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Modulation of Immunity by the Immunologic Zipper: The Role of Glucocorticoid-Induced Leucine Zipper in Extracoporeal Photochemotherapy

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By

Hao Feng

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ABSTRACT

Extracorporeal photochemotherapy (ECP) is a cellular therapy that is FDA approved for the treatment of a variety of T cell mediated diseases, including cutaneous T cell lymphoma, graft-versus-host disease, and solid organ transplantation rejection. Its ability to selectively suppress and stimulate immunity while causing minimal to no side effects in patients distinguishes it from other therapies including pharmacologic agents. Despite the clinical success of ECP, the exact mechanism by which ECP generates immunotolerance and immunogenicity in patients has been elusive. Since monocytes interact with platelets and serum proteins in the ECP chamber to undergo differentiation into dendritic cells (DCs), we examined whether 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) treatment modified the phenotypic and functional properties of these DCs in a laboratory model of ECP. In a dose-dependent fashion, 8-MOP/UVA treatment of ECP-induced antigen presenting cells induced the expression of glucocorticoid-induced leucine zipper (GILZ), a molecule shown to be both necessary and sufficient for tolerogenic DC phenotype and function. These GILZ expressing cells demonstrated a tolerogenic phenotype and down-regulated the expression of several co-stimulatory molecules including CD80, CD86, and ICAM1. Since the exposure of 8-MOP/UVA on ECP-induced dendritic antigen presenting cells is not uniform, a spectrum of DC is generated from the procedure. The dose-dependent induction of GILZ by 8-MOP/UVA may help to provide a molecular and mechanistic explanation of how ECP is capable of inducing immunosuppression and immunity with GILZ high DCs and GILZ low DCs respectively.

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INTRODUCTION

Solid Organ Transplant Rejection and Graft-Versus-Host Disease

Solid organ transplant (SOT) rejection and graft-versus-host disease (GVHD) are major problems in the field of transplantation that cause significant morbidity and mortality. Both disease processes are results of competent immune cells recognizing the presence of "non-self" antigens [1]. In humans, differences in human leukocyte antigens (HLA), the products of histocompatibility genes, are responsible for stimulating the adaptive immune system to respond and subsequently cause graft-versus-host disease and organ transplant rejection [2].

In SOT rejection, the immune system recognizes the alloantigen on the graft as foreign and mount an adaptive immune response that lead to its rejection via targeted cell death [3]. While both cellular and humoral mechanisms involving multiple cell types are responsible, T cells are central in the acute and chronic rejection of grafts [1, 4]. For heart transplant recipients, acute rejection affects 30-50% of patients in first year and 80% of surviving patients in 10 years [5]. In lung transplant patients, 50% and 74% of patients by 5 and 10 years post-transplant develop bronchilitis obliterans syndrome, a chronic rejection syndrome [5].

GVHD occurs after allogeneic stem-cell transplantation. It is the result of mature donor T cells recognizing the recipient tissue as foreign, causing a severe inflammatory disease that leads to host tissue destruction [1]. Acute GVHD occurs in about 60% of HLA-compatible cases and 80% of unrelated donor cases, and chronic GVHD occurs in about half of the patients despite immunosuppressants [4].

In both GVHD and SOT rejection, stimulation and proliferation of polyclonal T cells that recognize foreign antigens on the target tissue are at the center of both destructive processes [4]. Current pharmacologic immunosuppressive agents, such as tacrolimus and cyclosporine, target pathways involved in T cell activation and proliferation. Although effective, they induce global, non-specific immunosuppression that predisposes patients to malignancies and opportunistic infections. Additionally, side effects such as neurotoxicity, nephrotoxicity, and microangiopathy are common with these drugs.

Better therapies, especially those with minimal side effects and can selectively suppress the pathogenic clones, are desired. Extracorporeal photochemotherapy has been utilized extensively to induce sustained and specific immunological responses in patients, including those with GVHD and SOT rejection. Its mechanism of action, however, has remained elusive and is incompletely understood.

Extracorporeal Photochemotherapy

Extracorporeal photochemotherapy (ECP) is a cellular therapy that is FDA approved for the treatment of a variety of T cell mediated diseases. In this therapy [6], the plasma and leukocyte-rich fractions from the patient's peripheral blood are separated by centrifugation. The leukocytes are re-infused into the patient after *ex vivo* exposure to UVA light in the presence of a photo-activatable drug, 8-methoxypsoralen (8-MOP). The ECP procedure is demonstrated in **Figure 1**. When activated, 8-MOP causes apoptosis by cross-linking adjacent pyrimidine bases in all nucleated cells except monocytes for unclear reasons [7].

Although originally used to treat cutaneous T cell lymphoma (CTCL) [6], ECP has shown to be efficacious in treating GVHD and transplant rejection, which now accounts for over two thirds of its total use [4]. The clinical efficacy of ECP has been demonstrated in patients with acute and chronic GVHD after allogeneic hematopoetic stem cell transplantation [8-11]. Specifically, it prevents and treats graft rejection in renal, lung, and heart transplantations [12-20]. Additionally, it is effective in the treatment of other T cell mediated autoimmune disease that include type 1 diabetes mellitus [21], systemic sclerosis [22, 23], systemic lupus erythematosus [24], epidermolysis bullosa acquisita [25, 26], pemphigus foliaceous [27, 28], oral erosive lichen planus [29, 30], and nephrogenic systemic fibrosis [31-33]. Although many of the published reports noted above are not randomized control trials but rather case series or retrospective observations, it is clear that the use of ECP has been effective and broad across a spectrum of disease processes [34].

Maybe more important than the clinical efficacy is the lack of major side effects from and specificity of ECP. The most common described side effects include nausea, photosensitivity from psoralen, and cardiovascular effects relating to large intravascular fluid shifts [35, 36]. Patients who receive ECP treatments have no increased rate of infection or neoplasm [6, 37]. T and B cell responses to novel or recall antigen remain unchanged in patients after receiving the therapy [38]. In the setting of hematopoetic stem cell transplantation, ECP does not lead to higher relapse rates with preservation of graft versus leukemia effect, which is often diminished in conventional pharmacologic immunosuppression [37].

Despite ECP's clinical efficacy, the exact mechanism of its immunomodulatory effects has not been fully elucidated. Work done by others [39-42] and our group [43] have shown that dendritic cells (DCs) are generated during the ECP procedure and play a central role in mediating the seemingly divergent clinical effects of stimulating the immune system in CTCL and suppressing the immune system in settings of autoimmunity and transplantation.

Dendritic Cells

Dendritic cells (DCs) are professional antigen presenting cells derived from $CD34^+$ hematopoietic stem cells. They serve to link the innate and adaptive immunity and are regarded as critical regulators of immune reactivity [44, 45]. Thus, they are the ideal vehicles for inducing antigen-specific immunomodulation.

DCs possess both immuno-stimulatory and immuno-tolerogenic properties depending on its maturation state [46, 47]. Mature DCs are immuno-stimulatory, have high levels of co-stimulatory molecules (CD80, CD86) that elicit T cell response, and actively present antigen to activate T cells [46]. These mature DCs have been utilized to generate DC vaccines against cancer such as melanoma and ovarian cancer by up-regulating the immune response against cancer antigens [48, 49]. On the other hand, immature DCs are believed to be tolerogenic and can be used as "negative vaccines" to suppress the immune system against certain antigens.

Immature DCs have high phagocytic activity, lack the necessary CD80 and CD86 co-stimulatory signals to elicit T cell response, and render the antigen-specific responding T cells anergic and apoptotic [46]. Rapid uptake of apoptotic bodies in the absence of

danger signals (bacterial lipopolysaccharide, double-stranded RNA, etc) and inflammatory cytokines (IL-1beta, TNF-alpha, etc) have shown to induce tolerogenic phenotype with functional immunosuppression [47, 50]. In addition, the presence of anti-inflammatory cytokines, especially IL-10 and TGF-β, seems to blunt the maturation of DCs and allow for the potential to induce tolerance [51].

Utilization of DCs is a logical therapeutic strategy in the form of a "negative vaccine" to treat and prevent GVHD and SOT rejection by selectively suppressing the immune response to certain antigens [52-54]. Numerous studies suggest that immature DCs can regulate alloreactive T cell responses and promote antigen-specific tolerance in animal models. DCs regulate immune reactivity and promote tolerance by a variety of mechanisms that include induction of T cell anergy and apoptosis, promotion of regulatory T cells formation, and deviation of immune response toward Th2 type [44, 47, 52].

Because DCs comprise less than 1% of all mononuclear cells in the peripheral blood, isolation of monocytes and *in vitro* differentiation is an absolute requirement to utilize DCs therapeutically [52]. DCs can be derived from monocytes either by migrating through endothelium [55] or incubation with supra-physiologic levels of cytokine and growth factors such as IL-4 and GM-CSF [56]. Current efforts to induce tolerogenic DCs *in vitro* for clinical application in SOT rejection and GVHD face many limitations that include: 1) long time frame to generate DCs; 2) unclear form of donor alloantigen to be used; 3) *in vivo* maturation of injected immature DCs that cause sensitization; 4) issues of safety and ability to translate animal model studies to human subjects with clinical efficacy [52, 53].

ECP produces immature dendritic cells [57] and circumvents many of the current limitations that traditional methods of obtaining DCs with cytokine cocktails face. ECP is distinguished from other methods by its unique ability to generate large numbers of maturationally synchronized monocyte-derived DCs quickly and efficiently in one day, especially after overnight incubation [57]. Thus, modification of ECP with an overnight incubation step could allow for increased production of immature DCs that can be loaded with alloantigens or tumor antigens and utilized to selectively reduce or stimulate immunity. Additionally, during the ECP procedure, DC generation is done so in a physiologic manner by interacting with platelets and serum proteins [43]. Given its proven clinical efficacy, specificity to the host, and minimal to no side effects, ECP access and utilizes fundamental the same immunologic mechanisms with that of DC therapy. ECP, therefore, provides the ideal therapeutic background to learn from regarding the mechanism of immunologic actions. Studying this effect in a human model provides an opportunity to better understand and further refine this prevalent and effective selective immunotherapy for the future.

Mechanisms of Extracorporeal Photochemotherapy

The mechanism of ECP in the induction of immunotolerance remains incompletely characterized. There is evidence that ECP mediate its antigen-specific immunomodulatory effects via apoptotic lymphocytes, dendritic cells, and shifts in cytokine production. Additionally, regulatory T cells are implicated in GVHD and SOT rejection whereas clone specific anti-tumor responses are believed to be important in cutaneous T cell lymphoma.

In the treatment of CTCL, it is believed that ECP is inducing specific cytotoxicity against the monoclonal circulating low-grade, Non-Hodgkin's lymphoma T cells [58]. The report of the clinical success—sometimes, with complete response without remission—of ECP treatment for CTCL [6] offered support of a "vaccination" theory. Marks et al observed that malignant CTCL cells die via DNA damaged from the combination of 8-MOP and UVA [59]. These dying tumor cells may provide the individual's tumor-specific antigen for generation of a tumor response that is enduring and precise.

To help solve the enigma of the mechanism of action, studies have looked toward predictors of clinical response to ECP for clues. In patients with leukemic CTCL, a normal CD4/CD8 ratio and a normal absolute count of CD8+ cells in the peripheral blood at the start of ECP are commonly accepted criteria that help to predict a better outcome to therapy [60]. The presence of circulating Sezary cells seems to be another predictor of a satisfactory clinical response to ECP. It is reported that patients with circulating Sezary cells had a significantly better response to ECP than patients without circulating Sezary cells [61]. In fact, it is a common finding that patients with erythroderma respond best [6, 62, 63]. Heald et al. reported that patients with erythroderma who were heavily pretreated and received ECP late in the course of their disease did not respond as well as those who received ECP early [60]. These observations suggest that, in part, ECP works by causing apoptosis in the circulating malignant monoclonal CTCL cells which provide the tumor specific antigen that are taken up by the DCs generated on the ECP plate. These DCs then interact with CD8+ T cells to elicit a cytotoxic response against the malignant cells, causing clinical remission.

Overall, studies that examined characteristics of best CTCL responders to ECP produced criteria that reflect the need for a competent immune system that can mount an anti-tumor response in the patient. These criteria include fairly normal numbers of cytotoxic T cells, normal or close to normal number of natural killer cells, short duration of disease, absence of bulky lymphadenopathy, limited leukocytosis and the presence of a discrete amount of circulating malignant CTCL cells [58, 64]. This helps to support the immunostimulatory "vaccination" theory for ECP's response in patients with leukemic CTCL.

In the treatment of GVHD, the mechanism appears to be different and many ideas have been theorized. One proposed theory of ECP is that endogenous DCs in the liver and spleen are induced to acquire immunotolerizing phenotype and functionality secondary to uptake and processing of apoptotic lymphocytes delivered during ECP in the absence of danger signal [65, 66]. However, there is convincing evidence from our lab and others suggesting the mechanism behind ECP is more complicated than simply production of apoptotic lymphocytes.

Studies in mouse model of suppression of hapten-specific contact hypersensitivity that utilized 8-MOP and UVA (8-MOP/UVA)-treated cells showed that the cell-mediated inhibition of immunity was transferable, but was lost upon depletion of $CD11c^{+}$ DCs from the transferred cell inocula [67]. Previous work done in our lab have shown that there is a six-fold increase in the number of DCs after ECP and these DCs showed gene expression changes, some of which were shown to be linked to regulatory T cell development [57]. In addition, the process is independent of disease state and occurs with either peripheral blood mononuclear cells of normal donors or patients [57].

There is a growing body of evidence in animal and human models to suggest that the underlying mechanism of ECP-induced tolerance relies on antigen-specific $CD4^{+}$ $CD25^{+}$ $FoxP3^{+}$ regulatory T cells (Tregs) [68]. Tregs are a subpopulation of $CD4^{+}$ T lymphocytes, comprising 5% to 10% of the peripheral blood pool, that maintain immunological self-tolerance in the periphery by regulating and suppressing immune responses [69]. Tregs have been reported to protect against chronic cutaneous GVHD [70]. In ECP treated heart and lung allograft recipients, Treg blood levels are doubled compared to normal controls and the levels persisted for extended periods after ECP cessation [71]. Human studies showed the increase in circulating Tregs correlated with decreased organ rejection [72]. Experiments in mouse demonstrated the effect of ECP could be transferred to a non-ECP-treated animal via the infusion of purified Tregs [69]. In a murine model of immune tolerance, it was shown that Tregs were induced by ECP treatment [39]. Furthermore, these Tregs were able to suppress contact hypersensitivity reactions and that the suppression was lost when they were removed [39]. The details of how ECP induces Tregs remain unclear, but seem to involve immature DCs presenting antigens [73]. Therefore, it is very possible that tolerogenic DCs are responsible for the clinical efficacy of ECP by generating antigen specific Treg that suppress the alloreactive T cells responsible for producing the disease state.

Because CTCL and GVHD are distinctive diseases and ECP seems to have paradoxical effects in which it improves the cytotoxic function in CTCL while improves the regulatory function in GVHD, there is speculation whether Treg is the effectors cells that are effective in CTCL. Tregs are thought to be capable of inhibiting immune response against a variety of antigens, including those expressed by malignant cells [74].

In addition, the degree of Treg infiltration in CTCL was correlated to patient survival and Treg cells can directly suppress the function of malignant T cells [75].

Rao et al [76] studied and compared the in vivo effects of ECP on FoxP3 Tregs in patients with CTCL and GVHD. They found small but statistically significant increase in TGF-beta in both patient groups while a small decrease in pro-inflammatory cytokine IL-17 in the GVHD group following ECP. Interestingly, the mean level of TGF-beta was on average three times higher in the GVHD than the CTCL patients, but the Treg faction in GVHD ptatients was a level close to healthy control while the CTCL patients had Treg level more than twice as high. This was in contrast to other findings of equal Treg level in CTCL patients and controls [77] and paucity of FoxP3 Treg in peripheral blood of Sezary patients [78]. The authors explained that immunologically, CTCL is a more indolent disease than GVHD and elevated Treg might represent a homeostatic proliferative reaction to curb expansion of malignant T cells whereas the elevated serum TGF-beta in GVHD patients could reflect the very strong immune reaction taking place. The authors found tremendous individual variations in cytokine profile; that the profile for IL-4, IL-6, IL-10, IL-17, and IFN-gamma did not change; and that the local milieu and concentrations of cytokines are decisive for immune regulation without necessarily being reflected in serum of these patients.

While many possibilities exist as presented above, the seemingly opposite effect of ECP is likely dependent on the DC functional properties. The properties are affected by many factors including the DC’s maturational state, stimulating modalities, cytokine environments, co-stimulatory molecules, and other therapy used in the clinical conditions. Therefore, many factors must be taken into account in order to explain the

immune response to ECP and its paradoxical immunogenic and tolerogenic effects. In fact, the different clinical response times of ECP treatment resulting in CTCL after 2-3 months and in allograft rejection after only a few days to weeks suggests that multiple mechanisms of action may be applicable [79]. One possible explanation would be the production of both immunogenic and tolerogenic DCs during the same procedure [80], which is the result of plasma proteins, platelets, sheer stress, and 8-MOP/UVA light interacting with ECP processed antigen presenting cells [43, 80].

8-Methoxypsoralen (8-MOP) and Ultraviolet A (UVA)

Given the effectiveness of 8-MOP/UVA treatments in a variety of dermatologic diseases [36] and its use in ECP, it is possible that 8-MOP/UVA may play an important role in modifying DC function. For unclear reasons, perhaps relating capacity of parent monocytes to quench free-radicals caused by incident ultraviolet energy, monocytes and DCs are relatively resistant to irradiation-induced apoptosis. This resistance to apoptosis is important because long-term survival would allow for significant window of opportunity for ECP-treated antigen presenting cells to contribute directly *in vivo*. In other words, it contributes to the idea that their survival is linked to their active role in mediating immunomodulatory effects.

There exist tremendous evidence to suggest 8-MOP/UVA may play an important role in modulating immunologic function of antigen presenting cells. Because the IL-12 secretion of DCs decreases in an irradiation dose-dependent manner [81], this leads to the question of whether there could be a direct effect of 8-MOP/UVA on DCs. Ultraviolet B light (UVB) is known to cause immune suppression via release of soluble factors after

membrane damage [82, 83] and to promote an immature DC phenotype that leads to decreased T cell proliferative response in mixed leukocyte reactions [81]. Because 8-MOP/UVA exposure is an important step in ECP and results in similar apoptotic damage to cells as UVB, we hypothesize that 8-MOP/UVA may be critical in creating a tolerogenic DC phenotype and similar factors as found with UVB studies. There exist several lines of evidence in support of the idea that 8-MOP/UVA might be important in the mechanism of ECP.

Many cytokine changes have been documented after 8-MOP/UVA treatment of mononuclear cells [84]. There is evidence of increased anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist in untreated mononuclear cells when they are co-incubated with 8-MOP/UVA -treated cells [85]. In addition, there is increased IL-10 and IL-4 with clearance of apoptotic cells by DCs in the absence of danger signals [85, 86] and they subsequently decrease pro-inflammatory cytokine production [65]. While the production of these immunosuppressive cytokines does not explain the selective action of ECP, they might still play an important role such as production of Tregs and immature DC and maintenance of peripheral tolerance [39, 87]. To better understand the complete mechanism, more work needs to be done to clarify their roles.

8-MOP/UVA has been implicated in the efficacy of ECP in not only generating apoptotic lymphocytes but also truncating the maturation of the DCs. Legitimo et al showed that combining mixed culture of 8-MOP/UVA treated and untreated monocytes at ratio of 1:9, similar to the fact that only 10% of circulating cells are exposed to 8-MOP/UVA, 8-MOP/UVA did not influence monocyte to DC differentiation. However, the immature DCs generated in the mixed culture had significantly higher phagocytic

activity and lower HLA-DR with respect to untreated DC with both groups preserving the capability to mature in the presence of LPS [88]. In addition, mature 8-MOP/UVA-treated DCs showed a lower ability to induce T cell proliferation [88]. The simultaneous increase in antigen uptake activity and MHC Class II molecule expression may induce a reduction in the capacity to induce T cell proliferation. The 10% of 8-MOP/UVA treated cells did not seem to inhibit DC generation but did induce DCs with tolerogenic phenotype and function.

The findings above was consistent with finding by studies led by Spisek [40] and Holtick [89]. The investigators found that DCs in ECP product were in the immature state with respect to the phenotypic and functional characteristics. In contrast to the *in vitro*-generated monocyte-derived DCs and DCs not treated by 8-methoxypsoralen and UVA, they produced significant amounts of interleukin-10 (IL-10). They efficiently captured apoptotic lymphocytes, did not induce proliferation of T lymphocytes, and preserved the capacity to be activated by polyriboinosinic polyribocytidylic acid and lipopolysaccharide [40]. Holtick et al found that DCs become apoptotic but that monocyte-derived DC's phenotypic and functional properties are modulated by in vitro 8-MOP/UVA before undergoing apoptosis with a shift in the pattern of stimulated T cells toward a Th2 profile [89].

Given the results of the above studies, there is possibly an important role of 8-MOP/UVA on modulating DC phenotype and function, especially in a tolerogenic manner. Because one can control the degree and concentration of photo-activation by 8-MOP/UVA, it is among the most finely titratable and focusable pharmacologic agents in clinical use. It would be important to study the effect of 8-MOP/UVA on ECP-associated

monocyte-to-dendritic cell activation and maturation in a human model. This is especially true given recent findings from our lab showing induction of GILZ by 8-MOP and UVA light in cytokine generated DCs [80]. Further studies with ECP-induced DCs would give clinical relevance to the findings by relating to the antigen presenting cells that are important in clinical efficacy and produced in a physiologically manner by ECP.

Glucocorticoid-Induced Leucine Zipper

Glucocorticoid-Induced Leucine Zipper, GILZ, was initially found to be a gene induced by dexamethasone in thymocytes that was responsible for glucocorticoid-induced apoptosis [90]. Now, it is known be expressed by other cells of the immune systems and those in the peripheral tissues [91]. GILZ helps to mediate functions of glucocorticoids including modulation of T-cells [92-94], controlling protein trafficking and signaling, regulation of T-helper cell differentiation [95, 96], increase of epithelial sodium channel mediated sodium transport in the kidney [97, 98], and control of malignant transformation [94].

Recently, GILZ has gained attention because of its immonomodulatory effects in the immune system. On a molecular level, in immune cells, GILZ directly binds nuclear factor-κB (NF-κB) subunit and prevent its nuclear translocation and dependent transcription [92] (**Figure 2**). It also inhibits other pro-inflammatory factors including AP-1, Raf-1, and MEK and ERK phosphorylation [99]. Not surprisingly, in terms of its inflammatory properties and functions, GILZ is considered to be a key mediator of immunosuppressive effects of glucocorticoids. In addition to glucocorticoids, IL-10 [100,

101] and TGF-β [101] also induces GILZ and has the capability to induce tolerogenic DCs.

In human DCs, GILZ upregulation induces a tolerogenic phenotype characterized by decreased expression of CD80, CD83, and CD86, and MHC class II. In addition, molecules that are involved in the peripheral maintenance of tolerance, such as PDL1 and ILT-3, are increased [101]. GILZ also played a role in glucocorticoid induced IL-10 production and decreased production of pro-inflammatory chemokines by DCs [101]. Functionally, DCs that express GILZ showed a reduced ability to stimulate a CD4+ T cell response and generation of antigen-specific Tregs. The ability of glucorticoid-induced DCs to function in a tolerogenic manner in fact depended on GILZ as knockdown of GILZ by siRNA abolished their immunosuppressive effects. Additionally, over-expression of GILZ in DCs reproduced the effects of glucocorticoids. Together, the findings indicated that GILZ is both necessary and sufficient for induction of tolerogenic DCs and serves a key mediator of immunosuppressive stimuli [101, 102].

The important role of GILZ in regulating the phenotype and function of DCs has been verified by many studies. Lebson et al [103] observed that induction of GILZ limited the efficacy of DC vaccines and GILZ was highly upregulated in DCs found at site of tumor. The downregulation of CD86 with dexamethasone can be blockaded with silencing of GILZ, which enhanced T cell effector function in vivo. This was reflected by the observation that DCs with GILZ knockdown significantly prolonged survival of mice with a preexisting tolerogenic tumor [103]. Similarly, Catheline et al [104] observed that silencing of GILZ led to a decrease of the PD-L1 expression associated with an increase in their IL-12 secretion and T-cell induction capability. Ultimately, treatment with GILZ

siRNA induced stronger memory T-cell response in terms of the number of IFN-γ-secreting cells and improved the immunogenicity of clinical-grade mature dendritic cells [104]. Additionally, GILZ has also been shown to be immunosuppressive in a Th1-mediated model of colitis [96], hypoxia-induced inflammation and cyclooxygenase-2 expression [105], endothelial cell adhesive function implicated in inflammatory leukocyte recruitment [106], human airway epithelial cells injury repair [107], mice models of inflammation and arthritis [108, 109], and human alveolar macrophages activation induced by toll-like receptor [110].

8-MOP/UVA, Apoptotic Lymphocytes, and GILZ

Given that 8-MOP/UVA exert immunosuppressive effects and that GILZ induction may be a common mechanism by which DCs are committed towards a tolerogenic phenotype and functional state, we explored the possibility that 8-MOP and UVA directly upregulated the expression of GILZ in DCs. Since apoptotic cells deliver immunosuppressive signals [111] and 8-MOP and UVA renders lymphocytes apoptotic, we also hypothesized that apoptotic lymphocytes may indirectly induce the expression of GILZ in antigen presenting cells [80].

Using cytokine generated DCs, we demonstrated that the above premises to be true [80]. 8-MOP and UVA light directly up-regulated GILZ and induced a tolerogenic phenotype that is reflected by decreased co-stimulatory molecules of CD80 and CD86, resistance to toll-like receptor-induced maturation, and increased immunosuppressive and decreased pro-inflammatory production. Furthermore, knockdown of GILZ with siRNA

reduced IL-10 and increased IL-12p70 production, demonstrating that GILZ is critical for this tolerogenic profile [80].

While these findings provided a novel link between 8-MOP/UVA and GILZ expression, they may not necessarily apply to ECP because the findings are in cytokine generated DCs. It is well known that cytokine generated DCs have failed to produce significant benefits in various human clinical trials while ECP has over 25 years of worldwide clinical experience and well-documented clinical efficacy. The difference likely lies within ECP's unique ability to produce DCs in a physiologic manner that does not depend on supra-physiologic levels of cytokines [112]. Because of differences in the cytokine- and ECP-generated DCs, our findings of 8-MOP and UVA in cytokine DCs need to be verified and confirmed in ECP-induced DCs. Doing so will make our findings more clinically relevant and may help provide a molecular basis for ECP's observed clinical effects.

STATEMENT OF PURPOSE

Despite ECP's worldwide clinical use, unusual safety profile, and proven clinical efficacy, its exact mechanism is not completely clear. DCs play crucial role in antigen presentation and possess tremendous functional plasticity in its unique ability to induce and suppress the immune system in an antigen specific manner. Due to unknown factors to target, ECP has not changed or advanced since its invention. The goal of the project is to clarify the mechanism of ECP in a human model by examining the role of GILZ in ECP-induced DCs. We hope to utilize the knowledge gained to provide scientific evidence and basis to refine the procedure, circumvent issues that exist within current therapies, and design novel immunotherapy utilizing the principles behind ECP.

Hypotheses

1. ECP causes DC differentiation and apoptotic cell generation, which are critical to inducing tolerance.
2. Photo-activation of 8-MOP with UVA light inhibits maturation of ECP-induced DCs and enables antigen-specific T cell tolerization.
3. 8-MOP and UVA induce ECP-induced DCs to become tolerogenic via upregulation of GILZ.
4. DCs with GILZ upregulation show a reduced ability to stimulate a primary T cell response.

Specific Aims

1. To evaluate the effect and role of 8-MOP and UVA on ECP-induced DC maturation by phenotype and functionality.
2. To study the effect of 8-MOP and UVA on the induction of GILZ expression in ECP-induced DCs.
3. To demonstrate *in vitro* transferable, antigen-specific immunosuppression with ECP-induced DCs.

MATERIALS AND METHODS

Patient Sample

Yale Human Investigations Committee approval was obtained prior to the initiation of this study. Informed consent was provided to normal blood donors according to the Declaration of Helsinki. Donors were compensated financially for their blood donation with the amount deemed appropriate by the Yale Human Investigations Committee.

Monocyte Isolation and Enrichment

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of normal donor blood over a Ficoll-Hypaque gradient. Monocytes were subsequently enriched by either (1) plastic adherence or (2) $CD11c^+$ magnetic bead positive selection.

Generation of ECP-Induced DCs

A laboratory model of ECP was set up in attempt to replicate the main features of the clinical apparatus (**Figure 3**). Normal donor blood was initially spun in centrifuge at 1000 rpm for 15 minutes. Subsequently, the top two thirds of the plasma fraction was removed and used to coat the clinical ECP plate. The plasma contained serum proteins and platelets that are important for monocyte to DC differentiation and maturation [43]. After reconstituting the removed plasma volume with RPMI-1640, PBMCs were isolated via Ficoll-Hypaque gradient.

After obtaining PBMCs, 100-200 ng/mL of 8-MOP was combined with PBMCs and circulated through an ECP UVA exposure plate. RPMI-1640 media without phenol

red was utilized to ensure the coloring did not affect the effect of UVA exposure. Upon exposure of 0.5-2 J/cm^2 UVA light, 8-MOP became activated and caused apoptosis in lymphocytes. Monocytes differentiated into DCs through the passage of the exposure plate and the perturbation from interaction with platelets and plastic walls of the device [43, 113]. We hypothesized that 8-MOP/UVA exposure along with phagocytosis and processing of apoptotic lymphocytes in the absence of danger signals further induced DCs to acquire an immature phenotype.

After passage through the UVA plate, the products were collected and re-suspended in RPMI-1640 medium and 10% pooled human AB serum [114]. Following incubation, cells were harvested and either stained for flow-cytometry or underwent monocyte enrichment by positive selection using CD11c microbeads and magnetic cell separator (Miltenyi) for RNA expression analysis.

Generation of Cytokine DCs

DCs were also derived from monocytes using traditional cytokine cocktail for comparison to ECP-generated DCs and for functional studies. Monocytes was cultured in RPMI-1640 (Gibco, Carlsbad, CA), 2.05% mM glutamine, 15% pooled human serum (Gemini, Sacramento, CA), and 1% penicillin/streptomycin (complete media). 800 IU/mL recombinant human GM-CSF and 1000 IU/mL recombinant human IL-4 (R&D Systems, Minneapolis, MN) were added for 20-36 h to induce monocyte to DC differentiation [115].

Cytokine Generated DC and Apoptotic Lymphocyte Co-cultures [80]

Non-adherent cells removed after plastic adherence consisted primarily of lymphocytes, which were rendered apoptotic with 8-MOP (100 ng/mL) and UVA light (1 J/cm^2), and co-cultured in complete media for 24 hours with either 8-MOP/UVA-treated or untreated cytokine generated DCs. Some cytokine DCs were treated for 24 hours with dexamethasone (100 nM) (Sigma, Ronkonkoma, NY) and served as the positive control. To ensure that RNA was not isolated in significant quantities from lymphocytes, DCs were re-purified using CD11c magnetic bead positive selection before any functional experiments.

8-MOP and UVA Treatment of Cytokine DCs

Cells were incubated with 8-MOP (20 μg/mL) (Therakos, OH, USA) for 30 min in the dark, and then irradiated with a desktop UVA (320–400 nm) light box containing a series of 12 linear fluorescent tubes. The UVA irradiance was measured using a photodiode. Therapeutic doses of 8-MOP (100–200 ng/mL) and UVA light (1–2 J/cm^2) were used for this study [35].

Role of 8-MOP and UVA and Apoptotic Lymphocyte on DC Phenotype and Functionality

To study the effects of ECP and specifically 8-MOP/UVA on DC phenotype and functionality, aliquots of the following cells with and without overnight incubation were procured: cells before ECP (PBMC), cells passed through UVA exposure plate with 8-MOP/UVA (ECP), and cells passed through plate without 8-MOP/UVA (PP: plate passed). In plate passed cells exposed to 8-MOP/UVA, there would be apoptotic lymphocytes generated with the ability to interact with DCs. At times, a mixed group

consisting of 8-MOP/UVA-treated and untreated PBMCs were combined at 1:1 ratio. DC phenotype and functionality was subsequently assessed by monoclonal antibody staining and functional stimulation of antigen-specific T cell clones.

Lymphocyte Proliferation Assay

The technique has been well described and is utilized to ensure delivery of proper dosing of 8-MOP and UVA that abrogates T cell proliferation [116]. After PBMCs were isolated, they were either held as controls or passaged through the plate and exposed varying doses of UVA exposure (0.25-2 J/cm^2) with constant dosage of 8-MOP at 100 ng/mL. Control cells were incubated with the psoralen solutions, wrapped in tin foil, and placed in the incubator during the longest irradiation exposure period. After manipulation and irradiation, the cells were washed and 200,000 cells from each group were plated in 96-well plates. Each group was done in replicates of five containing RPMI-1640 medium with 10% pooled human serum.

After plating, phytohaemagglutinin (PHA) at 5 ug/mL was added into some of the wells to be used as mitogen to stimulate T cell proliferation. The cultures were incubated at 37 °C for three days under a 5% CO_2 atmosphere. The cultures were subsequently pulsed with 1 μCi of [^{3}H]-thymidine, either 6 or 18 hours prior to harvesting with an automated sample harvestor. Incorporation of [^{3}H]-thymidine was measured by liquid scintillation analysis in a beta counter.

Immunophenotype

Multicolor flow-cytometry analysis was used to determine DC immunophenotype and maturation status by measuring immunofluorescence of samples incubated with monoclonal antibodies specific for monocytes and DC. They include CD14 (LPS receptor: monocytes); CD11c (marker for monocyte lineage); HLA-DR (class II MHC molecule: monocyte and DCs); CD83 (DC marker: immature DC-cytoplasm, mature DC-membrane); CD80 and CD86 (B7.1 and B7.2 co-stimulatory molecules); ICAM-1 (crucial to immune cell migration and antigen presenting function); PLAUR; and TNFR-1 (a tumor necrosis family receptor, together with TNF play important roles in inflammation and autoimmunity) [57]. Immature tolerogenic DCs were identified by low levels of CD80 and CD86, presence of cell surface HLA-DR and CD11c [57]. Apoptosis was assessed using the Annexin-V Apoptosis Detection Kit (eBioscience, San Diego, CA), with 7-AAD substituting for PI as the cell viability dye.

The following gating strategy was utilized to ensure uniformity and consistency of analyzing viable antigen presenting cells: 1) lymphocytes and monocytes are gated based on forward and side scatter profiles; 2) doublet cells are exclude based on width; 3) viable cells are selected using viability dye (7-AAD negative); 4) $CD11c^+$ cells with high side scatter profile are gated (**Figure 4**). The last subset of cells are subsequently analyze with immunophenotyping using the various fluorescence-conjugated antibodies specific for monocytes and DCs.

Gene Expression Analysis Using Quantitative Real-Time PCR

RNA was isolated from purified $CD11c^+$ ECP-induced DCs using the RNeasy Mini Kit with on-column DNase I treatment (QIAGEN, Hilden, Germany). RNA yield

and purity were assessed with a NanoDrop spectrophotometer. cDNA was obtained with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Middletown, CT). Pre-designed and validated Taqman Gene Expression Assays were used to detect GILZ, IDO, and PDL1 transcripts, with HPRT-1 serving as reference genes (Applied Biosystems, Middletown, CT). Samples were run in triplicate on a 7500 Real Time PCR System. Fold change was calculated using the delta–delta C(t) method.

Stimulation of MART-1 Antigen Specific CD8^{+} T-Cells [80]

Cytokine DCs from the co-cultures were re-purified and used as antigen presenting cells for stimulating naïve autologous CD8^{+} T-cells. 0.4×10^{6} DCs were co-cultured in complete media with 4×10^{6} autologous lymphocytes, enriched by CD4/CD8 magnetic bead positive selection. A high frequency (>1 in 2500 CD8+ T-cells) of MART-1-specific CD8^{+} T-cells with a naïve phenotype are found circulating in HLA-A2^{+} healthy individuals, and these cells are capable of proliferating in response to MART-1 peptide cross-presentation. The MART-1$_{16\text{-}40(A27L)}$ HLA-A2-restricted peptide (10 μM, GHGHSYTTAEELAGIGILTVILGVL) [117] was added at the start of co-culture, with IL-2 (12.5 IU/mL) and IL-7 (5 ng/mL) added on day 3 (R&D Systems, Minneapolis, MN), and fresh media added every 2–3 days. After 9 days of co-culture, lymphocytes were harvested and incubated with the A*0201/ELAGIGILTV-MART-1$_{26\text{-}35(A27L)}$-PE dextramer, followed by an irrelevant peptide A*0201/KTWGQYWQV-gp100-APC dextramer control (Immudex, Copenhagen, Denmark). MART-1-specific live CD8^{+} T-cells were identified as CD8^{+}CD4^{-}MART-1^{+}gp100^{-} cells.

Statistical analysis

Student's *t*-tests were used for inter-group comparisons, with p-values < 0.05 considered to be statistically significant. Differential gene expression was considered statistically significant with a > 2.5-fold change and a p-value < 0.05.

RESULTS

Plate Passage Globally Activates Monocytes

ECP's physiologic induction of monocyte to dendritic cell differentiation and maturation is important in the clinical efficacy of ECP. In our laboratory model of ECP, we observed the effect of plate passage on monocytes as they interacted with serum proteins and platelets under sheer stress. Plate passage and overnight culture up-regulated several markers on the cell surface of monocytes. These included HLA-DR, PLAUR, and ICAM-1, three molecules that are important in antigen presentation and T cell biology. These markers shifted globally rather than in a subset of the monocyte population. While overnight incubation without plate passage induced increased expression of these markers, there was additional increase in expression as a result of plate passage. Upregulation of these markers were not necessarily consistent or stable. There was a drop in the expression of PLAUR on day 2 and day 3 following an increase on day 1 while the increase in ICAM-1 expression fell off after day 1. This can be seen **Figure 5**.

8-MOP and UVA Light Suppressed Lymphocyte Proliferation

Titration was conducted to ensure delivery of appropriate dosage of 8-MOP and UVA to inhibit mitogen-induced lymphocyte proliferation while inducing gentle apoptosis rather than necrosis. This was done to best replicate the dosages utilized used in the original studies of ECP and the clinical ECP apparatus [6, 116]. We observed that with 100 ng/mL of 8-MOP, 1-2 J/cm^2 of UVA light was the appropriate range to achieve the desired function (**Figure 6**), which correlated well with those used in the clinical ECP apparatus [35]. There was variation of the two donors tested, but both donors showed a

positive correlation between dosage of 8-MOP/UVA and inhibition of mitogen-induced lymphocyte proliferation. The trend of the results did not differ depend on the amount of time the cells were pulsed with [^{3}H]-thymidine. This data is shown in **Figure 6**.

CD11c$^+$ Antigen Presenting Cells Are Resistant to 8-MOP/UVA-Induced Apoptosis

Monocytes and DCs are resistant to 8-MOP/UVA-induced apoptosis as compared to lymphocytes. When 100 ng/mL of 8-MOP was used, lymphocytes quickly underwent apoptotic cell death starting at 0.5 J/cm^2 of UVA light. While both lymphocytes and antigen presenting cells demonstrated 8-MOP/UVA dose-dependent cell death, the rate of cell death was higher and at a much steeper rate in the lymphocyte group with application of increased UVA dosage. This is shown in **Figure 7**.

8-MOP and UVA Light Stimulated the Expression of GILZ and Suppressed the Expression of IDO and PDL1 RNA Transcript in ECP-Induced Antigen Presenting Cells

After showing 8-MOP/UVA induced GILZ expression in cytokine generated DCs [80], we examined whether the same phenomenon was true in DCs induced by ECP. While 8-MOP or UVA alone did not upregulate GILZ expression (data not shown), the combination of 8-MOP and UVA induced an average of three-fold change and four-fold change one and two days after manipulation respectively when normalized to that particular day's PBMC group (**Figure 8**). Furthermore, this upregulation was dose-dependent and present for several days after experimental manipulation (**Figure 11B**). At the highest level of 2 J/cm^2 of UVA, the level of GILZ expression in ECP-induced

antigen presenting cells was comparable to that induced by dexamethasone. Unlike 8-MOP/UVA, plate passage alone did not significantly alter the expression of GILZ.

The induction of GILZ by 8-MOP/UVA showed different kinetics than that by dexamethasone. While dexamethasone upregulated GILZ quickly within five hours after treatment (data not shown), 8-MOP/UVA induced GILZ much slower and required 24 hours.

Given the upregulation of GILZ by 8-MOP/UVA, we proceeded to examine its effect on two other immunosuppressive molecules that have been described to be important for tolerogenic dendritic cell function. Unlike GILZ, the RNA expression of IDO and PDL1 decreased with 8-MOP/UVA treatment and dexamethasone (**Figure 11B**). This change in expression occurred in a dose dependent fashion and the effect was present for three days after manipulation.

8-MOP and UVA Induced Antigen Presenting Cells with Tolerogenic Phenotype and Suppressed the Expression of Several Cell Surface Marker

In addition to upregulating GILZ expression, 8-MOP/UVA deviated antigen-presenting cells to take on an immature phenotype as defined by $CD11c^+$ $HLA\text{-}DR^+$ $CD83^-$ $CD14^-$ (**Figure 9A**). Similar to GILZ expression, the percentage of antigen presenting cells with an immature phenotype increased in an 8-MOP/UVA dose dependent manner (**Figure 9B**). Additionally, the percentage of cells with an immature phenotype may have increased over time. This was most apparent for those treated with either 1 or 2 J/cm^2 of UVA light (**Figure 9A, 9B**).

Treatment of cells with 8-MOP/UVA showed decrease in the expression of CD11c, CD86, HLA-DR, and CD14 on the cell surface of the induced DCs. The suppression was persistent and the effect was magnified from day one to day two. From the baseline expression of unmanipulated PBMCs, the decreased expression of CD11c was 54% and 68%, CD86 was 66% and 68%, CD14 was 67% and 78%, and HLA-DR was 25% and 65% one and two days after treatment respectively (**Figure 10A, 10B)**.

Because of very low expression of CD80 and CD83 at baseline, the effect of plate passage and 8-MOP/UVA on their expression was generally harder to quantify and therefore unclear in these sets of experiments. There was tremendous variability in the expression as indicated by high standard deviation (**Figure 10A, 10B**).

However, in an experiment looking specifically at the effect of varying dosages of 8-MOP/UVA on ECP-induced antigen presenting cells, 8-MOP/UVA decreased the expression of CD86, CD11c, CD14, and CD80 in a dose dependent manner while HLA-DR showed variable responses to 8-MOP/UVA (**Figure 11A**). This was consistent on days 1, 2, and 3 after treatment and sterile culture.

Markers upregulated by plate passage, namely ICAM1 and PLAUR, showed variable effect from 8-MOP/UVA. While ICAM1 was down-regulated by 8-MOP/UVA exposure, the expression of PLAUR seems to be magnified by 8-MOP/UVA 24 hours after treatment and sterile culture (**Figure 5B**). The effect of 8-MOP/UVA on PLAUR was unclear after 24 hours. While one experiment showed the change in expression of PLAUR approached that of plate passed but not 8-MOP/UVA-treated cells, another showed that PLAUR expression decreased precipitously to levels lower than plate passed cells. Results from the 8-MOP/UVA titration experiment correlated well with the above

findings showing dose-dependent reduction of ICAM1 by 8-MOP/UVA while unclear effect on expression of PLAUR (**Figure 11A**). For example, while 0.5 and 2 J/cm^2 of 8-MOP/UVA seems to decrease PLAUR when compared to plate passed cells, 1 J/cm^2 of 8-MOP/UVA increased PLAUR expression on all three days.

Cytokine Generated DCs with High GILZ Expression Demonstrated Mixed Ability to Stimulate MART-1-Specific $CD8^+$ T Cells [80]

To analyze a primary T cell functional response, we investigated the ability of cytokine generated DCs expressing GILZ to cross-present the MART-$1_{16\text{-}40(A27L)}$ peptide and stimulate naïve autologous MART-1-specific $CD8^+$ T-cells. It was previously shown that dexamethasone-treated DCs, 8-MOP/UVA-treated DCs, and DCs exposed to apoptotic lymphocytes expressed high levels of GILZ whereas untreated DCs had low GILZ expression [80].

For one donor, dexamethasone DCs, 8-MOP/UVA-treated DCs, and DCs exposed to apoptotic lymphocytes all demonstrated a reduced ability to stimulate MART-1-specific $CD8^+$ T cells after 9 days of co-culture, as compared to untreated DCs. For two other donors, dexamethasone-treated DCs, 8-MOP/UVA-treated DCs, and DCs exposed to apoptotic lymphocytes all demonstrated an equal, or greater ability to stimulate MART-1-specific $CD8^+$ T cells [80]. The data is demonstrated in **Table 1**.

DISCUSSION

Summary of Findings

This study demonstrated plate passage globally activated monocytes and they upregulated the expression of GILZ when treated with 8-MOP/UVA. While plate passage did not alter the expression of GILZ, it upregulated molecules on the surface of $CD11c^+$ antigen presenting cells such as ICAM1, HLA-DR, and PLAUR, especially after 24 hours. The induction of GILZ by 8-MOP/UVA occurred in a dose dependent manner and was preserved to day two or three after treatment and culture. DCs with GILZ RNA induction displayed a tolerogenic phenotype characterized by down-regulation of several co-stimulatory molecules that include CD80, CD86, and ICAM1. While 8-MOP/UVA suppressed the cell surface expression of CD11c and CD14, its effects on HLA-DR, CD83, and PLAUR are variable. Although 8-MOP/UVA-treated cells displayed a tolerogenic phenotype both in terms of GILZ RNA and cell surface molecules, they showed a decreased RNA expression of IDO and PDL1 and demonstrated a mixed variability to stimulate a naïve primary $CD8^+$ T cell response using the MART-1 long peptide.

Kinetics and Molecular Basis of GILZ Induction

Interestingly, the kinetics of GILZ induction in DCs by dexamethasone and 8-MOP/UVA differed tremendously. Dexamethasone was able to upregulate GILZ RNA expression within two to five hours with lasting effects up to three days while 8-MOP/UVA required one day for induction of GILZ. The effects by dexamethasone likely relates to the well-characterized mechanisms and kinetics of steroid induced responses.

The steroid molecule rapidly enters the cell nucleus after associating with ligand receptors in the cytoplasm and subsequently upregulates gene expression via steroid response element binding. In fact, there is a glucocorticoid-response element associated with the GILZ gene and that is likely the site of binding for dexamethasone. GILZ induction by 8-MOP/UVA has a much different kinetics and required one day instead of a few hours. This implies a fundamentally different mechanism of GILZ induction by 8-MOP/UVA.

There are likely multiple pathways to upregulate GILZ expression. The induction of GILZ by 8-MOP/UVA likely occurs through two distinct mechanisms of direct effect and indirect effect of 8-MOP/UVA-induced apoptotic leukocyte [80]. It is likely that GILZ upregulation is at least partially a byproduct of 8-MOP/UVA-induced cellular stress response, which takes time. It is known that 8-MOP/UVA cross-links DNA and interact with mitochondria, RNA, and proteins to cause cellular stress. GILZ may be induced to produce a tolerogenic environment when there is a stress-induced cell death to ensure peripheral tolerance and inhibition of auto-reactivity. The molecular mediator of GILZ upregulation by 8-MOP/UVA is unclear but there is evidence that suggest p53 may be one of the key facilitators.

There are several lines of indirect evidence tying p53 to induction of GILZ. GILZ was initially discovered to be upregulated in lymphocytes during IL-2 deprivation and it was found to help lymphocytes in preventing IL-2 deprivation induced apoptosis. Using human antigen-specific T cell clones, a study of cytokine rescue found that IL-2 protected T cell clones from IL-2 deprivation apoptosis using a pathway in which p53 protein expression is upregulated [118].

In models of hypoxia, p53 is also indirectly linked to GILZ. In a study using macrophage cell line, it was shown that hypoxia not only significantly upregulated GILZ expression, but also significantly enhanced dexamethasone induced expression of GILZ in macrophages and the spleen of rats [119]. Given that hypoxia is known to induce p53 [120, 121], it is possible that p53 may be involved in GILZ upregulation in the macrophage cell lines. Additionally, ERK activity is found to be involved in the upregulation of GILZ induced by hypoxia [119]. In a study of doxorubicin-induced apoptosis of cardiomyocytes and H9c2 cells, it was found that ERK1/2 are functionally linked to p53 [122].

Maybe most convincingly, in a study published in PNAS in 2011, it was found that loss of p53 impaired repression of NF-kappaB target gene transcription by glucocorticoid [123]. Additionally, the loss of p53 also impaired transcription of glucocorticoid receptor target genes, whereas upstream signaling cascades remained intact. In fact, p53 knockout partially abolished dexamethasone-induced transcription of GILZ and p53 loss severely impaired glucocorticoid rescue of death in a mouse model of LPS shock. In summary, these findings showed that p53 has a crucial role in the repression of NF-kappaB and other functions by glucocorticoid and involved in GILZ upregulation [123]. This is important because a key action of GILZ is inhibition of NF-kappaB nuclear translocation [91].

In addition to the direct effect of 8-MOP/UVA, the indirect effect likely involves tolerogenic signals delivered by apoptotic leukocytes. Apoptotic lymphocytes have been described extensively as possessing anti-inflammatory properties when taken up by dendritic antigen presenting cells. 8-MOP/UVA quickly and efficiently induces apoptotic

lymphocyte, which can interact with antigen presenting cells induced by ECP or the DCs present in lymphoid organs such as the spleen and liver. Transimmunization, which added an overnight incubation step of the ECP-processed products, have shown to increase the clinical efficacy of the procedure [124]. This implies that increased interactions of the treated lymphocyte and dendritic cell populations are important. While the effects of apoptotic lymphocytes have been studied extensively, it is unclear whether the signals delivered by different leukocytes are equivalent. Recent research on apoptotic DCs provides some evidence that there may be key differences between different types of apoptotic cells.

The importance of maintaining peripheral tolerance through highly regulated apoptotic process is well known [125, 126]. DC apoptosis, however, may be different than that of lymphocytes. It was observed that transgenic mice with defects in DC apoptosis, but not T or B lymphocytes, developed systemic autoimmune disease [127-129]. Furthermore, Kushwah and colleagues found that only apoptotic, not necrotic, DCs can induce viable DCs to become resistant to LPS-induced maturation and secrete TGF-beta. [130, 131]. In particularly, TGF-beta not only helped to generate antigen-specific Foxp3+ Tregs, but also was the factor responsible for induction of Treg. Viable DCs secreted TGF-beta when engulfing apoptotic DCs but not other types of apoptotic leukocytes [66]. This implied that while phosphatidylserine may play a role in the processing of apoptotic lymphocytes by antigen processing cells [132], there might be other ligands exposed on apoptotic DCs that would induce viable DCs to produce TGF-beta [131]. One of the possibilities may be $\alpha v\beta 8$ integrins, which are important to induce Tregs and found in high concentrations on the surface of DCs [133]. Furthermore, the

molecules that induce TGF-beta production may be different depending on the type of antigen presenting cells as macrophages, but not DCs, have a phosphytidylserine-dependent pathway [134].

Regardless of the exact pathway of GILZ induction, it is conceivable that induction of tolerogenic DC at sites of cellular stress and damage might serve as a means to protect self against the development of systemic autoimmunity. GILZ would be upregulated by 8-MOP/UVA exposures to play an important role in mediating the transformation of DCs to a tolerogenic state.

Functional Studies of DCs Expressing GILZ

Correlating phenotype with function is important in the characterization of tolerogenic DCs. A hallmark feature of tolerogenic DCs is a reduced T-cell immunostimulatory capacity and the ability to generate antigen-specific Tregs [44]. We wanted to develop a functional assay that was robust, specific, and stringent. Using the MART-1 system to stimulate a naïve CD8+ response fulfilled those criteria.

Advantages of the MART-$1_{16\text{-}40(A27L)}$ long peptide include antigen-specificity and a functional requirement for internalization, processing, and cross presentation of the peptide. A high frequency (>1 in 2500 $CD8^+$ T-cells) of MART-1-specific $CD8^+$ T-cells with a naïve phenotype are found circulating in HLA-$A2^+$ healthy individuals, and these cells are capable of proliferating in response to long MART-1 peptide cross-presentation [117]. The requirement of engulfment and cross-presentation limits the observed response to DCs as they have the unique ability to initiate a primary response and are the major cell type known for possessing the proper cellular machinery for cross-presentation

[44, 45, 135]. The high frequency of MART-1 specific naïve $CD8^+$ T cells eliminates the need for re-stimulation by DCs and reduces the time required for the functional assay. The use of MART-1 specific tetramer allows for identification of the specific T cell clone with high specificity and sensitivity.

In this present study, cytokine generated DCs expressing GILZ demonstrated a mixed ability to stimulate autologous MART-1-specific $CD8^+$ T-cells [80]. However, dexamethasone treated DCs also exhibited a variable response, and the stimulatory capacity of dexamethasone treated DCs for all three donors mirrored that of DCs treated with 8-MOP/UVA or exposed to apoptotic lymphocytes. Combined with high intra-group standard deviation, these observations suggest that stimulation of MART-1-specific $CD8^+$ T cells may be influenced by multiple factors distinct from GILZ induction.

A possible explanation for the varied responses comes from the discovery of novel MART-1 HLA-DRB1-restricted epitopes recognized by $CD4^+$ T cells [136]. It is feasible that DCs from HLA-$A2^+$ donors with particular MHC-class II haplotypes would be unable to induce MART-1-specific $CD4^+$ Tregs. These DCs would therefore stimulate $CD8^+$ T cells even in the context of tolerogenic cell surface phenotypes and cytokine profiles. Adding to the complexity of $CD8^+$ T cell responses, a recent study revealed a critical role for Tregs in the induction of primary $CD8^+$ T cell responses and effective memory induction [137]. The authors propose that in the absence of Tregs, low-avidity T cells over-proliferate and impair the activation of high-avidity $CD8^+$ T cells [137]. These observations could help explain why we observed a variable $CD8^+$ T cell stimulatory capacity for all DCs expressing GILZ, including dexamethasone treated DCs, 8-MOP/UVA-treated DCs, and DCs exposed to apoptotic lymphocytes. Further studies will

be needed to clarify the reasons. Despite the variable results, other studies have clearly shown that GILZ-expressing DCs function in a tolerogenic manner and its inhibition increase the immunogenic properties of the DCs [102-104].

A limitation of this particular protocol is that the $CD8^+$ T cell response does not reveal which specific tolerogenic mechanisms are operating. In addition, it is unclear whether low $CD8^+$ response is the result of tolerogenic or lack of immunostimulatory capacity on the antigen presenting cells. Therefore, future studies will be required to determine the production of IFN-γ by MART-1-specific $CD8^+$ T-cells, as well as whether 8-MOP/UVA-treated DCs expressing GILZ are capable of generating Tregs in a similar fashion as DCs generated by immunosuppressive pharmacologic agents like dexamethasone [102]. Once these issues are resolved, studies on ECP-induced DCs should be conducted to further characterize their functional properties and capacity to provide clinical relevance.

Divergent Effect of 8-MOP/UVA on PDL1 and IDO Versus GILZ

Similar to GILZ, both IDO and PDL1 have been implicated in induction of tolerance and suppression of immunogenicity. IDO, or indoleamine 2,3-dioxygenase, is an enzyme that degrades the essential amino acid tryptophan. It has been implicated in inhibition of T cell proliferation [138, 139], suppression of alloreactive T cells [140], protection of tumor lines against rejection [141], and protection of lung allograft [142]. PDL1 regulates peripheral tolerance by interacting with its receptor PD1 on the surface of T cells, which upregulate PD1 expression upon activation. PDL1 has been implicated to

induce co-inhibitory signal in activated T cells and promotes T cell apoptosis, anergy and functional exhaustion [143-145].

Interestingly, whereas 8-MOP/UVA and dexamethasone stimulated GILZ RNA expression, they suppressed the expression of PDL1 and IDO in a dose dependent manner. This highlights that there may exist two general classes of tolerogenic molecules. One class includes GILZ, where an immunosuppressive molecule is upregulated by an immunosuppressive signal such as glucocorticoids, IL-10, and TGF-beta. Another class, which includes IDO and PDL1, are suppressive counter-regulatory molecules that are expressed to control inflammation. This class of molecules can be upregulated by inflammatory signals such as IFN-gamma and TLR agonists including LPS [146, 147]. Thus, IDO and PDL1 may be expressed to control and limit the duration and level of inflammation in attempt to prevent development of autoimmune diseases. This may help to explain why we see the different trend of GILZ versus IDO and PDL1 RNA expression in our experiment. An important molecule that could potentially be involved is IFN-gamma, which deserves further examination in future experiments. It is also important to note that while RNA expression is important, post-transcription regulation, translation, and transportation to surface are additional factors to consider.

Implication of Findings for ECP Mechanism

One of the great mysteries behind ECP has been its divergent ability to stimulate the immune system to combat leukemic CTCL and to suppress the immune response in settings of transplantation and autoimmunity. With the discovery and recognition that ECP is capable of efficiently inducing monocyte-to-DC maturation in the absence of

suprapharmacologic concentrations of cytokines commonly used to generate DC *in vitro*, the cloud of the mystery became clearer. Because DCs are key regulators of immunity and are capable of promoting or suppressing T-cell responses, it became highly likely that DCs perform a critical role in ECP's immunotherapeutic clinical effects.

Overall, we suspect that during the ECP procedure, monocytes are induced to undergo differentiation to DCs while 8-MOP/UVA exposure modifies the properties of these antigen presenting cells via upregulation of GILZ. Furthermore, because the processed monocytes are not homogeneously exposed to 8-MOP/UVA, the procedure is producing both immunogenic and tolerogenic DCs.

Our laboratory has found that ECP's induction of DC involves discrete physiologic interactions between platelets that are tightly adherent to the UVA-exposure chamber and the processed monocytes, which flow through that chamber [43]. Utilizing a laboratory ECP model, this study shows that 8-MOP/UVA may truncate the DC-oriented maturation of the platelet-stimulated monocyte.

We suspect that 8-MOP/UVA exposure of monocytes is critically important in directing a major subset of ECP-processed monocytes into the tolerogenic mode, while relative protection from 8-MOP/UVA may permit simultaneously processed monocytes to mature into immunogenic DC. As monocytes flow through the 1 mm thick ECP exposure plate, they are not uniformly exposed to 8-MOP/UVA. Broadly, the passaged monocytes fall into two categories: one which is exposed to a degree sufficient to compromise their survival past several days and another which is exposed to a sufficiently low level that their survival and function appears uncompromised. The heavily exposed cells upregulate GILZ expression and become tolerogenic whereas the

least exposed go on to become immunogenic due to low GILZ induction. Therefore, the ECP procedure physiologically produces a spectrum of antigen presenting cells capable of inducing immunomodulatory effects in patients with CTCL, GVHD, allograft rejection, and autoimmune disease. Observations from the clinical outcomes of ECP support this claim. ECP's reversal of acute GVHD and organ transplant rejection commonly occurs significantly faster than its immuno-therapeutic impact on CTCL [112]. This observation suggests that, following the therapeutic ECP procedure, tolerogenic short-lived maturationally truncated DCs might dominate early, while immunogenic longer-lived mature DC would be expected to dominate later [112].

The Future of ECP

Studying the mechanism of ECP has tremendous value because understanding may provide the scientific rationale for the development of a novel immunotherapeutic strategy to combat cancer, autoimmune diseases, and transplant rejection. The results from this study coupled with our previous observation in cytokine generated DCs [80] provides an initial step towards a potential molecular explanation for ECP's immunomodulatory effects *in vivo*. The ability to replicate similar results in a laboratory model of ECP with ECP-induced DCs gives credence to the clinical relevance of our previous findings.

GILZ may be that important factor to manipulate in order to deviate the function of DCs to either stimulate or suppress the immune system. After over 25 years of clinical use as a FDA-approved therapy, ECP has not fundamentally changed from its original design. The roadblock to change has been the unclear mechanism of this enigmatic

therapy and what factors to modify. The finding of 8-MOP/UVA induction of GILZ may be an important step toward improving the therapy. Given that 8-MOP/UVA is extremely titratable, it could potentially be utilized to finely titrate the level of induced GILZ expression in ECP-induced DCs or even cytokine generated DCs to skew their function. Future studies on DC therapy focusing on altering the level of GILZ production should also be explored.

Combined with the concepts of DC therapy, ECP offers not only the possibility of effective treatment of GVHD and SOT rejection by regulating alloreactive T cell responses, but allows for prevention in the future. In one model of preventing SOT rejection, one could produce immature DCs via ECP with close monitoring of GILZ expression levels. These GILZ expressing DCs can be loaded with graft alloantigens, which will be derived from apoptotic 8-MOP/UVA-treated lymphocytes that we wish to be tolerated by the host. Because almost all nucleated cells possess the same set of HLAs, the immune response generated against HLAs on apoptotic lymphocytes should be identical to that against the graft. The antigen-loaded DCs will be incubated with recipient purified T cells to induce formation of Tregs that selectively suppress immunity. *In vitro* suppression assays can be conducted to evaluate for the presence and functioning of immunosuppressive cells. Following convincing *in vitro* results, clinical trials with periodic injection of antigen-loaded DCs and Tregs into recipients to induce *in vivo* tolerance could be conducted before transplantation. Delayed hypersensitivity reaction, as evaluated clinically and by biopsy, could then be used to test for immune activation as a monitor for *in vivo* antigen-specific suppression efficacy. Modifications can be made based on the *in vitro* and *in vivo* response by manipulating GILZ until the desired

outcomes has been achieved. Additionally, changes to this model could be utilized for GVHD and other diseases.

Even with its high potential, ECP faces hurdles including its incomplete immunomodulatory effects. Despite clinical response and decreased steroid use in transplant patients on ECP, there may be *in vivo* mechanisms blocking a complete immune tolerance. CTLA-4, which is located on the surface of T cells, has been found to function in suppressing immune response and maintaining self-tolerance [148, 149]. It binds to the co-stimulatory molecules CD80 and CD86, and suppresses T cell activation, proliferation, and IL-2 gene transcription [148, 150]. Because of its critical role in inducing tolerance, it may be useful to simultaneously utilize a CTLA-4 agonist, especially in patients without a response or one with incomplete response. This strategy has been utilized effectively to inhibit T cell activation and autoimmune diabetes in mice [150]. In addition to targeting CTLA-4, multi-modality therapies should be explored to augment or synergize with the effects produced by ECP [151].

Implication for DC Therapy

Given the important role of GILZ, it can be viewed as a molecular switch and mediator in determining the function and fate of DCs. Instead of upregulating GILZ via immunosuppressive therapies such as glucocorticoids that have unwanted side effects, selective targeting of GILZ in DCs may be the future of DC immunotherapy. Although no agents with selective GILZ-inducing capacities have been identified, regimens that do so may represent a promising option to promote tolerance in settings of autoimmunity and transplantation.

In addition to induction of GILZ, novel therapies may also be based on simulating the molecular actions of GILZ [152]. For example, in vivo inhibition of pro-inflammatory mediators such as NF-κB nuclear translocation could produce similar effects as GILZ. In fact, a peptide mimic derived from the p65 binding carboxyl terminus of GILZ inhibits T cells and prevents autoimmune encephalomyelitis, a model of human multiple sclerosis [153]. Another method would be selective induction of GILZ via gene therapy, which may be effective in redirecting the DCs to possess the desired immunosuppressive functionalities [101]. DC-SIGN has recently been identified as negatively regulating the function of GILZ [154] and may serve as another potential target to increase GILZ activity. Finally, further analysis of GILZ protein structure and identification of its targets could reveal targets for synthetic GILZ mimetics [152].

Concluding Remarks

ECP holds enormous promise as a personalized, immunological modulating therapy. Precise elucidation ECP's full mechanisms of action will allow this treatment to optimally harness its full potential in the treatment of a spectrum of T cell mediated diseases. Through physiologic induction of DCs, ECP is capable of inducing antigen-specific tolerance without the global immunosuppression seen with pharmacologic immunosuppressive therapy. It has minimal to no side effects and is very well tolerated by patients. The results from this study may help to elucidate the mechanism of ECP to generate antigen-specific tolerance and immunogenicity.

Overall, understanding the mechanism behind ECP will allow for better patient care by maximizing factors responsible for clinical responsiveness. Once the clarification

of mechanism is reached, there can be refinement in the system that provides ECP enormous potential to expand its treatment applications to other lymphocyte-mediated diseases. GILZ is a strong candidate that provides a molecular and mechanistic explanation of ECP's clinical effects. This acquired knowledge will be useful in solving current limitations that prevent both immunogenic and tolerogenic DC vaccines from reaching its full potential and clinical efficacy in human subjects.

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FIGURES

Figure 1: Clinical ECP Procedure

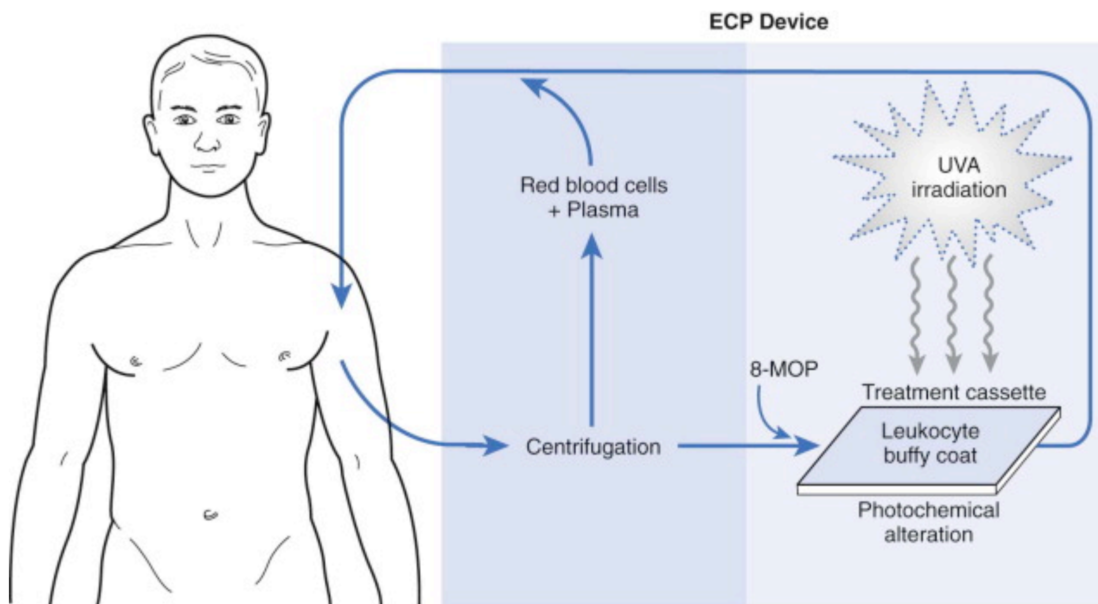


Figure 1. In the ECP procedure, venous access is established, usually with a 16-gauge needle. Whole blood is drawn from the patient and the leukocyte-rich buffy coat is harvested through several discontinuous pheresis cycles whereas the red blood cells and plasma are re-infused back to the patient. Psoralen is added to the isolated leukocytes, which are held in a sterile collection bag. After equilibration, the leukocytes are pumped through the transparent ECP plate and exposed to ultraviolet A (UVA) light at a dosage of between 1-2 J/cm^2. After treatment, these photochemically altered leukocytes are then reinfused into the patient. The entire procedure takes approximately three hours to complete.

Figure obtained from: Choi, Jaehyuk, Heald, Peter W., Girardi, Michael - Comprehensive Dermatologic Drug Therapy, 291-298.e2

Figure 2: The Role of GILZ as Mediator in Immune Signaling Pathways

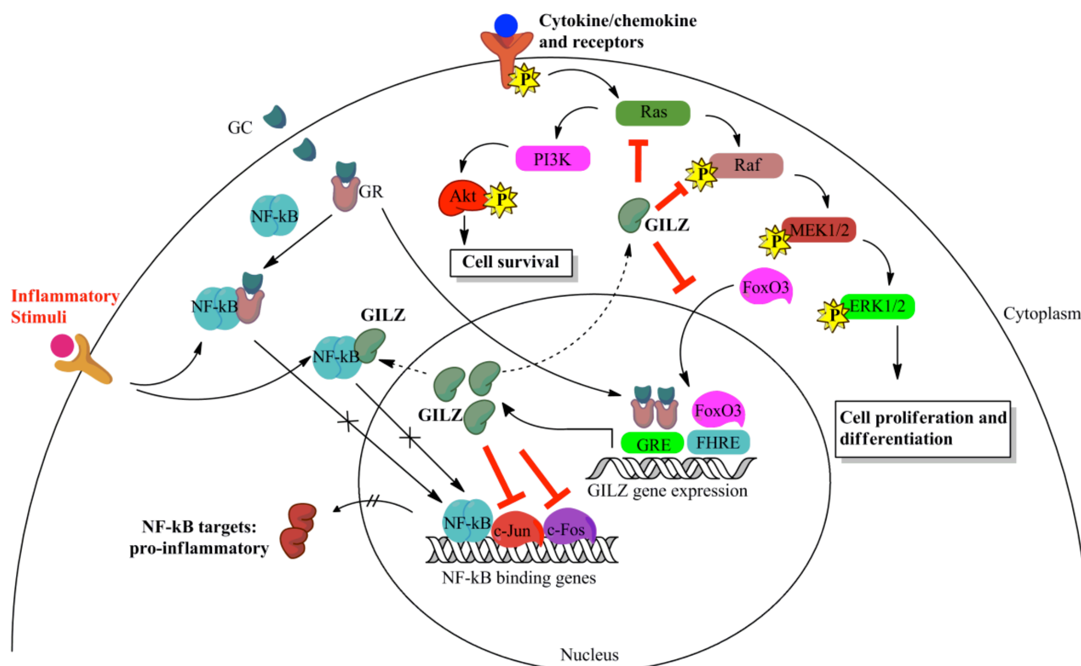


Figure 2. GILZ expression is induced by glucocorticoids (GC) binding to glucocorticoid response elements (GRE). GILZ mediates its anti-inflammatory effects by inhibiting a variety of pathway and signaling factors including Ras, Raf, MEK1/2, ERK1/2, c-JUN, c-FOS, and nuclear translocation of NF-kB.

Figure obtained from: Huapeng Fan and Eric F. Morand (2012). The Role of GILZ in Anti-Inflammatory and Immunosuppressive Actions of Glucocorticoids, Glucocorticoids - New Recognition of Our Familiar Friend, Dr. Xiaoxiao Qian (Ed.), ISBN: 978-953-51-0872-6, InTech, DOI: 10.5772/52027.

Figure 3: Laboratory Model and Procedure of ECP

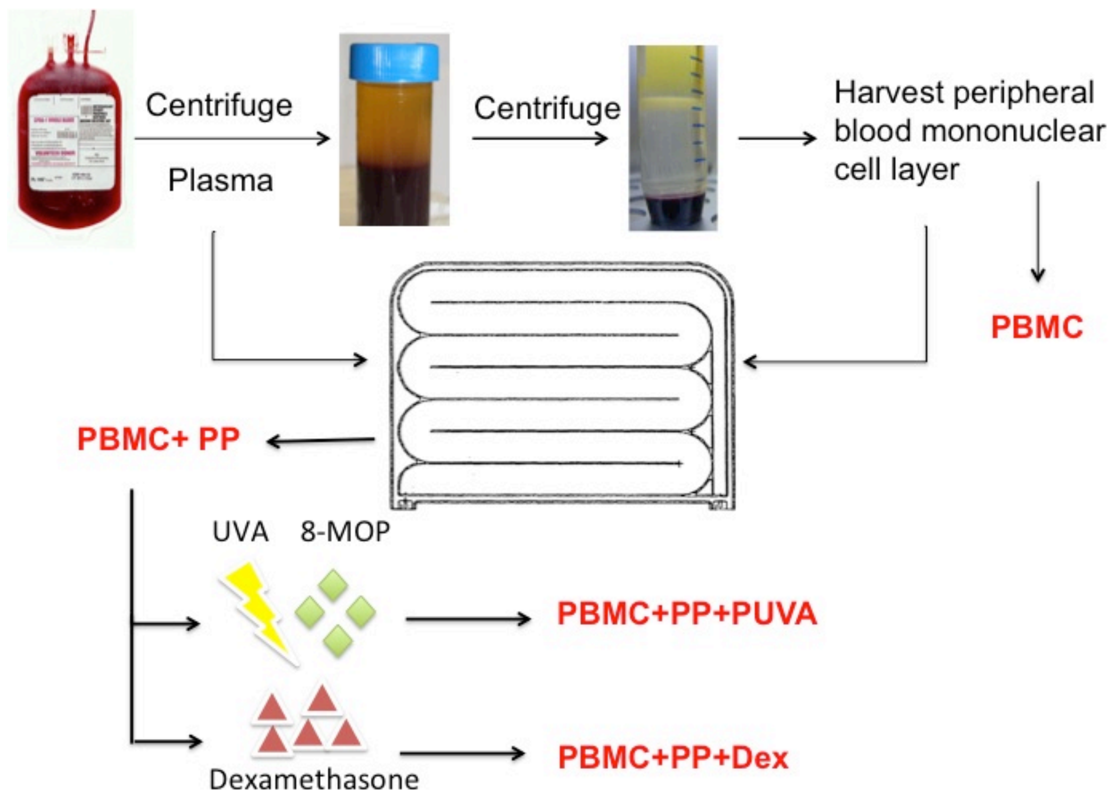


Figure 3. A laboratory model of ECP is established to simulate the important features of the clinical ECP device. After whole blood was obtained from healthy donors, plasma was obtained via a low speed centrifugation and utilized to coat the clinical ECP plate. After reconstituting the volume, the peripheral blood mononuclear cells (PBMCs) are isolated using a Ficoll-Hypaque gradient. Some of the PBMCs are set aside while others are passed through the plate where they interact with serum proteins and platelets to undergo monocyte-to-dendritic cell differentiation and maturation. After PBMCs are plate passed (PBMC+PP or PP), some are treated with dexamethasone (PBMC+PP+Dex or Dex) or 8-MOP and UVA (PBMC+PP+PUVA or ECP) of various dosages. These groups of cells are subsequently collected and analyzed phenotypically via flow cytometry and for RNA expression by TaqMan RT-PCR.

Figure 4: Flow Cytometry Gating Strategy for Antigen Presenting Cells

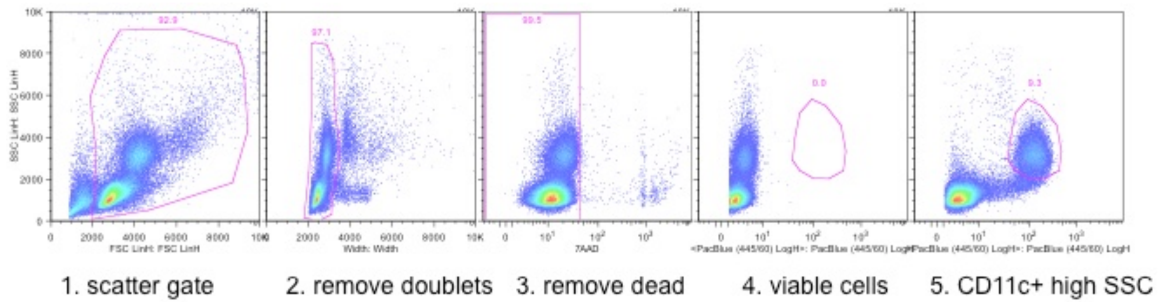
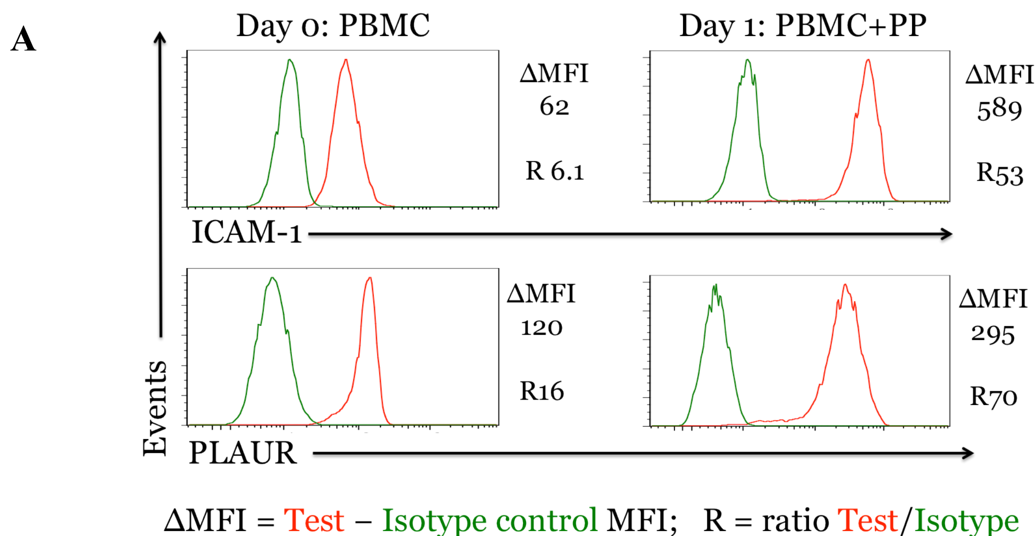


Figure 4. Gating strategy utilized in analyzing the phenotype of the antigen presenting cells are as follows:

1. Gate on lymphocytes and monocytes based on forward and side scatter profiles
2. Exclude doublets based on width
3. Select viable cells using viability dye (7-AAD negative)
4. Gate on CD11c+ cells with high side scatter profile
5. Subsequently analyze with immunophenotyping with fluorescence-conjugated antibodies

Figure 5: Plate Passage Globally Activates Monocytes with Increased PLAUR and ICAM-1 Expression

A



B

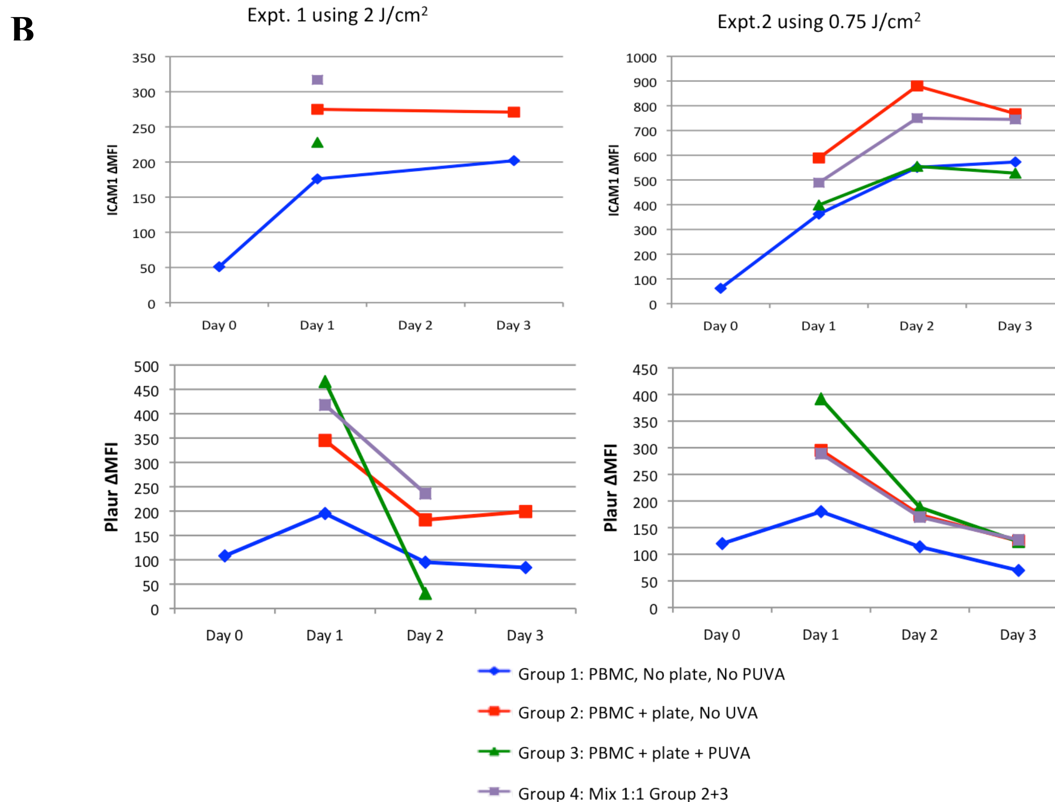


Figure 5. The expression of PLAUR and ICAM-1 on the surface of CD11c^{+} antigen presenting cells increased after plate passage and overnight culture. The increase in expression can be seen as the curve shifts toward the right and correspondingly with higher MFI, ΔMFI, and ratio of test and control MFI (A). The increase in expression of

PLAUR is not stable or consistent as there is decline in expression for PLAUR whereas ICAM1 expression continue to increase after day 1 (B). PUVA inhibited the expression of ICAM1 but increased the expression of PLAUR on day 1.

Figure 6: 8-MOP/UVA Inhibited Lymphocyte Proliferation

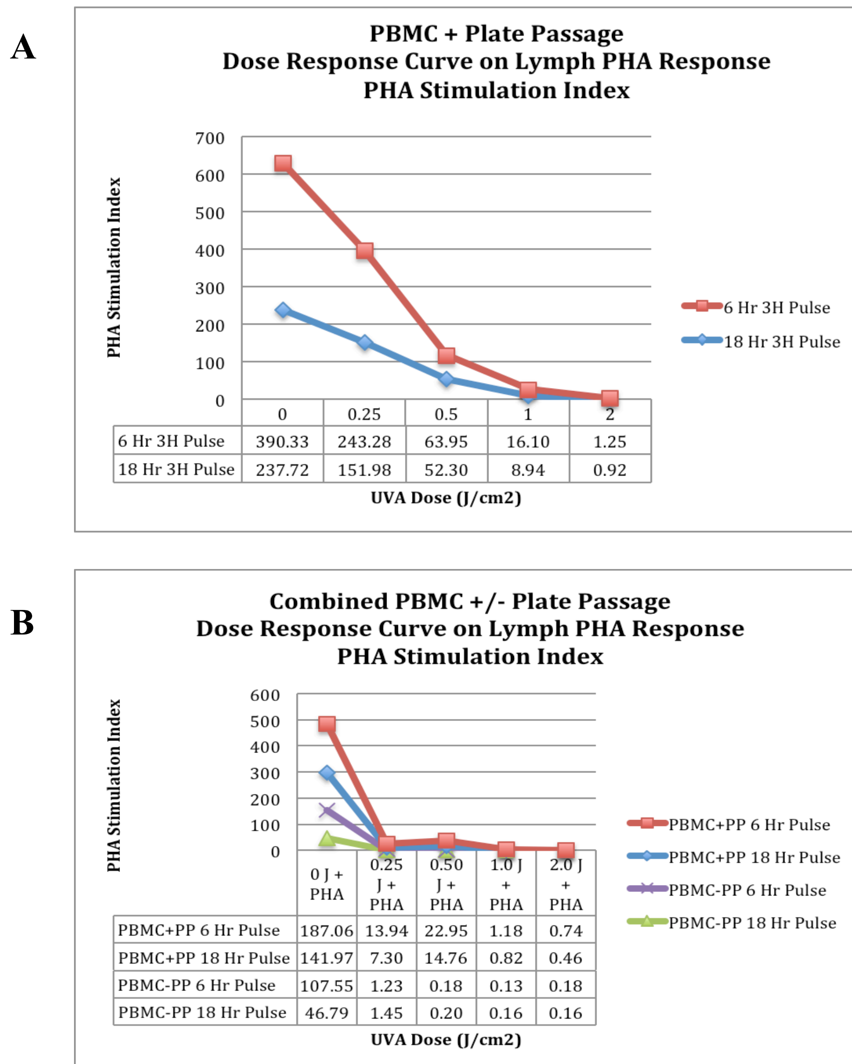


Figure 6. Dose response curve comparison of the effect of varying concentrations of UVA light with 100 ng/mL of 8-MOP on lymphocyte PHA response. In the two experiments conducted (A, B), 8-MOP/UVA was able to inhibit PHA-induced lymphocyte proliferation in a dose dependent manner and it took approximately 1 J/cm^2 to 2 J/cm^2 to completely inhibit lymphocyte proliferation of plate passed cells. There was excellent correlation between 6 hour and 18 hour [^{3}H]-thymidine pulse.

PHA Stimulation Index = (Mean CPM of PHA Stimulated Cells) / (Mean CPM of Unstimulated Cells). A PHA Stimulation Index of 1 is equivalent to complete suppression of proliferative ability of lymphocytes to PHA stimulation.

Figure 7: CD11c$^+$ Antigen Presenting Cells are Resistant to 8-MOP/UVA-Induced Apoptosis

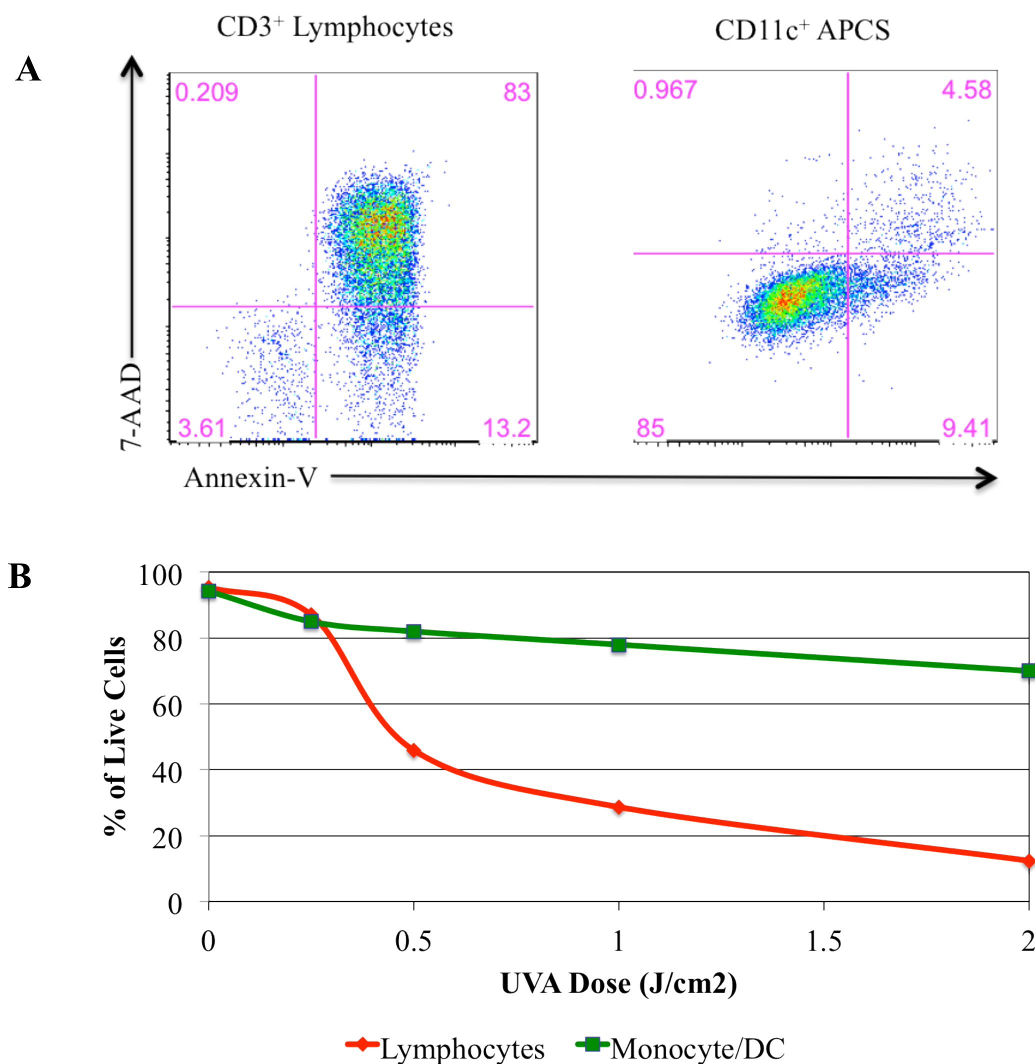


Figure 7. 8-MOP/UVA induce apoptotic cell death of both antigen presenting cells (monocytes and DCs) and T lymphocytes. However, antigen presenting cells are resistant to 8-MOP/UVA-induced cell death compared to lymphocytes at 24 hours post-exposure (A). Early apoptotic cells are defined by being annexin-V$^+$ 7-AAD$^-$ and late apoptotic cells are annexin-V$^+$ 7-AAD$^+$. There is a dose response of 8-MOP/UVA and leukocyte cell death at 24 hours post-exposure for both cell types (B).

Figure 8: Relative GILZ Expression Level of CD11c+ Antigen Presenting Cells

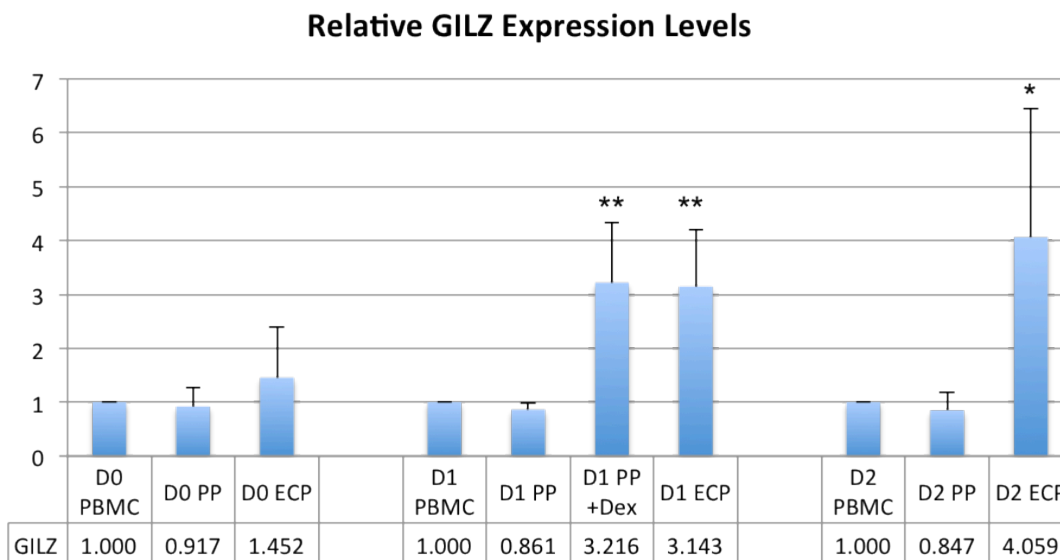


Figure 8. ECP-induced DCs upregulated GILZ when treated with dexamethasone and 8-MOP/UVA. GILZ mRNA expression in $CD11c^+$ ECP-derived DCs as a fold change relative to that particular day's PBMCs. Data represent mean ± standard deviation for at least three independent experiments in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the PBMC group of that day. D0: day 0; the day of experimental manipulation. D1: day 1; one day after experiment and sterile culture. D2: day 2; 2 days after experiment and sterile culture.

Figure 9: 8-MOP/UVA Induces DCs with Immature Phenotype

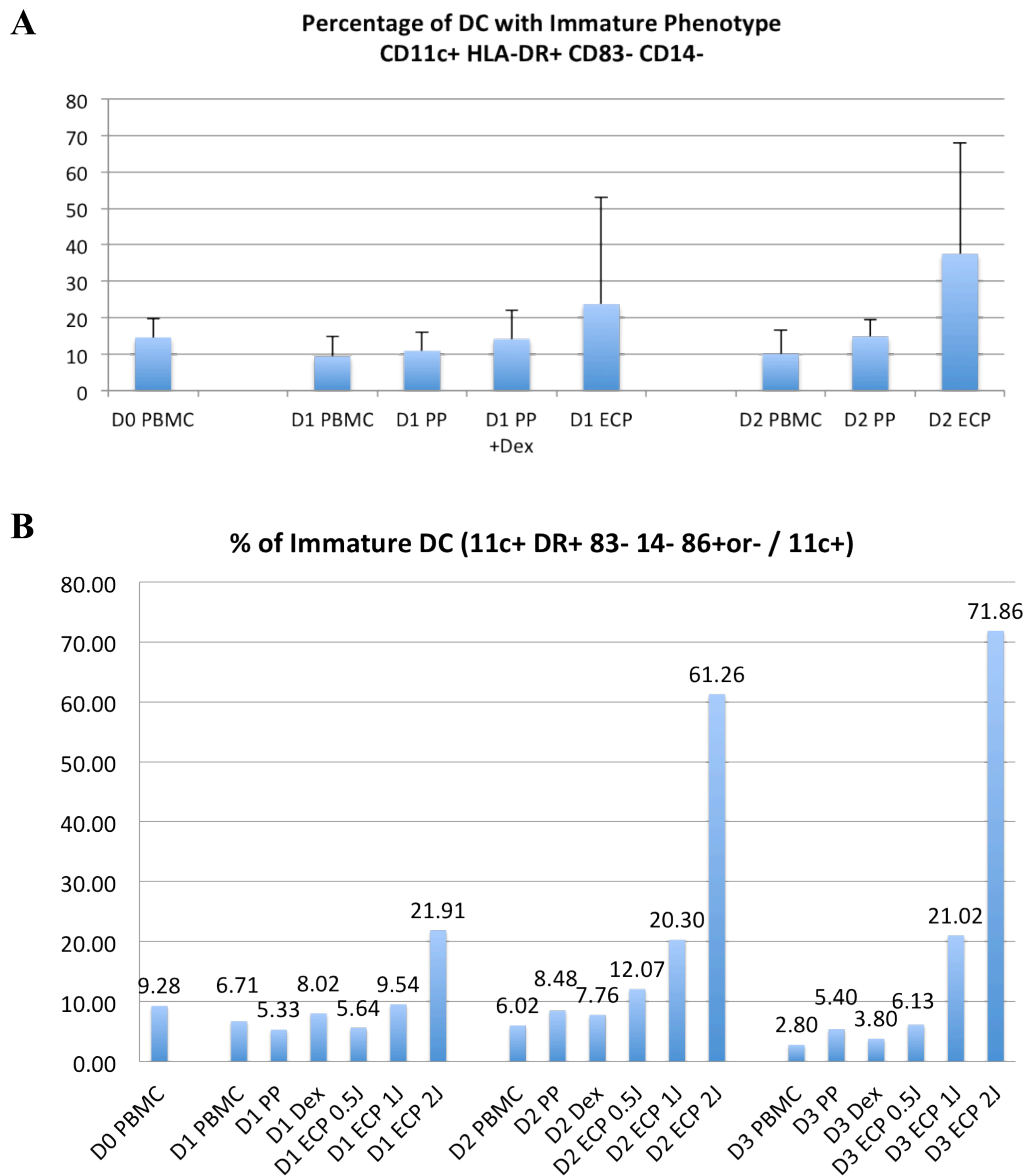


Figure 9. 8-MOP/UVA, but not plate passage alone, induce DCs with an immature phenotype (A) in a dose dependent fashion (B). The induction of immature phenotype is long-last and persistent over several days after 8-MOP/UVA treatment. Immature phenotype is defined as $CD11^{+}$ HLA^{-} DR^{+} $CD14^{-}$.

Figure 10: Effect of Plate Passage and 8-MOP/UVA on DC Markers

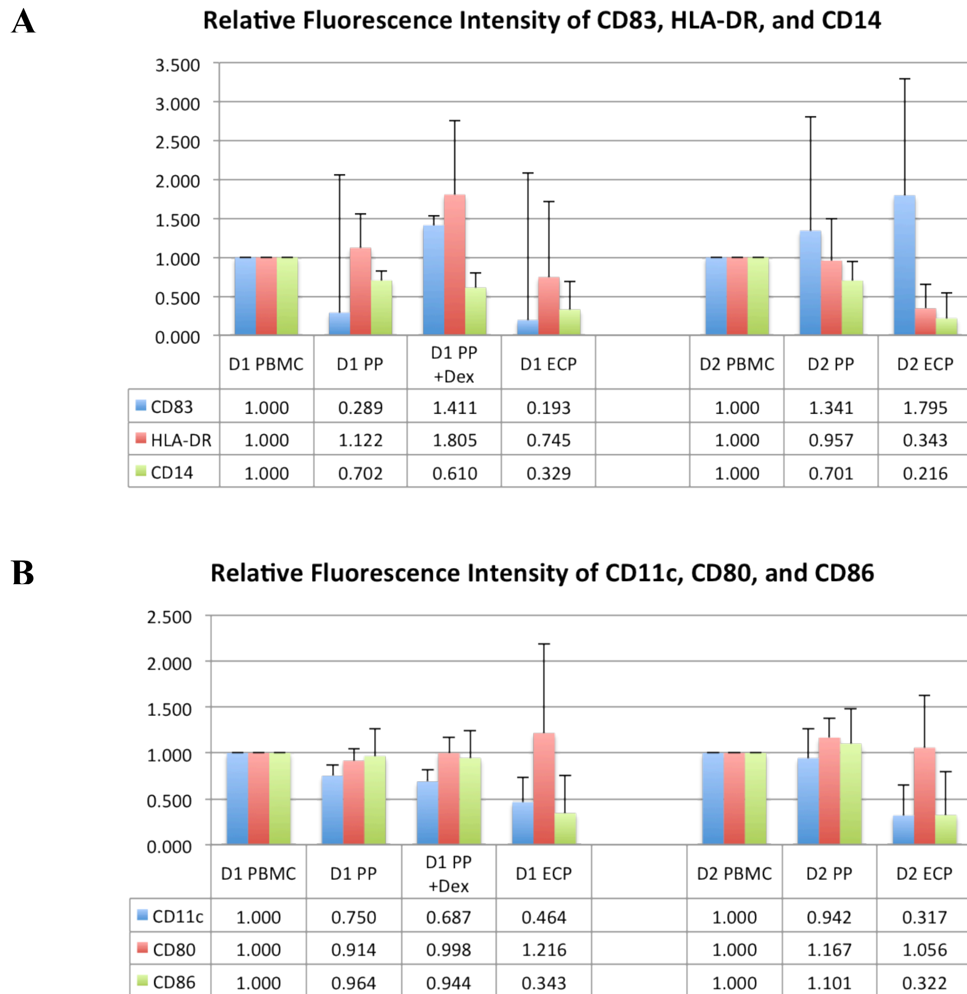


Figure 10. Relative fluorescence intensities for membrane expression of HLA-DR, CD83, and CD14 (A) and CD11c, CD80, and CD86 (B) are presented for the different groups one or two days after treatment and sterile culture. All data represent mean ± standard deviation of at least three independent experiments.

Figure 11: Dose Response Effect of 8-MOP/UVA

A

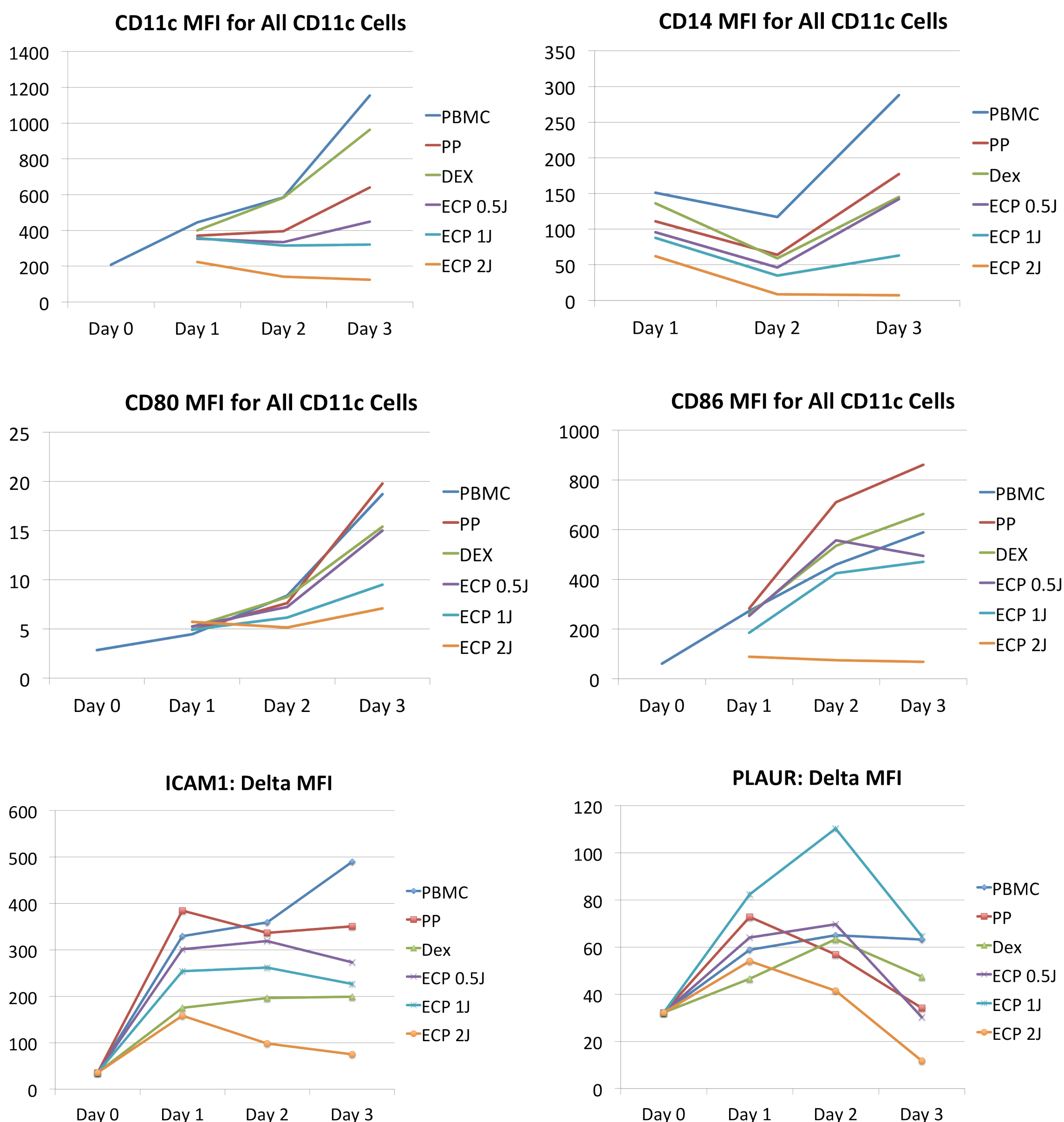


Figure 11A. 8-MOP/UVA exposure modified the expression of several cell surface markers on the CD11c$^+$ antigen presenting cells. In this experiment, 8-MOP/UVA decreased the expression of CD11c, CD14, CD80, and CD86 in a dose dependent fashion with the general trend holding true to day 3. Whereas plate passage increased the expression of ICAM1, 8-MOP/UVA suppressed its expression in a dose depedent fashion. The effect of plate passage and 8-MOP/UVA was variable and inconsistent for PLAUR.

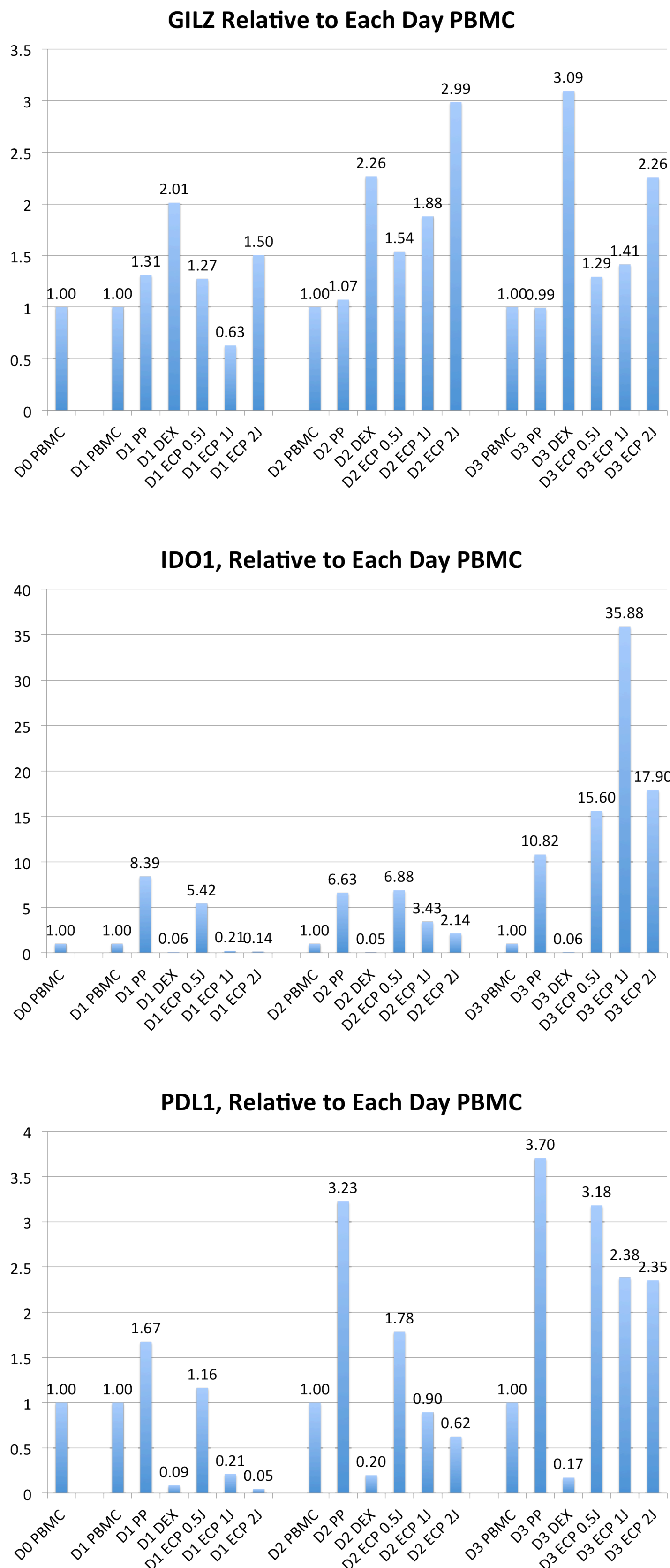


Figure 11B. 8-MOP/UVA increases the RNA expression of GILZ and decreases the RNA expression of IDO1 and PDL1. The effect of 8-MOP/UVA occurs in a dose dependent fashion and mirrors that of dexamethasone. The effect of 8-MOP/UVA is persistent over three days following treatment. Relative expression levels are normalized to the PBMC group of that particular day. RNA for analysis is derived from purified $CD11c^{+}$ antigen presenting cells for all groups. 100 ng/mL of 8-MOP was used in this set of experiment.

TABLES

Table 1: DCs Expressing GILZ Showed Mixed Ability to Stimulate MART-1 Specific CD8$^+$ Primary Response

	% CD8$^+$CD4$^-$MART-1$^+$gp100$^-$ T-cells (mean ± intra-group SD)		
Antigen-Presenting Cells	**Donor A**	**Donor B**	**Donor C**
Freshly Isolated Monocytes	1.8 ± 0.3	3.5 ± 1.3	1.1 ± 0.2
Untreated DCs	2.9 ± 0.4	5.1 ± 2.1	2.6 ± 0.1
8-MOP/UVA -DCs	2.2 ± 0.7	6.3 ± 1.8	3.5 ± 0.6
Untreated DCs + ApoL	2.3 ± 0.1	9.8 ± 2.1	2.5 ± 0.1
8-MOP/UVA-DCs + ApoL	1.8 ± 0.2	9.1 ± 0.2	3.4 ± 0.3
Dex-DCs	0.8 ± 0.1	7.0 ± 0.7	2.5 ± 0.5

Table 1. Cytokine generated DCs expressing GILZ at high levels demonstrated a mixed ability to stimulate autologous MART-1-specific CD8$^+$ T-cells. DCs were re-purified from cultures described in Materials and Methods and used as antigen presenting cells for stimulating T cells. DCs were co-cultured in a 1:10 ratio with autologous lymphocytes in the presence of MART-$1_{16\text{–}40(A27L)}$ peptide, IL-2 (12.5 IU/mL) and IL-7 (5 ng/mL). After 9 days of co-culture, the percentages of MART-1-specific CD8$^+$ T cells were quantified as a percentage of live cells displaying a CD8$^+$CD4$^-$MART-1$^+$gp100$^-$ phenotype. The percentages of live cells displaying a CD8$^+$CD4$^-$MART-1$^+$gp100$^-$ phenotype are listed for three independent experiments with three different HLA-A2$^+$ donors. Each experiment was performed in triplicate, and the intra-group SD is presented.

The expression of GILZ was highest in dexamethasone treated DCs (Dex-DCs) and 8-MOP/UVA-treated DCs fed with apoptotic lymphocytes (ApoL). The expression of GILZ was lowest in untreated DCs and freshly isolated monocytes. The expression of GILZ was for untreated DCs fed apoptotic lymphocytes and 8-MOP/UVA-treated DCs was in between the previously stated two groups.