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Activation of GILZ gene by photoactivated 8-methoxypsoralen: Potential role of immunoregulatory dendritic cells in extracorporeal photochemotherapy

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Abstract

Extracorporeal photochemotherapy (ECP) is a widely used method for either immunization against cutaneous T cell lymphoma or immunosuppression of graft-*versus*-host disease and organ transplant rejection (OTR). Leukapheresed blood is routed through a chamber, in which 8-methoxypsoralen is activated by ultraviolet energy (PUVA), thereby causing DNA crosslinks in processed leukocytes. Return of ECP-processed mononuclear leukocytes to the patient then modulates aberrant T cell immunity. Since interaction with the ECP flow chamber induces monocyte-to-dendritic antigen presenting cell (DC) maturation, we examined the possibility that PUVA may direct the most heavily exposed monocytes to differentiate into tolerogenic DC, while the least exposed DC might remain immunogenic. Expression of the glucocorticoid-induced leucine zipper (GILZ) gene is a distinguishing marker of tolerogenic DC. We report that PUVA directly stimulates GILZ expression. PUVA-exposed DC up-regulated GILZ, down-regulated costimulatory CD80 and CD86, became resistant to Toll-like receptor-induced maturation, increased IL-10 production and decreased IL-12p70 production, all features of immunosuppressive DC. Knockdown of GILZ with siRNA reduced IL-10 and increased IL-12p70 production, demonstrating that GILZ is critical for this profile. PUVA-induction of GILZ expression by DC may help explain how ECP suppresses GVHD and OTR. Conversely, those ECP-processed monocytes minimally exposed to PUVA may mediate ECP's immunogenic effects.

Keywords

Extracorporeal photochemotherapy; Dendritic cells; GILZ; Regulatory T cells; Immunoregulation

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.transci.2013.10.003>.

1. Introduction

Extracorporeal photochemotherapy (ECP) is an immunotherapeutic and immunomodulatory therapy used in a variety of clinical settings, including cutaneous T-cell lymphoma (CTCL), graft-*versus*-host disease, and solid-organ allograft rejection [1]. ECP consists of the passage of leukapheresed blood mononuclear cells through a plastic chamber in which they are exposed to an ultraviolet A (UVA) light-activated DNA-crosslinking drug, 8-methoxypsoralen (8-MOP) [1]. Upon absorption of UVA light, 8-MOP forms covalent DNA interstrand crosslinks, culminating in lymphocyte apoptosis over the course of 48 h [2]. Since inactive 8-MOP is biologically inert, and because the photoactivated form lasts only millionths of a second, the drug is uniquely titratable and its direct impact is limited solely to the UVA-irradiated cells.

ECP's clinically relevant capacity to immunize against CTCL antigens on the one hand, while tolerizing to tissue antigens in the autoreactive setting, indicates that its underlying mechanism(s) is potent, multifactorial, multidirectional and complex. Because dendritic antigen presenting cells (DCs) are pivotally positioned in both antigen-specific T cell immunity and tolerance, a central mechanistic role for them has been suggested [3]. That proposal is strongly supported by the finding that passage through the ECP exposure chamber broadly activates the processed monocytes and causes substantial rapid monocyte-to-DC differentiation as revealed by DC gene activation and expression of the protein products of those genes [4]. Recognition that ECP can augment numbers of regulatory T-cells (Tregs), likely reflects ECP-induction of tolerogenic DC [5].

DCs are versatile antigen-presenting cells (APCs) capable of eliciting robust immunogenic and tolerogenic immune responses [6]. Tolerogenic DCs are phenotypically semi-mature [7], characterized by low co-stimulatory molecule expression, active production of immunosuppressive molecules, and a reduced allostimulatory capacity [8]. Tolerogenic DCs maintain peripheral tolerance by inducing T-cell anergy and deletion, and by generating Tregs [9].

Murine models of ECP demonstrate that an infusion of ECP-treated cells reverses graft-*versus*-host disease [10], prolongs cardiac allograft survival [11], and inhibits contact hypersensitivity [12]. Moreover, apoptotic cells deliver immunosuppressive signals to APCs [13]. After internalizing apoptotic cells, DCs remain phenotypically immature, increase production of IL-10, and become poor stimulators of naïve T-cells [11,14]. The molecular mechanisms mediating these tolerogenic signals remain unclear.

The glucocorticoid-induced leucine zipper (GILZ) is both necessary and sufficient for the generation of dexamethasone-induced tolerogenic DCs [15]. In addition, DCs expressing GILZ generate CD4⁺CD25⁺Foxp3⁺ Tregs that inhibit T-cell responses in an antigen-specific fashion [16]. GILZ codes for an intracellular protein that inhibits a wide variety of signaling pathways, including the pro-inflammatory transcription factor NF-κB [17]. Immunosuppressive stimuli, including glucocorticoids, IL-10 and TGF-β, all up-regulate GILZ, suggesting that GILZ induction may be a common mechanism by which DCs are committed towards a tolerogenic phenotype and functional state [15].

ECP has been shown to mediate antigen-specific immunosuppression and increase the number of circulating CD4⁺CD25⁺Foxp3⁺ Tregs in patients with graft-versus-host disease [18]. Given these observations, we hypothesized that 8-MOP plus UVA light (PUVA) directly induces GILZ expression in DCs, thereby polarizing them towards a tolerogenic phenotype and functional state. We further hypothesized that exposure of DCs to apoptotic lymphocytes (ApoL) indirectly induces GILZ expression. We developed an *in vitro* model of ECP using human monocyte-derived immature DCs (MoDCs) and ApoL to help decipher the direct and indirect immunosuppressive effects of PUVA, and herein report laboratory support for both of these postulates.

2. Materials and methods

2.1. Monocyte enrichment

Whole blood was acquired from healthy HLA-A2⁺ donors under the guidelines of the Yale Human Investigational Review Board, and informed consent was provided according to the Declaration of Helsinki. Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque centrifugation, and monocytes were enriched by: (1) plastic adherence (CD14⁺: 71.6 ± 5.6%); (2) CD14 magnetic bead positive selection (CD14⁺: 88.1 ± 3.5%); or (3) Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) (CD14⁺: 83.8 ± 3.8%).

2.2. Generation of MoDCs

Adherent monocytes were cultured in a monolayer at 5×10^6 cells/mL in RPMI-1640 (Gibco, Carlsbad, CA), supplemented with heat-inactivated 15% human AB 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 36 h to induce monocyte to DC differentiation.

2.3. 8-MOP and UVA light treatment

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/cm²) used during ECP were chosen for this study [1].

2.4. MoDC/lymphocyte co-cultures

Non-adherent cells removed after plastic adherence will now be referred to as lymphocytes (CD3⁺: 66.0 ± 4.5%). Lymphocytes were rendered apoptotic with 8-MOP (100 ng/mL) and UVA light (1 J/cm²), and co-cultured in complete media for 24 h with either PUVA-treated or untreated MoDCs. MoDCs treated for 24 h with dexamethasone (100 nM) (Sigma, Ronkonkoma, NY) served as the positive control. For some experiments, MoDCs and ApoL were separated via a semi-permeable membrane (pore size: 0.8 µm) (Sigma, Ronkonkoma, NY), or incubated with a neutralizing IL-10 monoclonal antibody (eBioscience, San Diego, CA). To ensure that RNA was not isolated in significant quantities from lymphocytes, MoDCs were re-purified using CD11c magnetic bead positive selection (CD11c⁺: 96.4 ± 1.0%). Re-purified MoDCs were then plated in complete media and stimulated with LPS

(100 ng/mL) (Sigma, Ronkonkoma, NY). 24 h after LPS stimulation, MoDCs were harvested for RNA isolation and immunophenotyping, and supernatants were collected for cytokine quantification.

2.5. GILZ knockdown

Silencer select pre-designed and validated GILZ siRNA (Invitrogen, Carlsbad, CA), with off-target prediction algorithms, was used to transiently knockdown GILZ expression. GILZ siRNA and scramble siRNA were used at a final concentration of 50 nM. MoDCs were transfected using the Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad CA). Transfected MoDCs were treated in an identical fashion as described for the MoDC/lymphocyte co-cultures.

2.6. Stimulation of antigen-specific CD8⁺ T-cells

MoDCs from the co-cultures were re-purified and used as APCs for stimulating naïve autologous CD8⁺ T-cells. 0.4×10^6 MoDCs 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 Melan-A/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 Melan-A/MART-1 peptide cross-presentation. The Melan-A/MART-1_{16-40(A27L)} HLA-A2-restricted peptide (10 μ M, GHGHSYTTAEELAGIGILTVILGVL) [32] 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-Melan-A/MART-1_{26-35(A27L)}-PE dextramer, followed by an irrelevant peptide A*0201/KTWGQYWQV-gp100-APC dextramer control (Immudex, Copenhagen, Denmark). Melan-A/MART-1-specific live CD8⁺ T-cells were identified as CD8⁺CD4⁺MART-1⁺gp100⁺ cells.

2.7. Immunophenotype

Monoclonal antibodies included HLA-DR, CD80, CD8, CD83, CD3, CD4, CD86, CD14, CD11c and GILZ (Beckman-Coulter, Brea, CA and eBioscience, San Diego, CA). 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. Dual membrane and intracytoplasmic staining was performed using the IntraPrep fix and permeabilization kit (Beckman-Coulter, Brea, CA). Background staining was established with appropriate isotype and FMO controls. Cells were fixed and immunofluorescence was analyzed using the FACSCalibur L (BD Biosciences, San Jose, CA).

2.8. Quantitative real-time PCR

RNA was isolated from CD11c⁺ MoDCs 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, CD80, and CD86 transcripts,

with HPRT-1 and GAPDH 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.

2.9. Cytokine quantification

Culture supernatants were analyzed in multiplex format using magnetic beads specific for IL-6, IL-8, IL-10, IL-12p70, IFN- γ , TNF- α , RANTES, MCP-1, MIP-1 β (BioRad Laboratories, Hercules, CA), and enzyme-linked immunosorbent assays for IL-10 (R&D Systems, Minneapolis, MN) and IL-12p70 (Enzo Life Science, Farmingdale, NY).

2.10. Statistical analysis

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

3. Results

3.1. GILZ is down-regulated upon monocyte-to-DC differentiation

Freshly isolated monocytes rapidly down-regulated expression of GILZ mRNA (Fig. 1a), and protein (Fig. 1b), as they differentiated into immature MoDCs in the presence of GM-CSF and IL-4. GILZ down-regulation correlated with the acquisition of an immature MoDC phenotype (Fig. 1b), as evidenced by increased expression of cytoplasmic CD83 (immature MoDC marker), and low expression of membrane CD83 (mature DC marker) [19].

3.2. 8-MOP plus UVA light directly up-regulated GILZ and induced a tolerogenic phenotype

We next examined if PUVA had a direct effect on GILZ expression. While 8-MOP or UVA light alone did not significantly induce GILZ expression in MoDCs, the combination of 8-MOP and UVA light (PUVA–DCs) increased GILZ expression 5.5-fold (Fig. 1c). GILZ induction after PUVA treatment exhibited a prolonged time course, peaking 24 h after treatment, whereas dexamethasone maximally up-regulated GILZ 2 h after treatment (Dex-DCs) (Fig. 1d). Furthermore, MoDCs up-regulated GILZ in a PUVA dose-dependent fashion. GILZ expression increased 3.0-fold as the product of 8-MOP and UVA reached 100, and 4.8-fold as the product increased to 200 (Fig. 2a).

Dexamethasone up-regulates GILZ, truncates DC maturation, and induces a tolerogenic phenotype [15]. Similarly, the PUVA dose-dependent induction of GILZ correlated with the acquisition of a tolerogenic cell surface phenotype. As the product of 8-MOP and UVA exceeded 50, the expression of CD83, CD80 and CD86 decreased by 31%, 30% and 54% respectively (Fig. 2c and d). The PUVA dose-dependent reduction in the percentages of HLA-DR⁺CD83⁺ and CD80⁺CD86⁺ MoDCs can be observed on flow cytometry dot plots (Fig. S1a and b).

The ability of PUVA to induce MoDC apoptosis is uncertain, with studies showing both a resistance [20], and sensitivity [21] to apoptosis. The percentage of early apoptotic MoDCs increased at 2 J/cm² and 200 ng/mL; however, as observed on flow cytometry dot plots, the

percentages of early and late apoptotic MoDCs remained low for all other 8-MOP and UVA doses analyzed (Fig. S2a). Treatment with 0.5 J/cm² did not effect GILZ expression, and treatment with 4 J/cm² resulted in high levels of non-specific cell death (data not shown). When quantified, there was no difference in the percentages of early or late apoptotic MoDCs 24 h after treatment with 1 J/cm² and 100 ng/mL, as compared to untreated MoDCs (Fig. S2b). In comparison, 44.3% of lymphocytes displayed early apoptotic markers 24 h after PUVA treatment, and will be generally referred to as ApoL (Fig. S2c).

3.3. MoDCs exposed to ApoL up-regulated GILZ and were resistant to full maturation

MoDC and ApoL co-cultures were established to analyze the direct and indirect effects of PUVA. GILZ was up-regulated in an ApoL dose-dependent fashion (Fig. 3a). GILZ was not induced when ApoL were separated from MoDCs via a semi-permeable membrane (Fig. 3b), and adding a neutralizing IL-10 monoclonal antibody did not reduce GILZ expression (data not shown). Moreover, PUVA-DCs exposed to ApoL expressed GILZ at higher levels than did untreated MoDCs exposed to ApoL (6.7-fold compared to 3.6-fold) (Fig. 3c). GILZ protein was increased at least 1.5-fold above untreated MoDCs in all groups demonstrating GILZ mRNA up-regulation (Fig. 3d). GILZ induction was not related to an significant increase in apoptosis, as there were less than 12% early and late apoptotic MoDCs (ranges 3.8–11.4% and 6.3–11.5%, respectively) in all groups demonstrating GILZ up-regulation.

Immature MoDCs exposed to Toll-like receptor (TLR) ligands rapidly mature and increase membrane expression of CD83, CD80 and CD86 [22]. MoDCs expressing GILZ 2.5-fold higher than untreated MoDCs demonstrated a resistance to full maturation after exposure to the TLR ligand lipopolysaccharide (LPS). Among MoDCs up-regulating GILZ, LPS increased CD80 expression to 50%, and increased CD86 expression to 45% of the levels observed after LPS stimulation in untreated MoDCs (ranges 48–57% and 42–47%, respectively) (Fig. 3e). A similar phenomenon was observed for HLA-DR and CD83 (ranges 23–57% and 47–65%, respectively) (Fig. 3f). The resistance to full maturation observed in MoDCs expressing GILZ can be visualized on flow cytometry dot plots (Fig. S3a and b). CD80 and CD86 expression was also confirmed at the RNA level; MoDCs up-regulating GILZ expressed 6% of the CD80 mRNA of untreated MoDCs, and 50% of the CD86 mRNA (ranges 4.5–7.5% and 12.4–85.1% respectively, $p < 0.05$).

3.4. MoDCs expressing high levels of GILZ displayed a tolerogenic cytokine profile

Tolerogenic DCs are characterized by increased production of IL-10 and reduced production of numerous pro-inflammatory cytokines and chemokines [8]. Dex-DCs up-regulated GILZ 4.3-fold (Fig. 3c), increased production of IL-10, and decreased production of all pro-inflammatory molecules analyzed, except MIP-1 β (Fig. 4a–e). PUVA-DCs up-regulated GILZ 2.8-fold (Fig. 3c), also increased production of IL-10, and decreased production of all pro-inflammatory molecules analyzed, except TNF- α and IFN- γ (Fig. 4a–e). Both PUVA-DCs and untreated MoDCs, when co-cultured with ApoL, expressed GILZ at higher levels than PUVA-DCs cultured alone (Fig. 3c). These groups increased production of IL-10 and decreased production of all pro-inflammatory molecules analyzed (Fig. 4a–e).

3.5. siRNA-mediated knockdown of GILZ reduced the IL-10 to IL-12 ratio

To investigate whether GILZ was responsible for this tolerogenic cytokine profile, GILZ was transiently knocked down with siRNA. Transfection with GILZ siRNA reduced GILZ expression by 65% (range 57–69% of non-transfected MoDCs) (Fig. 5a). MoDCs expressing GILZ 2.5-fold higher than untreated MoDCs produced more IL-10; knockdown of GILZ reduced IL-10 production by 39% (range 34–48%) (Fig. 5b). MoDCs expressing GILZ 2.5-fold higher than untreated MoDCs also produced less IL-12p70; knockdown of GILZ increased IL-12p70 production by 188% (range 149–214%) (Fig. 5c). Treatment with scramble siRNA had no appreciable effect on the production of IL-10 or IL-12p70. The IL-10 to IL-12 ratio increased in all groups demonstrating up-regulation of GILZ, and knockdown of GILZ reduced the IL-10 to IL-12 ratio (Fig. 5b and c).

3.6. MoDCs expressing high levels of GILZ demonstrated a mixed ability to stimulate Melan-A/MART-1-specific CD8⁺ T-cells

To analyze a primary T-cell functional response, we investigated the ability of MoDCs expressing GILZ to cross present the Melan-A/MART-1₁₆₋₄₀(A27L) peptide and stimulate naïve autologous Melan-A/MART-1-specific CD8⁺ T-cells. For one donor, Dex-DCs, PUVA-DCs, and MoDCs exposed to ApoL all demonstrated a reduced ability to stimulate Melan-A/MART-1-specific CD8⁺ T-cells after 9 days of co-culture, as compared to untreated MoDCs (Fig. 6a). Flow cytometry dot plots are presented for donor A, demonstrating reduced percentages of CD8⁺CD4⁺MART-1⁺gp100⁺ T-cells when MoDCs expressing GILZ at high levels were used as stimulator cells, as compared to untreated MoDCs (Fig. 6b). For two other donors, Dex-DCs, PUVA-DCs, and MoDCs exposed to ApoL all demonstrated an equal, or greater ability to stimulate Melan-A/MART-1-specific CD8⁺ T-cells (Fig. 6a).

4. Discussion

One of the most intriguing mysteries about ECP's mechanism is how it can be immunostimulatory in the CTCL setting, while being tolerizing in the context of GVHD and organ transplant rejection. With the recognition that ECP efficiently induces monocyte-to-DC maturation in the absence of the suprapharmacologic concentrations of cytokines commonly used to generate DC *in vitro* [4], it became highly likely that DCs perform a critical role in ECP's immunotherapeutic clinical effects. For two convergent reasons, we have suspected that PUVA exposure of monocytes may be a critical factor in directing a major subset of ECP-processed monocytes into the tolerogenic mode, while relative protection from PUVA may permit simultaneously processed monocytes to mature into immunogenic DC.

First, ECP processed monocytes are not homogeneously exposed to PUVA in the 1 mm film through which they flow in the PUVA exposure chamber. 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.

Second, separate ongoing studies in our laboratory, detailed in an accompanying paper, revealed that ECP's induction of DC involves discrete physiologic interactions between platelets that are tightly adherent to the ECP UVA-exposure chamber and the processed monocytes, which flow through that chamber. In a laboratory ECP model, we found that PUVA may truncate the DC-oriented maturation of those platelet-stimulated monocytes.

The experiments described in this paper were initiated to explore that suggested dichotomy. Specifically, we hypothesized that a PUVA-maturationally truncated, relatively short-lived population of ECP-processed monocytes might be tolerogenic to those antigens displayed on their surface. A longer lived, fully matured DC population, produced in parallel by ECP, might be responsible for the immunogenic effect recognized in treated CTCL subjects. Our findings support that premise.

We have shown that PUVA acts directly on MoDCs, and indirectly through the generation and uptake of ApoL, to induce expression of GILZ. MoDCs, after up-regulating GILZ down-regulated co-stimulatory molecule expression, became resistant to full maturation, displayed a tolerogenic cytokine profile, and demonstrated a variable ability to stimulate antigen-specific CD8⁺ T-cells. GILZ clearly contributed to the conversion of MoDCs to this tolerogenic state, as demonstrated by their reduced IL-10 and increased IL-12 production following siRNA-mediated knockdown of GILZ. PUVA and apoptotic cells may now be included in the list of immunosuppressive stimuli, including glucocorticoids, IL-10, and TGF-, that lead to the induction of GILZ.

The molecular pathways mediating PUVA's direct induction of GILZ are presently unclear, but may be initiated by DNA damage and cellular stress. Interestingly, IL-2 deprivation of T-cells stimulates GILZ expression and protects them from apoptosis [23]. Our induction of GILZ expression most likely reflects a molecular protection of MoDCs from PUVA-induced apoptosis. We cannot definitively exclude the possibility that interactions between viable and apoptotic MoDCs were responsible for GILZ induction [24]. However, GILZ was induced when there was no corresponding increase in the percentage of apoptotic MoDCs, and this study supports prior reports documenting a relative resistance of MoDCs to the apoptotic effects of PUVA [20]. We suggest that local production of tolerogenic DC at sites of tissue damage might be biologically advantageous means of protecting against the development of systemic autoimmunity.

While this paper provides the first evidence that PUVA exposure of human DC induces expression of GILZ message, a phenomenon “necessary and sufficient” to secondarily induce T_{regs} and T_{H17}, it will be important to experimentally link these phenomena in future planned experiments [16]. Now that our laboratory has preliminarily demonstrated that PUVA exposure induces ECP-induced DC to similarly express GILZ message, those determinations will be performed ECP-processed monocyte DC precursors to firmly establish relevance to the clinical ECP procedure.

Inflammatory stimuli induce DC maturation through activation of NF-κB [25]. Many immunosuppressive stimuli, including apoptotic cells, inhibit NF-κB and generate tolerogenic DCs [8]. This present study implicates GILZ as a potential mediator of the

immunosuppressive effects of apoptotic cells. The signaling pathways linking apoptotic cells to the induction of GILZ are unknown, but likely include receptors involved in the recognition of apoptotic bodies. Direct contact between DCs and apoptotic cells inhibits IL-12 and increases IL-10 production [26,27]. Physically separating MoDCs and ApoL with a semi-permeable membrane prevented GILZ up-regulation, suggesting that cell-to-cell contact, rather than a soluble factor, is necessary for GILZ induction. Future studies will address how the recognition and internalization of apoptotic cells contribute to the induction of GILZ.

One intriguing possibility is that TAM receptors may provide the link between apoptotic cells and GILZ induction. TAM receptors recognize phosphatidylserine on the surface of apoptotic cells, inhibit TLR-induced inflammation, and transduce immunosuppressive signals from apoptotic cells [28], a possibility that we are currently exploring. Moreover, DCs co-cultured with apoptotic cells demonstrate TAM-dependent inhibition of NF- κ B [29]. Curiously, GILZ and TAM knockout mice have a similar testicular phenotype, characterized by defects in the phagocytic clearance of apoptotic cells [15,28], an unexplained phenomenon that highlights the necessity of deciphering the precise mechanism by which GILZ expression induces its multiplicity of effects.

A hallmark feature of tolerogenic DCs is a reduced T-cell immunostimulatory capacity and the ability to generate antigen-specific Tregs [8]. In this present study, MoDCs expressing GILZ demonstrated a mixed ability to stimulate autologous Melan-A/MART-1-specific CD8⁺ T-cells. However, Dex-DCs also exhibited a variable response, and the stimulatory capacity of Dex-DCs for all three donors mirrored that of MoDCs treated with PUVA or exposed to ApoL. Combined with high intra-group standard deviation, these observations suggest that stimulation of Melan-A/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 Melan-A/MART-1 HLA-DRB1-restricted epitopes recognized by CD4⁺ T-cells [30]. It is feasible that MoDCs from HLA-A2⁺ donors with particular MHC-class II haplotypes would be unable to induce Melan-A/MART-1-specific CD4⁺ Tregs. These MoDCs 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 [31]. 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 [31]. These observations could help explain why we observed a variable CD8⁺ T-cell stimulatory capacity for all MoDCs expressing GILZ, including Dex-DCs, PUVA-DCs, and MoDCs exposed to ApoL.

Advantages of the Melan-A/MART-1₁₆₋₄₀(A27L) long peptide include antigen-specificity and a functional requirement for internalization, processing, and cross presentation of the peptide [32]. A limitation is that the CD8⁺ T-cell response does not reveal which specific tolerogenic mechanisms are operating. Future studies will be required to determine the production of IFN- γ by Melan-A/MART-1-specific CD8⁺ T-cells, as well as whether

PUVA-DCs expressing GILZ are capable of generating Tregs in a similar fashion as Dex-DCs [16].

This study is an initial step towards a potential molecular explanation for the immunosuppressive signals delivered by PUVA and apoptotic cells, and may help explain how ECP generates antigen-specific immunomodulation *in vivo*. Our recent preliminary findings indicate that we can replicate, and extend, this line of investigation with ECP-induced DCs, supporting the likely relevance of our findings to clinical ECP. Moreover, on the basis of findings reported here, we have developed a new UVA exposure flow system, scalable from mouse to man, that will permit laboratory manipulation of the ECP system and potential skewing of the resulting DC to either immunostimulatory or immunosuppressive populations, preliminary to human testing.

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Abbreviations

ECP	extracorporeal photochemotherapy
8-MOP	8-methoxypsoralen
UVA	ultraviolet A
DCs	dendritic cells
APCs	antigen-presenting cells
Tregs	regulatory T-cells
GILZ	glucocorticoid-induced leucine zipper
PUVA	8-methoxypsoralen plus UVA light
ApoL	apoptotic lymphocytes
MoDCs	monocyte-derived dendritic cells
Dex-DCs	dexamethasone-treated immature MoDCs
PUVA-DCs	8-methoxypsoralen and UVA light-treated immature MoDCs

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Fig. 1.

Expression of GILZ was rapidly down-regulated as monocytes differentiated into immature MoDCs, and up-regulated after dexamethasone or PUVA treatment. Total RNA was isolated from MoDCs. (a) GILZ mRNA expression in CD11c⁺ MoDCs is presented as a fold change relative to freshly isolated monocytes. (b) Median fluorescence intensities of intracellular markers (GILZ, CD83) and cell surface markers (CD11c, CD14, CD83) in CD11c⁺-gated MoDCs after 36 h of culture with GM-CSF and IL-4. (c) GILZ expression in CD11c⁺ MoDCs 24 h after treatment is presented as a fold change relative to untreated MoDCs. (d) GILZ expression over time in CD11c⁺ MoDCs is presented as a fold change relative to untreated MoDCs. All data are expressed as mean $\pm$ standard deviation for a minimum of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the untreated MoDC group. NS, nonsignificant.

Fig. 2.

Immature MoDCs treated with 8-MOP and UVA light up-regulated GILZ in a PUVA dose-dependent fashion and acquired a tolerogenic phenotype. (a) GILZ mRNA expression in CD11c⁺ MoDCs 24 h after PUVA treatment is presented as a function of the 8-MOP concentration (0–200 ng/mL) multiplied by the UVA light dose (1 or 2 J/cm²). (b) Relative fluorescence intensities for membrane expression of HLA-DR and CD83, and (c) CD80 and CD86, are presented as a function of the 8-MOP concentration multiplied by the UVA light dose 24 h after PUVA treatment. All data represent mean $\pm$ standard deviation of at least four independent experiments. * $p < 0.05$, ** $p < 0.01$, compared to the untreated MoDC group.

Fig. 3.

Immature MoDCs exposed to apoptotic lymphocytes up-regulated GILZ and were resistant to full maturation after LPS stimulation. (a) GILZ mRNA expression in CD11c⁺ MoDCs 24 h after co-culture is presented as a fold change relative to untreated MoDCs. (b) GILZ expression in CD11c⁺ MoDCs 24 h after being separated from ApoL via a semi-permeable membrane. (c) GILZ mRNA expression and (d) protein expression in CD11c⁺ MoDCs 24 h after co-culture is presented as a fold change relative to untreated MoDCs that were cultured alone. (e) Relative fluorescence intensities post- to pre-LPS (100 ng/mL) stimulation for CD80 and CD86, and (f) HLA-DR and CD83, were calculated as follows:

$(\text{MFI}_{\text{treated after LPS}} - \text{MFI}_{\text{treated before LPS}}) / (\text{MFI}_{\text{untreated after LPS}} - \text{MFI}_{\text{untreated before LPS}})$. Data represent mean $\pm$ standard deviation for at least 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, compared to the untreated MoDC group.

Fig. 4. MoDCs expressing GILZ at high levels increased production of IL-10, and decreased production of various pro-inflammatory cytokines and chemokines. MoDCs harvested from co-cultures described in Fig. 3 were re-purified and stimulated with LPS (100 ng/mL). Culture supernatants were collected 24 h after LPS stimulation, and cytokines levels were quantified using magnetic bead multiplex immunoassays for (a) IL-10, and the pro-inflammatory cytokines (b) IL-12p70, IFN- γ , and (c) IL-6, TNF- α . The same analyses were performed for the pro-inflammatory chemokines (d) IL-8, and (e) MCP-1, MIP-1 β , RANTES. Preliminary data are presented as mean $\pm$ standard deviation of three independent experiments. * $p < 0.05$ compared to the untreated MoDC group. NS, non-significant.

Fig. 5. siRNA-mediated knockdown of GILZ reduced IL-10 production and increased IL-12 production. MoDCs were transfected with GILZ or scramble siRNA and cultured as described in Fig. 3. **(a)** GILZ mRNA expression in CD11c⁺ MoDCs transfected with GILZ or scramble siRNA is presented as fold change relative to non-transfected, untreated MoDCs that were cultured alone. * 2.5-fold change and $p < 0.05$, compared to identically treated MoDCs not transfected with siRNA. Quantification of **(b)** IL-10 and **(c)** IL-12p70 protein levels in culture supernatants 24 h after LPS stimulation. A calculation of the IL-10 to IL-12 ratio for **(b)** non-transfected groups and **(c)** GILZ siRNA transfected groups is listed above each corresponding group. Data represent mean $\pm$ standard deviation of three independent experiments. * $p < 0.05$, compared to identically treated MoDCs not transfected with siRNA.

Fig. 6.

MoDCs expressing GILZ at high levels demonstrated a mixed ability to stimulate autologous Melan-A/MART-1-specific CD8⁺ T-cells. MoDCs were repurified from cultures described in Fig. 3 and used as APCs for stimulating T-cells. MoDCs were co-cultured in a 1:10 ratio with autologous lymphocytes in the presence of Melan-A/MART-1₁₆₋₄₀(A27L) peptide, IL-2 (12.5 IU/mL) and IL-7 (5 ng/mL). After 9 days of co-culture, the percentages of Melan-A/MART-1-specific CD8⁺ T-cells were quantified as a percentage of live cells displaying a CD8⁺CD4⁺ MART-1⁺gp100⁻ phenotype. **(a)** 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. **(b)** Representative flow cytometry dot plots from donor A are presented, illustrating the percentages of MART-1⁺gp100⁻ dextramer staining for CD8⁺CD4⁺-gated T-cells. SD, standard deviation.