA reduces DC costimulatory molecule (CD80/CD86) expression (4.2%) compared to no8MOP/UVA population (29.5%). Figure 5b shows the means, where 8MOP/UVA decreased CD80/CD86 coexpression 7-fold on average (8MOP/UVA: 4.1+/-3.6 vs no8MOP/UVA: 27.5 +/-5.40) and was highly significant, $p<0.000001$. Mean expression CD80/CD86 in 8MOP/UVA DC (4.1%+/-3.62) was not significantly different from pretreatment value (2.38%+/-3.00, $p=0.43$). We found 76.73%+/-8.75 of positive control mature DC (generated by traditional 7-day IL4/GM-CSF tissue culture and matured with LPS) to express both CD80 and CD86, while pretreatment PBMC did not express either of these mature DC molecules as expected (2.38+/-3.00).

To test whether the decrease in costimulatory molecules might be associated with apoptotic body uptake, no8MOP/UVA generated cells were incubated for 18hours at 4:1 ratio with apoptotic CD4+cells and analyzed by two-color flow cytometry for mature DC markers CD80/CD86. As shown in Figure 5b, addition of apoptotic CD4+ lymphocytes decreases CD80/CD86 expression on no8MOP/UVA DC population. Addition of apoptotic cells resulted in a small, but statistically significant, decrease in costimulatory molecule expression compared to the no8MOP/UVA DC (19.25%+/-4.75 vs. 27.5+/-5.40, $p=0.033$).

Another set of mature DC markers, membrane HLA-DR and CD83 on the membrane were also reduced on 8MOP/UVA-exposed cells (Figure 5c). Negative control cells were PBMC incubated for 18hrs. Positive control DC were generated by traditional 7 day IL4/GM-CSF tissue culture and matured with LPS. Experiments were

repeated a minimum of 3times. The difference in double positive membrane CD83/HLA-DR expression after 18hr incubation between no8MOP (16.7+/-2.62) and negative control (5.05+/-1.50) is highly significant ($p<0.00001$) and did not differ from positive control Monocyte derived DC (MoDC) matured with LPS (20%+/-1.62). There was no statistical difference between 8MOP treatment group (5.38% +/- 1.45) and negative control (5.05+/-1.50).

Mature APCs elicit an antigen specific immunogenic response by presenting antigen to syngeneic CD4+ Tcells on HLA receptors. We tested our hypothesis that 8MOP/UVA prevented DC maturation and thus antigen presentation capabilities, by testing the cells generated overnight in antigen presenting assays. Figure 6a suggests that 8MOP/UVA treated, immature DC (green) are not capable of stimulating antigen-specific CD4 cells. The red line and dark blue line represents vigorous CD4 stimulation by no8MOP DC and Pos Co DC (IL4/GMCSF generated DC respectively, and is antigen dose dependent. Most significantly, when 8MOP/UVA DC were added as a third party to the pos Co reaction (blue) these DC actively suppressed the T cell response to the antigen (purple).

In addition, 8MOP/UVA DC were less efficient at stimulating allogeneic CD4+ cells in a one-way mixed leaukocyte reaction when compared to control stimulator cells (Figure 6b). The gamma irradiated stimulator control was significantly more efficient at stimulating CD4+ response than 8MOP/UVA treated cells ($13.2x10^3$ +/-$5x10^3$ vs. 6.0 $x10^3$ CPM+/-$5x10^3$, $p<0.0001$). An autologous CD4+ cell reaction was used as a negative control (responder +responder). Results are the mean and standard deviated of 5 replicate wells of a minimum of three experiments.

Discussion

The purpose of this study was to further elucidate the immunotolerizing mechanism of ECP in the treatment of GVHD and organ transplant rejection. Clinical and laboratory data suggest that ECP's immunoregulaory effect is mediated by tolerizing DC and Tregs, although the details of how ECP mediates this cellular effect were unclear.

Dendritic Cell Immunotherapy

Previous studies from our laboratory suggest that passage of normal human donor monocytes or patient cells through the 1mm ECP plate induces monocyte-to-dendritic cell conversion in a single day. DC identity was assessed by flow-cytometry analysis of dendritic cell markers and microarray data (6) is a significant finding, since the conventional approach for generating dendritic cells involves in vitro tissue culture with supra-pharmacologic concentrations of cytokines over the course of a week. Both processes generate cells in equal amounts, in the order of 54-316 $x10^6$ total cell number of functional dendritic cells, which can be reinfused to the patient (6, 55).

Using the human tabletop in vitro model of ECP, we were able to investigate the role of light activated drug in this important cellular immunotherapy. Findings from this study suggest that monocyte-to-dendritic cell differentiation during ECP occurs in the absence of 8MOP/UVA and thus, in the absence of any apoptotic cells or other antigen. It is the passage through the plate and/or incubation overnight that is required for expression of dendritic cell antigens, and these DC go on to mature and present antigen to T cells. Notably, this phenomenon is disease independent. Research into the mechanism of monocyte-to-DC differentiation during ECP is on going in our lab and data suggests interaction of plastic surface and platelets with monocytes, and activation of the

chemokine/adhesion pathway is critical for dendritic cell activation. in ECP-processed monocytes from CTCL, GVHD and normal subjects (56).

Importantly, Dendritic cell therapy has been advancing and is now most clinically relevant in oncology for the treatment of multiple cancers. Long-term survival has been demonstrated in the treatment of renal cell carcinoma with pulsed dendritic cells (57) as well as mesothelioma (58). INGN-225, a p53 modified DC vaccine has improved immune response in small cell lung cancer, and sensitizes the cancer cells to chemotherapy (59). There are studies ongoing testing DC vaccines in pancreatic cancer and glioblastoma multiforme, with anti-CD25 monoclonal antibody for treatment of stage III and IV melanoma, breast cancer, and prostate cancer (60-64). The potential for DC generated from monocytes not exposed to 8MOP/UVA as dendritic cell therapies is endless. In this study, we show these cells to be functional, antigen presenting, allogeneic to a patient, and can be generated in a single day. With future research, it could be feasible to passage a patient's PBMC's through the plastic plate with out 8MOP/UVA and with the addition of any antigen and proper costimulation create an allogeneic Dendritic cell anti-tumor therapy in a single day, that could be reinfused back to the patient.

Immature DC

In addressing our hypothesis that 8MOP/UVA impedes maturation of DC during ECP, we measured several known markers of DC. Notably, general DC markers CD83, cytoplasmic HLA-DR, were not significantly different among +8MOP/UVA and no8MOP/UVA cell populations. This suggests that both groups underwent monocyte to immature DC conversion, and the 8MOP/UVA cells were not frozen as monocytes or no

longer living. Molecules required for DC to function as mature APCs, costimulatory molecules CD80/CD86 and membrane MHCII HLA-DR was significantly reduced in the 8MOP/UVA cell population compared to those cells that received no drug/light exposure. These data indicate that 8-MOP does in fact impede the maturation of the exposed DC.

In a tetanus toxoid antigen recall assay, 8MOP/UVA treated immature DC and were not capable of stimulating antigen-specific CD4 cells, and most significantly, appear to actively suppress the T cell responses to this recall antigen when added as a third party to a positive response generated by Positive Control DC . The background of CD4+ cells + stimulators with out tetanus toxoid is likely secondary to human autologous lymphocyte reaction (AMLR), proliferation of T-cells to signals from autologous non-T cells (65). 8MOP/UVA DC were also less effective than control allogeneic stimulator cells at generating a T cell response in a one-way mixed leukocyte reaction. This result is consistent with our previous observations, since 8MOP/UVA DC expressed fewer class II MHC molecules and costimulatory molecules on their surface than non-treated cells.

The phenotypic and functional studies together suggest that 8-MOP/UVA DC are immune-tolerizing, and may be actively inhibitory of antigen presentation to T cells, either directly or indirectly. These results are consistent with ECP's immunotolerizing effect in the treatment of GVHD and solid organ transplant rejection, and support our hypothesis that the immature DC generated on the ECP plate are the active cells involved in the treatment mechanism.

Since the previous prevailing theory of ECP mechanism is that uptake of in vivo apoptotic bodies by splenic DC induces immune tolerization, we examined the effect of apoptotic bodies in vitro combination with our ECP generated DC. Research has

suggested that apoptotic cells produce anti-inflammatory cytokines, such as IL-10 or TGF-beta, an anti-inflammatory environment that stimulates immature dendritic cells to engulf the apoptotic cells, which then produced immunoregulatory cytokines (IL-10 or TGF-beta) and decrease their production of pro-inflammatory cytokines (e.g., TNF-alpha, IL-1, or IL-12). DC that engulf apoptotic bodies down regulate costimulatory molecules and activate regulatory T cells downstream. We hypothesized that the DC generated on the plate during ECP might be engulfing apoptotic allogeneic lymphocytes sensitive to 8MOP/UVA with down-stream immune tolerizing effects. To test this, we incubated apoptotic allogeneic CD4+ lymphocytes with no8MOP/UVA population (ratio 1:4) for 18hours and analyzed these cells for markers of DC maturation. We noted a decrease in expression of costimulatory molecules that was statistically significant, but not as striking as the effects of direct 8MOP/UVA exposure on PBMCs during ECP. Future studies will examine dose response of this phenomenon.

There is some data in the literature, however, to suggest that apoptotic body uptake by DC does not explain or is insufficient to induce an immature phenotype. First, in a model involving passing human leukocytes through a column with apoptotic cells, the apoptotic bodies have not induced immunotolerizing phenotype. Instead, these cells exhibited properties of increased immunogenicity. In addition, other furocoumarins other than 8-methoxypsoralen, 4,6,4′-trimethylangelicin, 4,8,5′-trimethylpsoralen, 1,4,6,8-tetramethyl-2H-furo[2,3-h]-quinolinone, and 5-methoxypsoralen fail to induce immune suppression and cannot be used for ECP. 4,6,4′-Trimethylangelicin has greater proapoptotic effects in vitro than 8-MOP, but does not induce immunosuppression in an animal model of ECP. These data combined suggests that uptake of apoptotic bodies or

self antigens in the absence of danger signals does not adequately explain the immunotolerizing phenotype of the 8MOP/UVA treated cells generated in our ECP plate model (5).

An alternative hypothesis is that 8MOP/UVA is eliciting a downstream immune tolerizing effect via a similar mechanism to that of Mitocycin C (MMC), gamma irradiation, or viral infection; by directly effecting DC gene and protein expression. Studies testing the effect of MMC on dendritic cells, suggests MMC enhances expression of GILZ, a transcription factor coded by the TSC22D3 gene, which inhibits NFκB transcriptional activity. GILZ determines the decision of DCs regarding whether to stimulate or suppress the T-lymphocyte response (66). As suggested by its name, the GILZ gene is upregulated by glucocorticoids, but also by other agents known to convert DCs into tolerizing DCs such as interleukin (IL)-10 or transforming growth factor-β. GILZ-expressing antigen-presenting cells upregulate inhibitory IL-10,molecules and downregulate costimulatory molecules CD80 and CD86 (66). When $CD4^+$ T lymphocytes are stimulated with GILZ-expressing DCs, they develop Treg properties inhibiting the antigen-specific responses of $CD4^+$ and $CD8^+$ cells (67). Future studies could investigate the appealing possibility that 8MOP/UVA is an additional upregulator of the gene GILZ, which would explain downstream effects on T cell differentiation. It is critical to differentiate between indirect (via uptake of apoptotic bodies) and direct (change in gene expression) control of DC maturation in the future so that these variables can be appropriately modulated as needed for patient treatment.

Just as antigen-specific immunogenic DC have many implications for the treatment of cancer in the form of vaccinations, self-antigen specific immune tolerizing

human DC generated by ECP have broad implications for the treatment and prevention of many T cell mediated autoimmune diseases. In this study, we have demonstrated that light activated drug exposure freezes ECP generated DC in an immature state and prevents antigen presentation activity to T-cells in normal blood donors. These cells can be generated in a single 18 hour period, far less time than the typical 7 day cell culture for DC used in vaccinations today. Multiple studies already discussed have demonstrated the efficacy of this cell therapy in the treatment of GVHD and transplant rejection, as well as the improved safety profile of this treatment over present medical immunosuppressant. Based on this study, the amount of drug and light would have a direct effect the immune tolerizing capacity of the dendritic cells generated during therapy. Thus, these particular variables could be manipulated to improve clinical outcomes in the treatment of GVHD and transplant rejection. ECP treated leukocytes should be investigated in any disease process that would benefit from antigen-specific tolerizing DC with downstream regulatory T cell activity.

Mechanistic conundrum

ECP has a paradoxical effect in that it is capable of eliciting an anti-tumor response in the Tcell malignancy CTCL and an immunomodulating effect in GVHD and solid organ transplant rejection. ECP's immune tolerizing effect and proposed mechanism has been discussed in detail in this report. Data to support ECP eliciting an anti-tumor immune response has be described by many investigators, who report that in Sezary patients, clinical and haematological improvement after ECP are associated with a shift in Th1/Th2 balance and the increase of Th1 cytokines and IL12 as well as cytotoxic CD8 cells (68). Thus ECP can enhance an anti-tumoral response by Th1 type specific

immune response. Additional research, however, has suggested a significant and sustained increase in regulatory T cells during treatment of CTCL with ECP (69). How is it possible for a single treatment to elicit both an antigenic response in cancer and a tolerizing response in GVHD and transplant rejection? One appealing hypothesis proposed for future investigation is that the amount or degree of 8MOP/UVA dictates the immune response during ECP.

Using our in vitro ECP model that allowed for control of UVA light exposure, we were able to generate two separate DC populations: one mature and immunogenic and one immature and immune tolerizing. Since UVA and 8MOP exposure can vary based on flow dynamics within the plate, as well as individuals hematocrit, we aim to investigate the hypothesis that two populations of DC are generated during ECP based on light and drug exposure.

Already referenced in this report, Maeda et al found CD11c+ cells generated during ECP to be critical to inhibition of a hapten immune response in a murine model of contact hypersensitivity, suggesting photopheresis exerts its primary immunomodulatory effect via tolerizing DC derived from ECP processed monocytes, with secondary induction of Ag-specific regulatory T cells (39). In this study, we discovered the immune tolerizing DC are the direct or indirect result of 8MOP/UVA exposure during the procedure. Whereas it has been hypothesized that immature DC are induced in vivo after ECP treatment, this is the first reporting to our knowledge, demonstrating immature DC converted directly from ECP treated peripheral blood monocytes. In the absence of 8MOP/UVA, monocyte-to-DC conversion still occurs and these cells progress to maturity. Our future studies will investigate the downstream affect on

Foxp3+CD25+CD4+ Tregulatory cell expression as well as immune tolerizing cytokines such as IL10. These results provide important insight into the role of ECP as a current and future immunosuppressive treatment for GVHD and solid organ transplant rejection patients.

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Figures

Figure 1

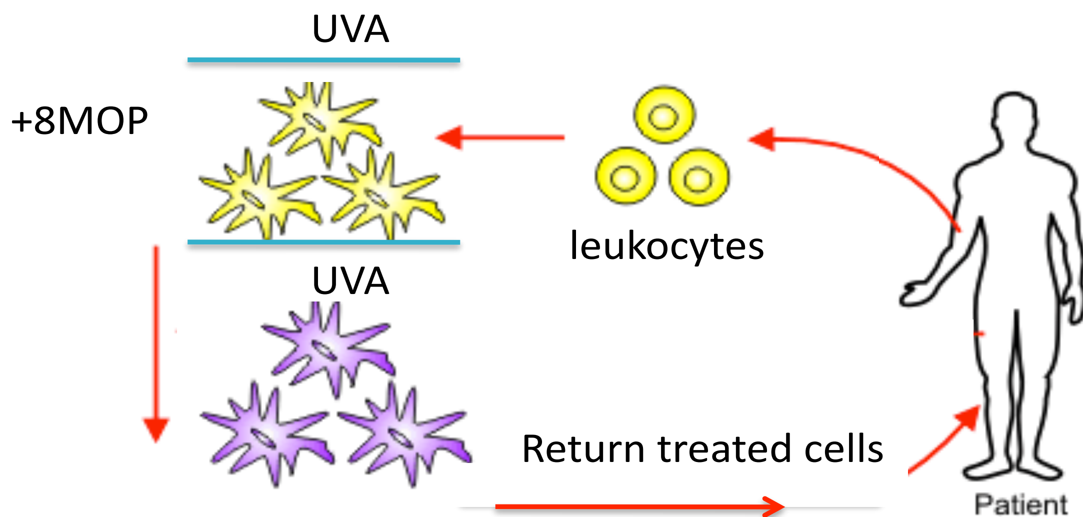

Figure 1. Extracorporeal Photochemotherapy (ECP). During ECP, a patients' leukapheresed blood is exposed ex vivo to an UVA activated chemotherapy agent, 8-methoxypsoralen (8-MOP) in a 1mm thick plastic exposure plate. Upon UVA exposure, 8-MOP intercalates in the DNA of nucleated cells and induces apoptotic in lymphocyte population. The processed leukocytes are then reinfused to the patient in a closed-loop system.

Figure 2

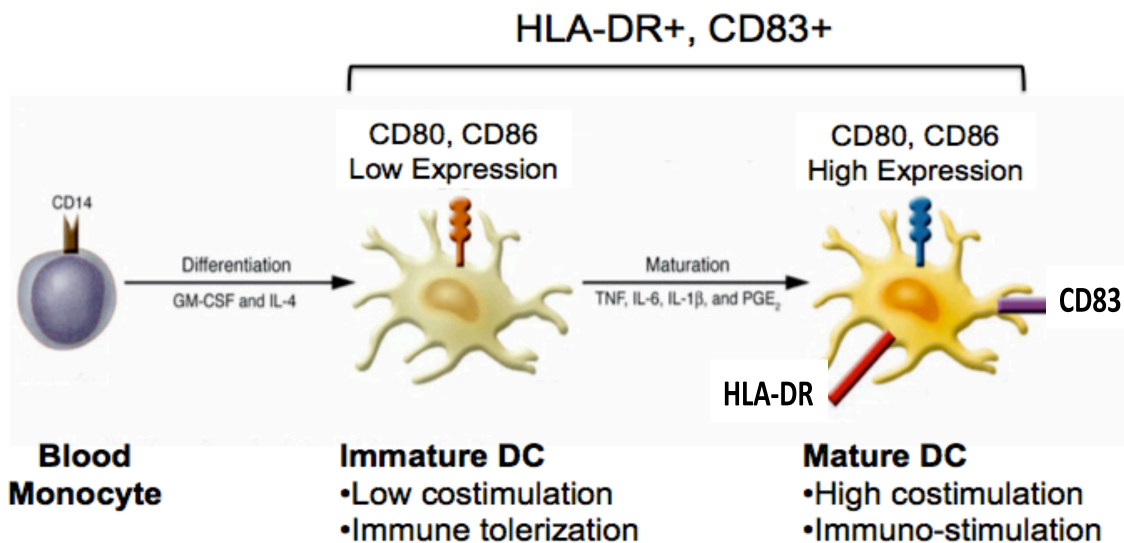

Figure 2. Relationship of Monocyte Derived Dendritic Cell Function to Maturation. Immature DC express low levels of co-stimulatory molecules CD80 and CD86 and are immune tolerizing. Mature DC are antigen presenting, immunogenic and express high levels of CD80/CD86. Cytoplasmic HLA-DR and CD83 is non-specific marker for DC, while mature DC express these molecules on their membranes.

Figure3

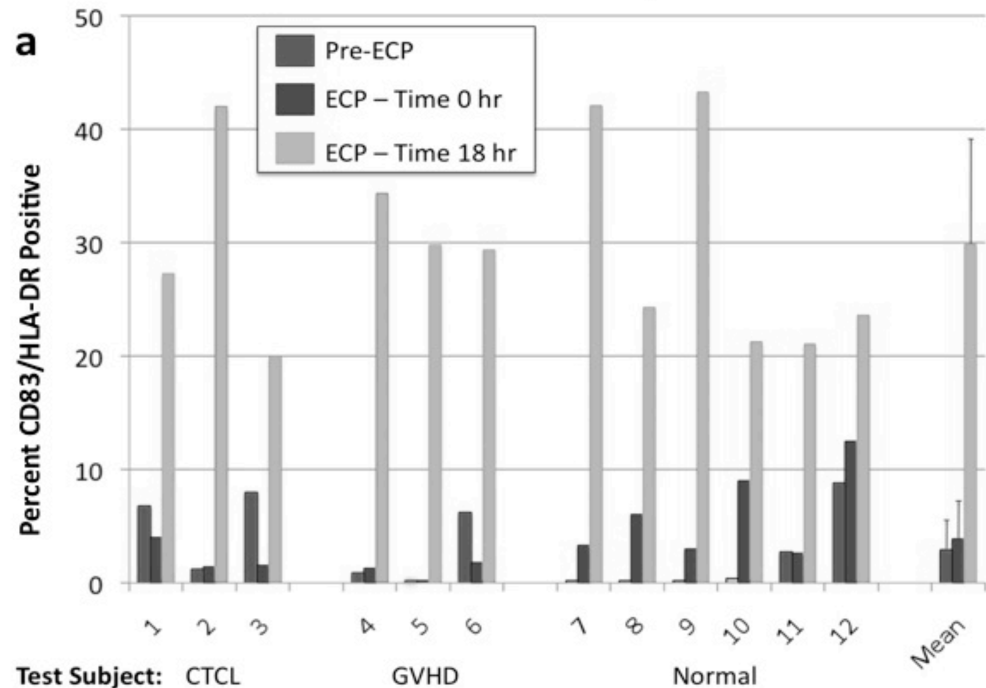

Figure 3a. Monocyte-to-DC conversion during ECP is generalizable phenomenon. Two-color flow cytometry analysis of 10,000 monocytes from 3 CTCL and 3 GVHD patients, as well as from 6 normal subjects, is shown at the three time points: from the leukapheresis harvest prior to ECP (Pre-ECP), immediately after ECP (ECP-Time 0 hr), and 18 hr after treatment (ECP-Time 18 hr) The monocyte/DC population (CD14+/CD11c+) was gated using forward and side scatter. The CD83 percentage was obtained by staining for fixed membrane expression of class II and permeabilized cytoplasmic staining of CD83 (immature DC). The last set of bars demonstrates the mean 30%+/- 8.0 for all twelve subjects, revealing significant enhancement in HLA-DR/CD83 expression after 18 hr incubation ($p<0.001$), compared to pre- and immediately post-ECP.

Figure 3

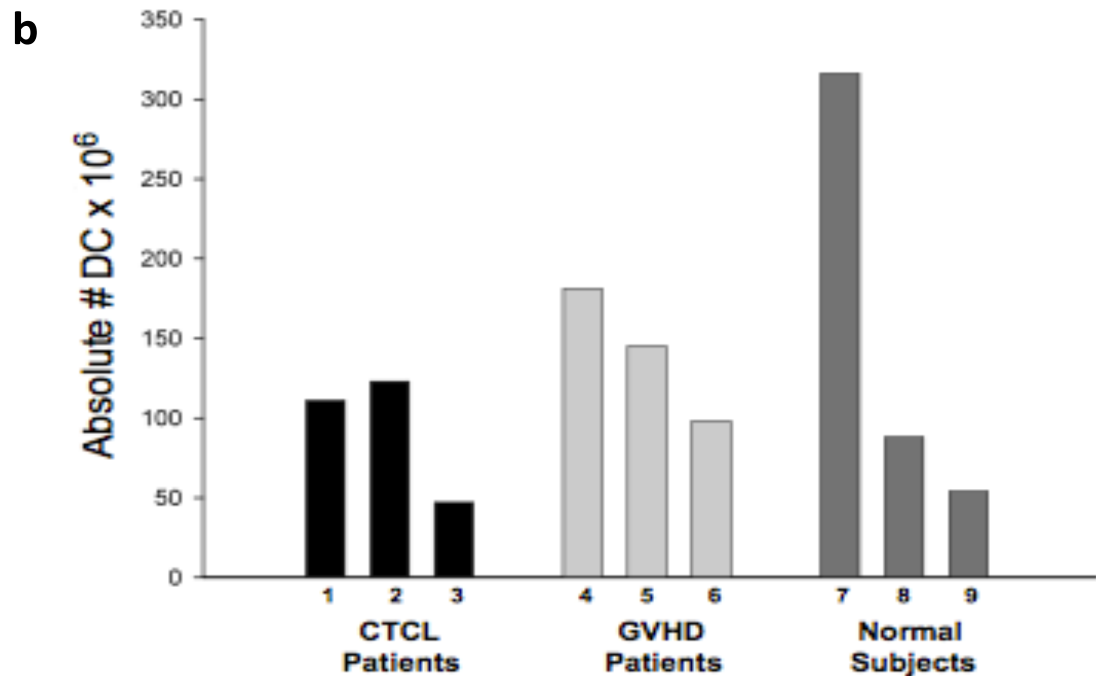

Figure 3b. Absolute number of DC generated by ECP in CTCL, GVHD, and normal blood donors. The absolute number of DC was calculated as the product of the percent of leukocytes cytometrically gating, by forward and side scatter, in the Monocyte/DC region and the total number of cells in that region displaying the CD14 or CD11c marker typical of monocytes and DC. The absolute number of induced CD83+ cells varied with white blood cell count of the subject, but exceeded 50 million in all but one CTCL patient and exceeded 300 million in one normal subject. The absolute number of DC was then calculated by multiplying the CD83+ percentage by the relevant total volume.

Figure 4

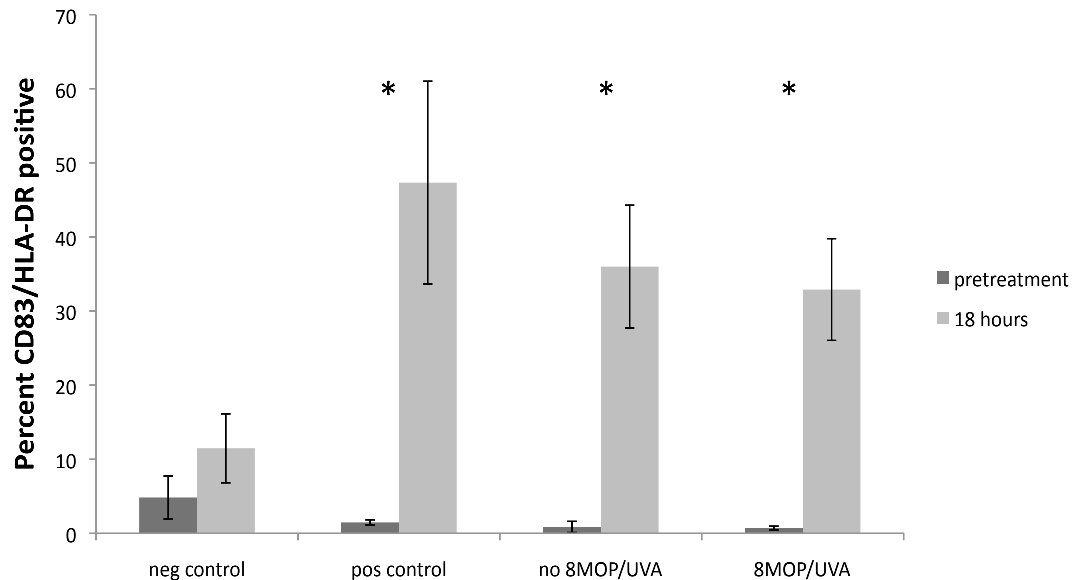

Figure 4. CD83/HLA-DR upregulation occur independent of 8MOP/UVA. PBMC ficoll-hypaque isolated from normal donors were treated with in vitro ECP apparatus, and collected (no8MOP/UVA) or exposed to 8MOP (100ng/ml) + 2J/cm2 UVA (8MOP/UVA) and analyzed by two-color flow cytometry for CD83/HLA-DR coexpression at time 0 and time 18hrs. The mean percentage of CD14+/CD11c+ cells positive for CD83+/HLA-DR+ (34.45%+/- 7.01) for all 6 experimental subjects after 18 hr incubation compared to pre-treatment (0.78%+/-0.51; p<0.001). There was no significant difference in percentage of CD11c+CD83+HLA-DR+ cells after 18 hr incubation between positive control DC generated from monocyte tissue culture with IL4/GM-CSF for 7 days (47.33% +/-13.69), no8MOP/UVA treatment (36%+/-8.29), and 8MOP/UVA treatment (32.9% +/-6.86). There was no significant change in CD83+/HLA-DR+ coexpression after 18hr incubation in negative control samples, PBMCs not exposed to ECP apparatus (4.83 +/- 2.91 vs. 11.47 +/- 4.65, p=0.12).

Figure 5

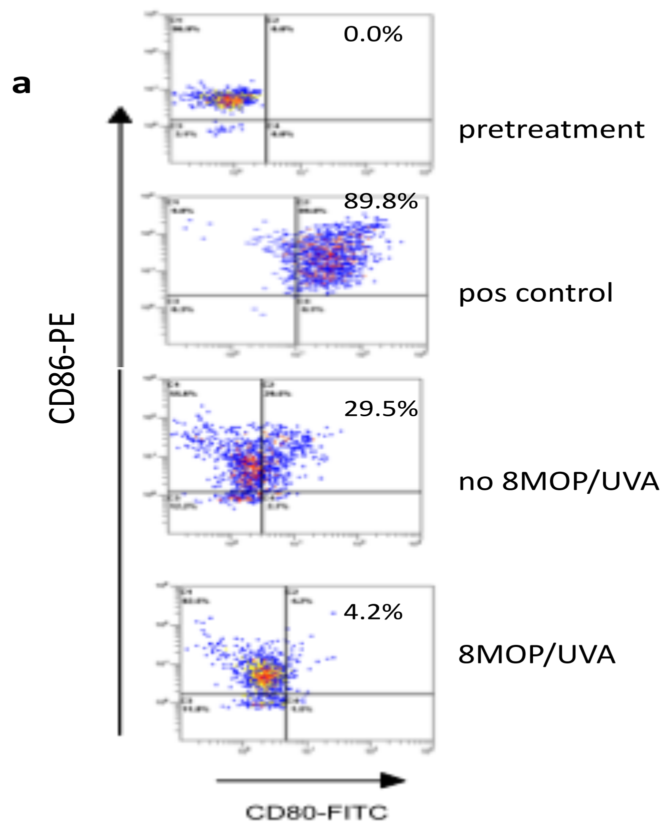

Figure 5a. 8MOP reduces expression of co-stimulatory molecules in normal subjects. Forward and side scatter gating, confirmed by CD11c and CD14 staining, was used to identify the monocyte population. Two-color membrane staining for CD80 (FITC) and CD86 (PE) was used to identify expression of co-stimulatory molecules on pos control DC (generated by traditional 7 day IL4/GM-CSF tissue culture and matured with LPS), pretreatment PBMC, and PBMC exposed to in vitro ECP apparatus in the presence (8MOP/UVA) or absence of 8MOP/UVA (no8MOP/UVA). 8MOP/UVA decreased CD80/CD86 coexpression 7-fold on average (8MOP/UVA: 4.1+/-3.6 vs. no8MOP/UVA: 27.5 +/-5.40) and was highly significant, $p<0.000001$. Selected image is demonstrative of a minimum of 3 experiments.

Figure 5

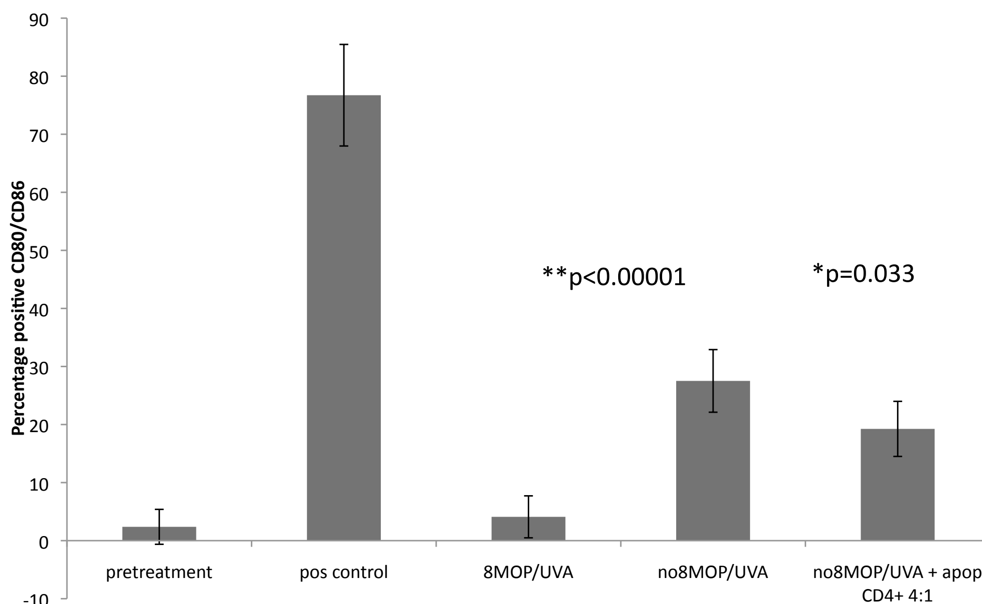

Figure 5b. Addition of apoptotic CD4+ lymphocytes decreases CD80/CD86 expression on no8MOP/UVA DC population. No8MOP/UVA generated cells were incubated for 18hours at 4:1 ratio with apoptotic CD4+cells and analyzed by two-color flow cytometry for mature DC markers CD80/CD86. Addition of apoptotic cells resulted in a small, but statistically significant, decrease in costimulatory molecule expression compared to the no8MOP/UVA DC (19.25%+/-4.75 vs. 27.5+/- 5.40, p=0.033). Mean expression CD80/CD86 in 8MOP/UVA DC (4.1%+/-3.62) was not significantly different from pretreatment value (2.38%+/-3.00, p=0.43).

Figure 5

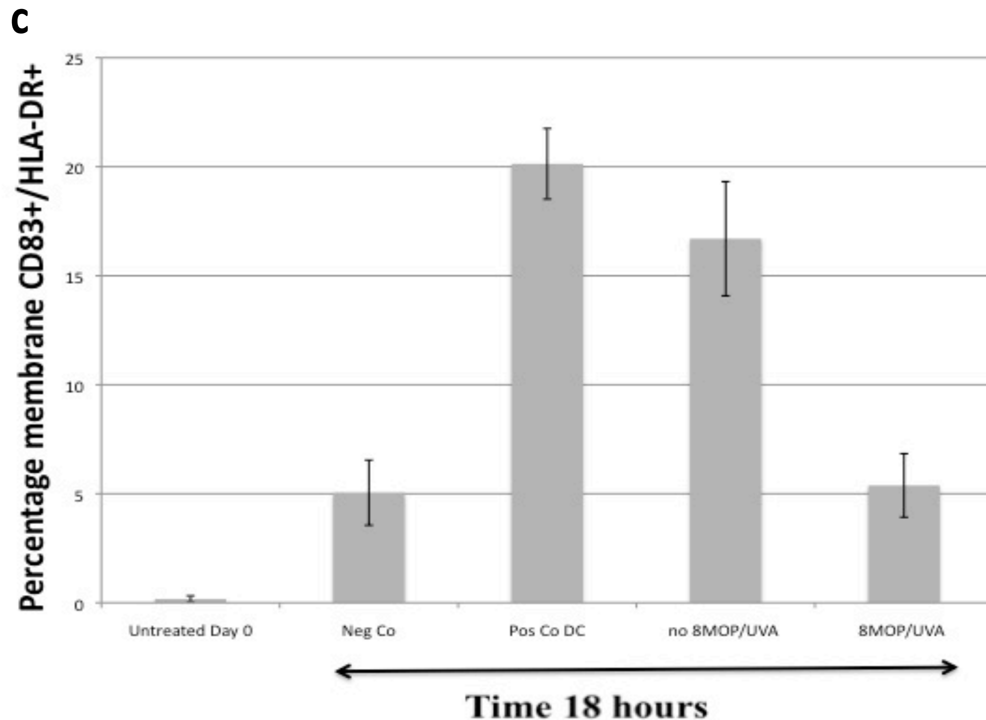

Figure 5c. 8MOP/UVA exposure decreases mature DC markers, membrane CD83/HLA-DR class II MHC, expression in normal subjects. Membrane HLA-DR (FITC) and CD83 (PE) on the membrane were used to identify maturing DC. Negative control (neg control) cells were PBMC incubated for 18hrs. Positive control (pos control) DC were generated by traditional 7 day IL4/GM-CSF tissue culture and matured with LPS. Experiments were repeated a minimum of 3times. The difference in double positive membrane CD83/HLA-DR expression after 18hr incubation between no8MOP and Neg Co is highly significant ($p<0.00001$) and these cells did not differ from Pos Co DC. There was no statistical difference between 8MOP treatment group and Neg Co.

Figure 6

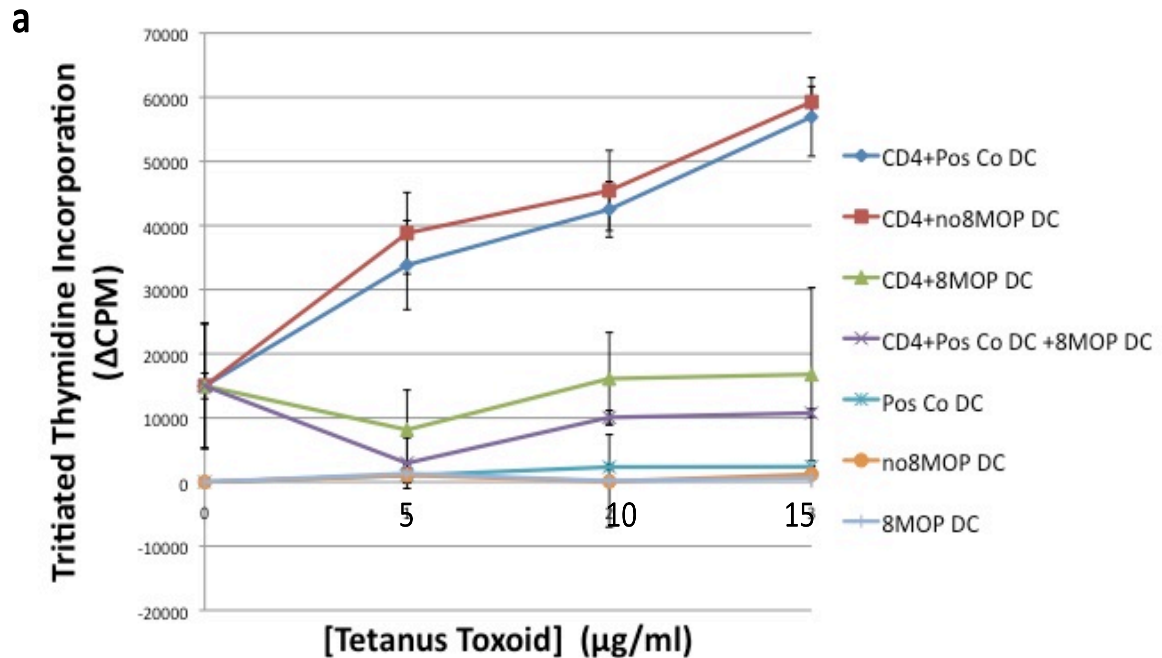

Figure 6a. No8MOP/UVA DC efficiently processed and presented tetanus toxoid antigen to responsive autologous CD4 T cells, while 8MOP/UVA DC may repress CD4 T cell stimulation. No8MOP DC (red) from a tetanus-immunized normal subject, efficiently processed and presented tetanus toxoid antigen to fresh autologous magnetic bead-purified CD4 T cells, and this response was comparable to Pos Co DC (blue) generated by cytokine tissue culture/matured with LPS and this response was tetanus toxoid dose dependent. 8MOP/UVA DC (green) did not elicit a T cell response to increasing doses of tetanus toxoid, and when these cells were added to Pos Co reactions (purple), appeared to repress the response. Autologous CD4 T cells alone or the DC alone yielded limited or no response. Data shown are the mean and standard deviation of 3 replicate wells of a minimum of 3 experiments.

Figure 6

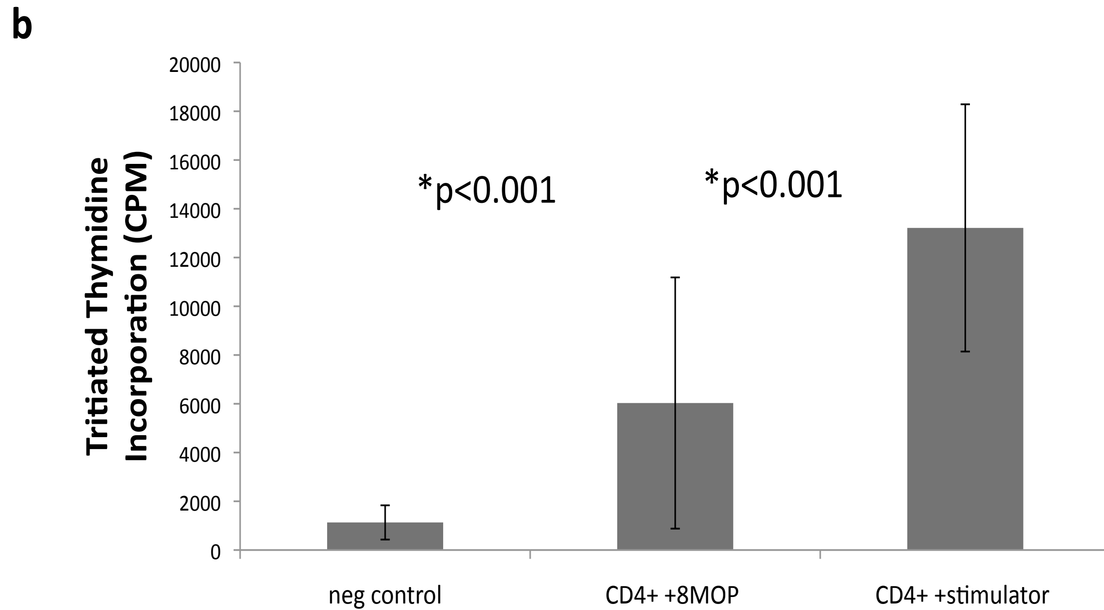

Figure 6b. 8MOP/UVA reduces allogeneic CD4+ stimulation in one-way Mixed leukocyte reaction. Magnetic bead-enriched CD4+ cells (responder) were added to allogeneic stimulator cell populations: either 8MOP/UVA treated PBMCs (8MOP) or gamma irradiated control cells (stimulator). The stimulator control was significantly more efficient at stimulating CD4+ response than 8MOP/UVA treated cells ($13.2x10^3$ +/- $5x10^3$) vs. 6.0 $x10^3$ CPM+/-$5x10^3$, $p<0.0001$). A syngeneic CD4+ cell reaction was used as a negative control (responder +responder). Results are the mean and standard deviated of 5 replicate wells of a minimum of three experiments.