Review

Extracorporeal Photochemotherapy: Mechanistic Insights Driving Recent Advances and Future Directions

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Dendritic cells (DCs) are professional antigen-presenting cells, necessary for the initiation and maintenance of antigen-specific immunity and tolerance. Decades of research have been driven by hopes to harness the immunological capabilities of DCs and achieve physiological partnership with the immune system for therapeutic ends. Potential applications for DC-based immunotherapy include treatments for cancer, autoimmune disorders, and infectious diseases. However, DCs have poor availability in peripheral and lymphoid tissues and have poor survivability in culture, leading to the development of multiple strategies to generate and manipulate large numbers of DCs *ex vivo*. Among these is Extracorporeal Photopheresis (ECP), a widely used cancer immunotherapy. Recent advancements have uncovered that stimulation of monocyte-to-DC maturation via physiologic inflammatory signaling lies at the mechanistic core of ECP. Here, we describe the landscape of DC-based immunotherapy, the historical context of ECP, the current mechanistic understanding of *ex vivo* monocyte-to-DC maturation in ECP, and the implications of this understanding on making scientifically driven improvements to modern ECP protocols and devices.

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play an integral role in the activation and regulation of the adaptive immune response [1,2]. In fact, DCs are necessary initiators and maintainers of antigen-specific T cell immunity and tolerance. Named for their unique branched projections, DCs are found in lymphoid and peripheral tissues exposed to the external environment, such as the skin (where they are known as Langerhans cells), respiratory tract, and gastrointestinal

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tract [3-6]. In these tissues, immature dendritic cells internalize the antigens with which they come into contact. Once they take up relevant antigen, the DCs subsequently mature, losing their phagocytic capacity and instead becoming uniquely potent stimulators of T cells [1]. During this process, the internalized antigen is processed into small peptides, called epitopes, that are able to be presented on the individual patient's distinctive set of MHC class I and class II proteins. These MHCs bind and frame epitopes that conform to the motifs of their peptide-binding grooves. Relevant T cell clones then recognize these MHC-peptide complexes in a patient-specific fashion. Because an individual's physiologic DCs automatically process antigens in precisely the way that their own T cells are able to recognize and respond, without needing the clinician or scientist to know the identity of the antigens or epitopes themselves, the ability to access and manipulate these cells would represent a tremendous clinical advantage.

Due to their central role in modulating adaptive immunity and maintaining tolerance, DCs have been of great interest in biomedical research. However, many barriers exist in attempts to investigate DC physiology. These include the low yield of DCs obtainable from peripheral tissues and poor survivability *in vitro* and *in vivo* of cytokine-derived DC due to spontaneous maturation, loss of responsiveness, and effector function "exhaustion" [7-12]. Additional challenges arise in the study of naturally-occurring human DCs, especially since the only readily accessible populations of DCs are in the blood, where they represent less than 1% of circulating leukocytes [13].

Despite these challenges, the DC's role as a professional APC, that is, to uptake, process, and present antigen and thus prime naive T cells and activate T cell-mediated immune responses, has made DCs a prime target for developing immunotherapeutic methods of modulating cellular immunity and tolerance. Among these methodologies for DC production for use as a cellular immunotherapy is Extracorporeal Photopheresis (ECP), otherwise known as "Extracorporeal Photochemotherapy" or "Extracorporeal Photoimmunotherapy." ECP was initially developed for use in patients with treatment-refractory cutaneous T cell lymphoma (CTCL)/Sezary syndrome, for which it received US FDA approval in 1988 [14]. Since then, it has been utilized for a number of other indications, including control of Graft-versus-host Disease (GvHD) and prevention of solid organ rejection. During ECP, whole blood is collected from the patient via a cubital vein or implanted catheter. Then, it is centrifuged to isolate the peripheral blood mononuclear cells (PBMCs), also called the buffy coat, which are subsequently exposed to ultraviolet-A (UVA) radiation in the presence of 8-methoxypsoralen (8-MOP), a photoactivatable DNA-crosslinking drug. These irradiated leukocytes are then reinfused back into the patient. Though the mechanism of action for this empirically-developed therapy remained a mystery for several decades, the fact that ECP treatment produces antigen-specific immunity and tolerance, processes of which DCs are necessary initiators, strongly suggested that DCs must be central to its mechanism. In fact, recent advances demonstrate that ECP triggers *ex vivo* monocyte-to-DC differentiation in a way that is thought to mirror physiologic processes [15-18]. In this paper, we discuss the current state of DC therapy, how ECP fits into this landscape, and the implications of recent mechanistic understandings of ECP on protocol and device design. We also present preliminary data indicating that this mechanistic understanding can lead to additional experimental modifications to the ECP device/cellular interface, potentially leading to optimization of differentiation and *in vivo* function of these physiologically-derived DC.

THE LANDSCAPE OF DC-BASED ANTI-TUMOR THERAPY

DC-based immunotherapies have generated intense clinical interest because they offer a number of advantages over other anti-neoplastic modalities, such as adoptive cell transfer (ACT) of tumor-infiltrating lymphocytes (TILs), chimeric antigen receptor-expressing T cells (CAR-T), and immune checkpoint inhibitors. While each of these immunotherapies has shown promising efficacy in a limited number of malignancies, they have also been associated with significant adverse side effects. Lymphodepletion before treatment is a standard procedure in ACT therapy – consequently, infection-related side effects are common [19]. CAR-T cell therapy has been associated with cytokine release syndrome and neurotoxicity [20]. Furthermore, immune checkpoint inhibitors have demonstrated marked toxicity in multiple organ systems, leading to dermatologic [21], cardiovascular [22], ophthalmic [23], pulmonary, gastrointestinal, and endocrine [24] adverse events among others. Conversely, DC-based immunotherapies, particularly ECP, have demonstrated a remarkably favorable safety profile. Over the last two decades, clinical trials of DC vaccination for cancer have shown that such treatments are well-tolerated by patients, with no evidence of autoimmunity or toxicity beyond local inflammation at the injection site [25,26]. The favorable safety profile of DC-based therapies is likely because they are exceptionally specific and therefore have little to no off-target effects. This specificity is inherent to DCs being the upstream initiator and qualitative controller of the adaptive immune response. For this reason, DC-based therapies, both alone and in tandem with other immunotherapeutic modalities, are the continued subject of significant investigative effort.

The earliest methods of DC culture arose from work on granulocyte-macrophage colony-stimulating factor (GM-CSF), which was initially identified to stimulate the differentiation of mouse hematopoietic stem cells (HSCs) into granulocytes and macrophages [27] and later discovered to increase the survivability and longevity of tissue-derived DCs in both mice and humans [8]. Other factors were often added to HSC DC cultures, including tumor necrosis factor a (TNFa), stem cell factor (SCF/c-kit ligand), and FMS-like tyrosine kinase 3 ligand (Flt3L), in order to achieve greater cell numbers and a more "dendritic" phenotype and function. Human umbilical cord blood was already being used as a rich source of allogenic HSCs in stem cell transplantation, making it preferable for potential clinical application of HSC-derived DC cultures [28,29].

However, HSCs for DC culture were still relatively scarce, so work continued to identify a method that would enable controlled production of DCs from a readily available source in sufficient numbers for clinical and research use. This culminated in the development of a method of stimulating easily-accessible human blood monocytes to differentiate into immature DCs following a 5-7 day incubation with GM-CSF and interleukin-4 (IL-4) and subsequent maturation upon exposure to inflammatory stimuli for another 1-3 days [30,31]. Just as in the HSC DC cultures, GM-CSF stimulated differentiation and maintained viability of DCs, while the addition of IL-4 was speculated to maintain DCs in an immature state. The GM-CSF/IL-4 DC culture method became the foundation both for research into human DC biology and for cell production in the majority of clinical trials of DC immunotherapy, particularly in developing cancer vaccination protocols. Further modifications of the GM-CSF/IL-4 DC culture have led to improvements in culture efficiency in terms of time and labor (*e.g.* "FastDC") and selectivity for immunogenic versus tolerogenic phenotypes. These modifications have recently been introduced into immunotherapy trials [32-34].

Despite advancements in the ability to generate large numbers of monocyte-derived DCs and to polarize them towards immunizing or tolerizing phenotypes, the relationship of these cells to endogenous DC populations is still unclear. Though phenotypically similar, they have been noted to be functionally distinct, especially in terms of their ability to induce T cell effector responses *in vitro* and *in vivo* [35,36]. Clinical trials of immunotherapies using these cytokine-derived DCs have demonstrated disappointing clinical outcomes [37-39], leading even those who developed *ex vivo* cytokine-based DC culture methods to call into question their biological integrity [40].

Beginning in the mid-late 1990s, the excitement generated by the clinical promise of cytokine-derived DC-based anti-cancer vaccination led to hundreds of clinical trials attempting to use these DCs to target nearly every tumor for which antigens were available in the form of a defined tumor-associated antigen, tumor lysates, or whole tumor cells. Of those, four tumor types were most commonly tested: malignant melanoma (>1250 patients), prostate cancer (>750 patients), malignant glioma (>500 patients), and renal cell cancer (>250 patients). Only in this limited number of malignancies did DC vaccines reach Phase III clinical trials, with generally disappointing results indicating objective response rates in the range of 5-15% [41,42].

Even with these limitations in mind, there remained substantial commercial interest in bringing DC-based immunotherapy to market. This culminated in the 2010 approval of the DC-based therapy PROVENGE (Sipuleucel-T) by the US FDA. PROVENGE is a treatment in which blood mononuclear cells are harvested from prostate cancer patients and incubated with recombinant prostatic acid phosphatase (PAP) tumor-associated antigen combined with GM-CSF as a GM-CSF/PAP fusion protein [43,44]. PROVENGE was used in the treatment of 30,000 patients with hormone-refractory prostate cancer. However, large-scale adoption of the immunotherapy was hampered by several issues, including relatively high costs ($100k USD), uncertainty regarding insurance reimbursements, limited survival benefit (~4 months), and questions regarding the clinical trial design that showed this observed benefit [45]. Other more recent commercial cytokine-derived DC-based vaccines include DCVax-L from Northwest Therapeutics, a vaccine loaded with autologous tumor lysate following surgery in patients with newly diagnosed glioblastoma [46], as well as APCEDEN® (APAC Biotech), a monocyte-derived DC-based autologous vaccine approved outside of the US for prostate, ovarian, colorectal, and non-small cell lung carcinomas [47]. WT-1 and PRAME are antigen-loaded "FastDC" vaccines for treatment of acute myeloid leukemia for which Medigene Inc. has recently presented data from its ongoing Phase I/II trial [48].

Many factors are currently hypothesized to contribute to the disappointing clinical efficacy in these first-wave DC immunotherapies, including loss of sensitivity to *in vivo* cytokine signals, restricted migration to lymphoid tissues, and reduced ability to mount effector responses [10,12,49]. These factors suggest that the method of production in cytokine-derived DCs may play a central role in their reduced effectiveness *in vivo*. These limitations have led to renewed interest in improving DC-based immunotherapeutic performance based on cell production which more closely mimics physiologic DC production *in vivo*. The recent clinical success of other immunotherapies such as checkpoint inhibitors and CAR-T cells have also spurred a revival of DC-based immunotherapy, both as vaccine monotherapies and as combination therapy

with other immunotherapeutic, chemotherapeutic, or surgical modalities. This indicates that an urgent need exists for improvements in DC production methodologies to keep up with the changing and increasingly synergistic immunotherapy landscape.

This recent wave of next-generation DC-based immunotherapy employs a number of novel strategies, including new cellular and antigenic components. These include rare but naturally-occurring DC populations, such as conventional DCs (cDCs) which can be isolated from patient blood without cytokine exposure; monocyte-derived DCs generated by more potent culture methods, combined with new antigenic sources including vaccines loaded with "neoantigens" specific to alterations present in the patients' unique tumor proteins or genetic material. New routes of administration are also being explored, including intranodal vaccination and *in situ* DC targeting [50].

Therapies based on naturally-occurring DCs, which are populations readily accessible directly from blood and tissue, represent the most pragmatic recent candidates for therapeutic use. These include cDC1 (CD141+) populations, which appear to be the most adept at cross-presentation and therefore at priming CD8+ T cells [51]; cDC2 (CD172a+) cells, which are the predominant DC subset present in the blood and have been shown to be potent CD4+ T cell activators and therefore proficient at inducing Th1, Th2, and Th17 responses [52]; as well as plasmacytoid DC (pDC), characterized by high type I IFN secretion, and epidermis-derived Langerhans cells. These DC populations are particularly amenable for therapeutic application, as they can be rapidly isolated from patient blood or tissue, activated, loaded with target antigen, and immediately reinjected back into the patient [53,54], giving such therapies significant advantages over monocyte-derived DC-based protocols dependent on 7-14 days of *ex vivo* culture.

Regimens utilizing naturally-occurring DC subsets have already demonstrated promising *in vivo* antitumor activity, with clinical studies of purified CD1c+ DCs administered intranodally following loading with HLA-A2.1-restricted tumor peptides in patients with stage IV melanoma (NCT01690377) showing improved progression-free survival [55]. Multiple CD141+ DC vaccine trials also demonstrated promise in the European "Professional Cross-priming for Ovarian and Prostate Cancer" (PROCROP) initiative (www.procrop.eu). Vaccination using cDC2 cells has also been shown to be feasible, safe, and effective in prolonging progression-free survival [50]. A recent trial of a pDC vaccine in patients with melanoma also demonstrated technical feasibility, safety, and resulted in evidence of enhanced T cell immunity [56]. Combination vaccines of both cDCs and pDCs are also being evaluated for whether this combination in-

creases efficacy against melanoma (NCT02574377) and prostate cancer (NCT02692976). Additionally, vaccines utilizing LCs derived from CD34+ hematopoietic stem cells are currently in Phase I clinical trials in melanoma patients (NCT01456104) and in multiple myeloma patients (NCT01995708).

In addition to the isolation of rare natural DC populations, updated and more potent methods for the controlled generation of DCs from myeloid or monocytic precursors are also being investigated in clinical trials, with the goal of modulating DC differentiation and function using different cytokines and growth factors. These alternative factors include M-CSF, FLT3L, TGF-β, type I IFNs, TNF, IL-15, and TLR/PRR agonists such as nucleic acids (CpG), Imiquimod, LPS, monophosphoryl lipid A, and BCG, which have been shown to generate DCs with more potent stimulatory capabilities *in vivo* [57-59].

Additional clinical effectiveness has also been associated with the use of more relevant antigen sources. DCs loaded with "personalized" neoantigens are especially promising, owing to the observation that therapeutic responsiveness in any immunotherapy is strongly associated with mutational load for that specific tumor type [60]. There are currently more than two dozen Phase I/II trials ongoing targeting neoantigens with a wide variety of vaccine platforms [61,62], resulting from landmark proof-of-principle trials of neoepitope vaccines in melanoma [63,64] and glioblastoma [65]. However, current neoepitope identification strategies have real shortcomings in terms of cost and availability, and do not address the reality that different malignancies vary greatly in mutational load, that the current lack of MHC II epitope targeting may severely limit the ability to mount CD8+ T cell responses, and that tumor heterogeneity will allow unstable subclones to avoid neoepitope targeting.

In this context, the critical importance of the type of cell death in rendering autologous tumor cells useful as potentially complex phagocytic substrates resistant to the aforementioned tumor escape mechanisms has become apparent, bringing the field of immunogenic cell death (ICD) to the forefront of DC-based immunotherapy. ICD is defined as a form of regulated cell death that is sufficient to activate an adaptive immune response in immunocompetent hosts. While DC recognition of and responses to apoptosis and necrosis are relatively well understood [66,67], recent evidence suggests that ICD, triggered by a specific subset of antineoplastic therapies and pharmacological agents, including 8-MOP in ECP, is particularly effective in generating DC antigen-loading substrates from autologous tumor cells [68-70]. Induction of ICD in tumor cells leads to the release of specific immunological danger signals, including damage-associated molecular patterns (DAMPs), which can enhance DC response to antigen and stimulate or restore *in vivo* immune target-

ing of tumors [68,71]. While the majority of the ICD inducers are known to induce immunogenicity through the specific spatiotemporal emission of these DAMPs, certain ICD-triggering therapies may also enhance the antigenicity of the dying cell. As such, loading DCs with patient-derived whole tumor or tumor lysates prepared by ICD triggers has the additional advantages of exposing DCs to a complete antigenic profile including all potential epitopes presentable by MHC I and II, defined and undefined, without the expense of extensive neoantigen identification strategies. This allows the DCs to sort and process these antigens themselves, with the concurrent stimulation of anti-tumor T cell responses [72]. A recent review of DC-based vaccines used in combination with chemotherapeutic agents highlights the importance of ICD induced by relevant agents in heightening anti-tumor immunity [73,74].

The current landscape of DC-based immunotherapy reveals both significant challenges and substantial opportunity for the development of improved DC-based immunotherapies. Given the limitations in the *in vivo* function of cytokine-derived DCs and the rarity of native DCs, an alternative favorable approach to generating therapeutic DCs may be to elucidate the physiologic mechanisms of DC generation from monocytes *in vivo* and then co-opt these pathways for experimental and therapeutic applications. Recent evidence suggests sites of immune challenge are also sites of rapid and direct conversion of abundant monocytic precursors in the circulation to DCs both *in vivo* and *ex vivo*. *In vivo*, exposure to pathogens or pathogen components triggers the rapid production of inflammatory DCs (infDCs) from blood monocyte precursors; *ex vivo*, it is observed in the large-scale conversion of blood monocytes to functional DC associated with the FDA-approved cellular immunotherapy extracorporeal photopheresis (ECP) [17,75]. The clinical promise of ECP as an effective, rapid, and cytokine-free method of generating therapeutic DCs and therefore as a platform on which future DC-based immunotherapies can be built upon is an exciting new chapter in the cellular armamentarium potentially targeting cancer, transplant rejection, and autoimmunity.

ECP AS A DC-BASED CELLULAR IMMUNOTHERAPY

Recent elucidation of the central mechanism underlying the efficacy of ECP has revealed that the key elements guiding its clinical responses are identical to those instrumental in adaptive immunity and tolerance. Awareness of that congruency has helped bilaterally, since investigation of ECP's scientific foundation is informed by an understanding of the physiologic immune system, while acquired insights about ECP's science has revealed several important previously unrecognized basic truths about the immune system as well [76].

We now know that DCs, the master-switch of antigen-specific T cell-mediated immunity and tolerance, are physiologically and abundantly induced by ECP [72]. Once these new DCs are formed, ECP then loads them with the relevant antigens. Clinical application of ECP was originally empirically driven by two distinctive features highly attractive to clinicians: exquisite specificity for the malignant cells and an exceptionally favorable safety profile. It was these two remarkable advantages that led to ECP's becoming the first FDA-approved immunotherapy for any cancer (CTCL) in 1988 and its adoption by a large number of university medical centers throughout the world [14]. Shortly after its clinical application to immunotherapy of leukemic CTCL, leading transplantation groups reported similar efficacy and safety in the management of heart transplant recipients whose rejection episodes were resistant to conventional immunosuppression. It was this bidirectionality, immunizing against tumor antigens in a serious cancer and tolerizing against histocompatibility antigens in allotransplants, that initially suggested that ECP was somehow clinically partnering with the equally bidirectional normal physiologic immune system.

Today, ECP is regularly administered, at a majority of university medical centers in the USA and Europe, for CTCL, organ transplant rejection, and post-allo-stem cell transplant graft-versus-host disease [77]. More than three million ECP treatments have been administered to more than 70,000 patients, extensively confirming and extending the original promising results. More than one thousand peer-reviewed publications document the intense worldwide search for ECP's elusive mechanism, motivated by the expectation that its identification would reveal a previously unknown set of scientific principles and potentially set the stage for treatment of a broad spectrum of immunogenic cancers, transplant reactions, and autoimmunity. Those goals may now finally be in reach, and this paper describes our attempts to significantly contribute to that international group effort.

ECP's name reflects the structure of the device by which it is administered. While the patient reclines comfortably for two hours on a blood donor-type chair, anti-coagulated blood is circulated from an antecubital vein of one arm into single-purpose apparatus which processes and then returns it in entirety. Alternatively, in some adult and many pediatric patients the placement of a central venous catheter is required in order to reproducibly obtain venous access, particularly during the "induction" phase of therapy in transplant patients undergoing twice weekly treatments for a minimum of 5 weeks. In either case, first the buffy coat, containing mainly mononuclear cells, is sequestered via centrifugation. Next, those leuko-

cytes are passed through a plate composed of two parallel transparent plastic sheets, as a film 1 mm thick. While in transit through that plate, the cells are exposed to a carefully titrated concentration of 8-methoxypsoralen which is activated by UVA radiation directed at the plate, transiently (millionths of a second) activating the drug from its resting inert state to one which covalently crosslinks pyrimidine bases of the exposed leukocyte DNA. This exquisitely controllable reaction produces slow cell death in the processed lymphocytes. Then, from this completely closed extracorporeal blood processing system, all of the treated blood is returned to the patient, through the same antecubitally-placed catheter. This entire process is comfortably accomplished in an outpatient setting, in less than half a day. A typical active ECP unit, such as ours at the Yale-New Haven Hospital, can treat up to 12 patients each day.

The collective ECP clinical experience framed the scientific questions. Since ECP, in the best responders, led to immunologic elimination of the malignant clone in CTCL patients without suppressing normal immune protection against opportunistic infectious agents, it was entirely selective for the patient's malignant T cells. The only way that could occur, based on established T cell biologic principles, is for DCs to initiate such reactions. So, question number one was, how does ECP induce processed monocytes to become healthy DCs? Since it was not known how monocytes normally become DCs, if this first question could be answered, then we might also learn how monocytes are normally triggered to transform into DCs, identifying an important fundamental principle in its own right.

Since ECP also selectively suppressed autoreactive T cells in transplant reactions, that also must be initiated by DCs. So, question number two was, how does the treatment induce tolerizing DCs when needed? Since antigen-specific T cell responses require DC processing of the target antigen, followed by the presentation of that antigen to selectively responsive T cells, principal question number three was, what are the sources of those DC-processed antigens? From extensive research, superimposed on the evolution of T cell biology itself over the three decades since ECP first serendipitously occurred, the answers to these questions are becoming clear.

We now know that ECP plate-passed platelets signal the simultaneously processed monocytes to enter the DC maturational pathway [15]. Fibrinogen, abundantly present in the plasma, first coats the plastic surface of the ECP plate, in a manner similar to how it adheres to collagen in wounds. Next, plate passed resting platelets, through receptors specific for the gamma chain of the plate-adherent fibrinogen, avidly stick to the fibrinogen, thereby instantaneously activating the platelets to display preformed P-selectin. When microscopically examined,

these plate-adherent platelets give an appearance reminiscent of a cobblestone road (Figure 2a). The monocytes now sail by, literally "jumping" from platelet to platelet, with the platelet-displayed P-selectin signaling monocyte displayed PSGL-1 (P-selectin glycoprotein ligand), signaling monocytes to proceed into the DC maturational pathway. In this way, by mimicking the way that sites of inflammation normally likely stimulate monocytes to become DCs precisely where they are needed, ECP conveniently reproduces the physiologic steps, thereby placing large numbers of new DCs in the physician's hands.

The relevant patient-specific cancer antigens are provided to the new DCs by 8-MOP injured cancer cells, which the DCs internalize and digest, and extract and display antigenic peptides to responsive T cells [78]. Because the plate-passaged monocytes/incipient DCs get variably exposed to the ultraviolet-activated 8-MOP's deleterious effects, some of the monocyte-derived DCs are not seriously damaged and retain immunizing capabilities. That normal DCs functioning permits them to immunize the CTCL patient against the relevant tumor antigens. Other plate-passaged DCs, also containing internalized and processed relevant antigens, are significantly damaged by the 8-MOP, and these compromised DC become selectively tolerogenic to the antigens they contain.

All of these steps are now well characterized, although there is still much to learn. The basic distillate, however, is that ECP did not "invent" anything new. Instead, it fortuitously borrows normal physiologic principles to place the clinician in charge of the pivot point, provided by the new antigen-loaded DCs, so that antigen-specific immunity and tolerance now become therapeutic options.

In this paper, we focus on the required platelet contribution to the cascade of physiologic induction of monocyte-to-DC maturation. We examine preliminary dose-response curves controlling that inductive process, striving to optimize the formation of genuinely functional cross-presenting DCs. The ultimate goal of this project is to establish the preferred methodology to optimize and maximize the production of functional physiological DC by ECP, with an eye on intelligently redesigning the ECP device interface in ways which will maximize *in vivo* T cell responses in both immunity and tolerance applications.

## "PHYSIOLOGIC" DC PRODUCTION THROUGH PLATELET-MONOCYTE INTERACTIONS

Recent work suggests that the mechanistic core of ECP mirrors *in vivo* physiologic processes by which blood monocytes are rapidly converted to inflammatory DCs (infDCs) in the absence of cytokines during and
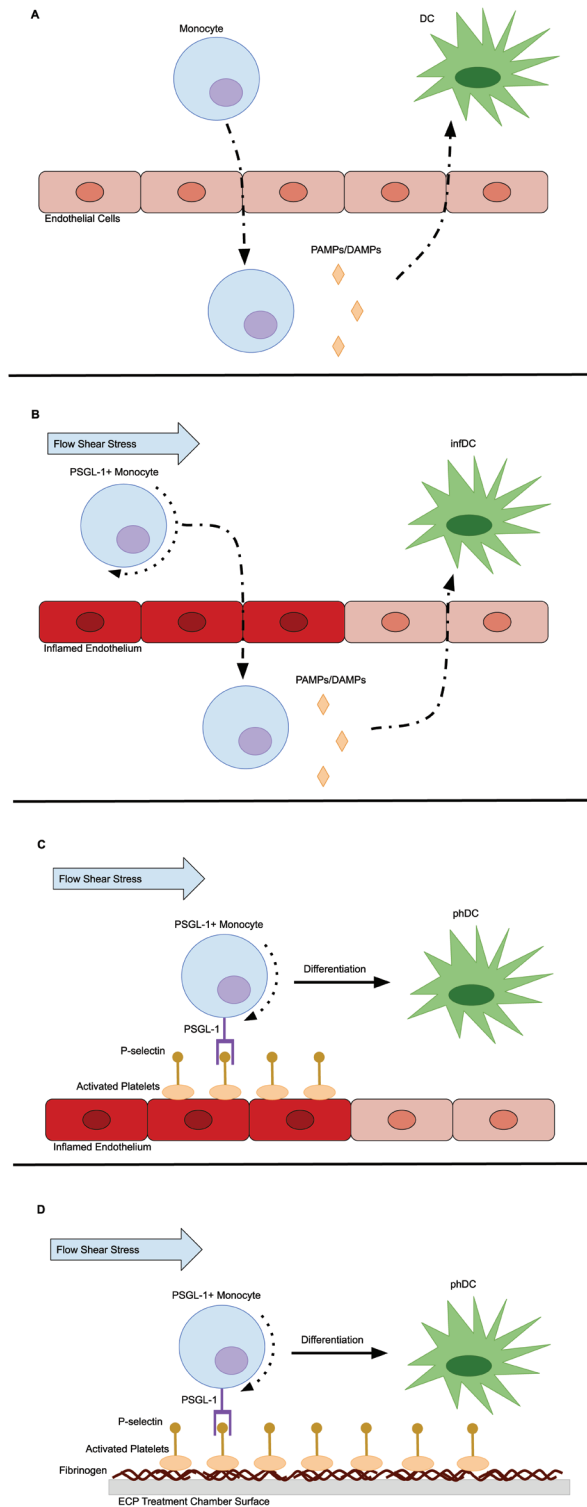
Figure 1. *Ex-vivo* **platelet-driven monocyte-to-DC differentiation mirrors current understandings of *in-vivo* physiological DC maturation**. **A-C.** Progression of models of physiological monocyte-to-DC maturation. **A.** Monocyte transepithelial migration followed by PAMP/DAMP stimulation and subsequent emergence as DC. **B.** Monocyte transepithelial migration triggered by interaction with endothelial luminal surface markers and resulting flow shear-mediated rolling, followed by stimulation and re-emergence as DC. **C.** *In vivo* platelet-mediated monocyte-to-DC differentiation via PSGL-1/P-selectin interaction. **D.** *Ex vivo* platelet-mediated monocyte-to-DC differentiation in the ECP treatment chamber via PSGL-1/P-selectin interaction.

following tissue transmigration (Figure 1). In an *in vitro* model of endothelial translocation, monocytes spontaneously migrated across cultured endothelial cell barriers, received stimulation from microbial pathogen-associated molecular patterns (PAMPS) in the subendothelial space, and reemerged by 24 hours with the characteristic phenotypic and functional features of DCs, including the capacity to potently stimulate allogeneic T cells *in vivo* [79]. This phenomenon was later confirmed in *in vivo* murine models of infDC production [80]. These infDCs act as "professional APCs" in ways that mirror cDC function and are able to strongly stimulate Th1 and Th2 immune responses, confirming that physiologically-derived DCs arising from migratory monocyte populations, especially those expressing Ly6C in mice or CD14^{high} in humans, are important components of the normal immune system's response against pathogens [81].

The inflammatory DCs resulting from rapid differentiation of monocytes could be considered a specialized inflammatory branch from monocytes' better-known role as circulatory precursors of tissue macrophages. Monocytes are major myeloid precursors that represent 10-15% of mononuclear leukocytes found in human blood. They can be routinely signaled to transmigrate across endothelial barriers by luminal surface markers, such as adhesion molecules E- and P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), as well as tethered chemokines expressed by activated endothelial cells in response to inflammation or tissue damage [82-84]. Using their complementary ligands to these signals, monocytes interact with the activated endothelium and adhere despite the high shear stress caused by blood flow near the vessel wall. PSGL-1, one of these monocyte ligands, is expressed at a significantly higher level by inflammatory Ly6C^{hi} monocytes than by resident Ly6C^{low} monocytes, suggesting that PSGL-1 and Ly6C^{hi} monocytes are strong candidates for key interactions leading to infDC production versus macrophage differentiation [85]. While most of the necessary and sufficient signaling events that enable this type of
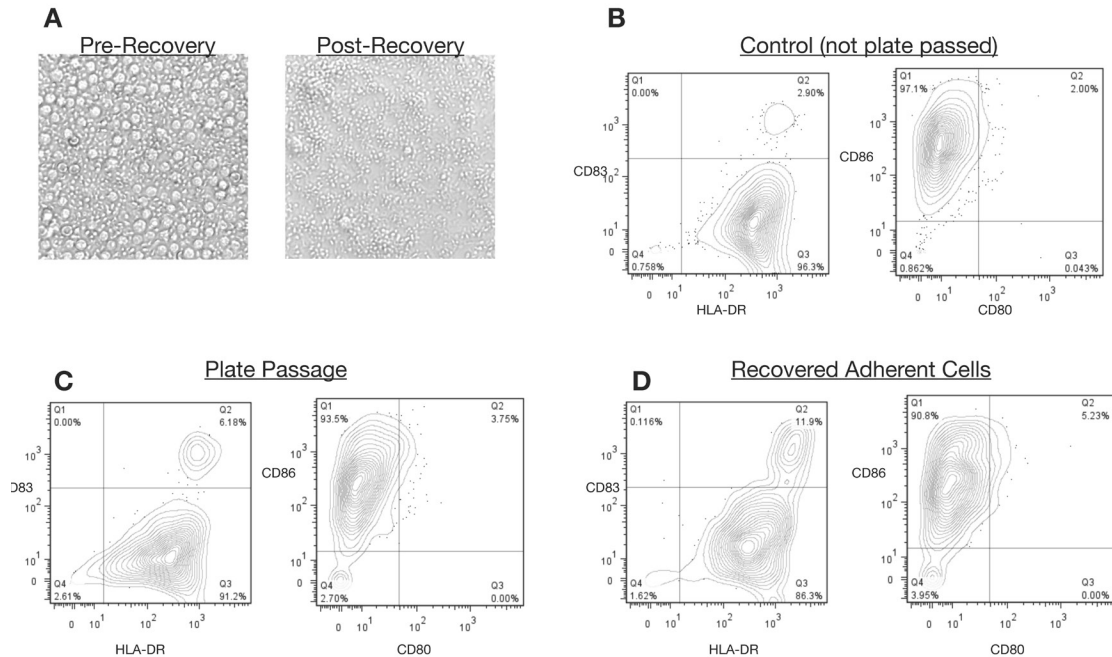
Figure 2. **Dendritic cells are activated and preferentially adherent after plate-passage**. Panel A (left) shows a significant number of large granular cells adherent to the plate after plate passage using light microscopy. Panel A (right) shows that most of these cells can be recovered by increasing the fluid shear stress at the end of traditional plate passage. Panel B shows the control group with low levels of surface HLA-DR, CD83, CD80, and CD86 markers. Panel C shows significant activation of these same surface markers after plate-passage. Panel D shows that the recovered adherent cells have the highest levels of activation of these classical dendritic cell phenotypic markers. All of these experiments represent human cells that were collected, plate passed (except for control group), incubated overnight, and then phenotyped the next day. Cells were gated on mono-dendritic cells based first on forward scatter and side scatter properties, next based on CD11c positivity, and then the above markers (HLA-DR and CD83, CD80 and CD86) were plotted on the respective axes. Results shown were from a single donor but representative of three experiments from multiple donors.

monocyte-to-DC differentiation are still not completely understood, clear evidence points to the possibility that the resulting physiologically-derived DCs may act as APCs with similar potency as cDCs.

There has also been increased awareness of the role of activated platelets in the signaling cascade by which monocytes are triggered to differentiate into infDCs during transendothelial migration. Activated platelets have been shown to act as "pathfinders" for bloodborne monocytes *in vivo*, where they mark sites of extravasation across endothelial layers [86,87]. This suggests that, in addition to their well-known role in hemostasis, platelets likely serve a previously unrecognized role in blood monocyte recruitment, homing, activation, and differentiation into DCs during diapedesis to inflamed tissues. More than two decades ago, it was noted that activated platelets have the capacity to form an integrin synapse with circulating monocytes, triggering the release of monocyte-derived cytokines including MCP-1, TNFa [88], IL-8 [89], MMP-9 [90], and CXCR-5 [91]. Additionally, artificial surfaces coated with P-selectin caused

monocytes allowed to interact with these surfaces under static or flow conditions to produce the aforementioned monokines as well as IL-1b, IL-6, IL-8, IL-12, and MIP-1b [88,92]. Interactions between platelet P-selectin and its corresponding monocyte ligand, PSGL-1, have also been demonstrated to induce monocyte NF-kB and COX-2 signaling [93]. Furthermore, platelets have been shown to preferentially bind with monocytes over other PBMCs [94]. Such experiments identify P-selectin and PSGL-1 as key components of a critical tethering and signal transduction junction linking platelets and monocytes.

If the rapid generation of infDCs at sites of potential pathogen infiltration or injury represents a type of immunological "emergency" response, then platelets are uniquely well situated to serve a central role in this reaction, not only in terms of their location and number but also in regard to their physiology. Limited experimental evidence regarding the true complexity and function of platelets' repertoire of signaling molecules is presently available, though a recent proteomic study determined that 827 proteins are present within platelet granules
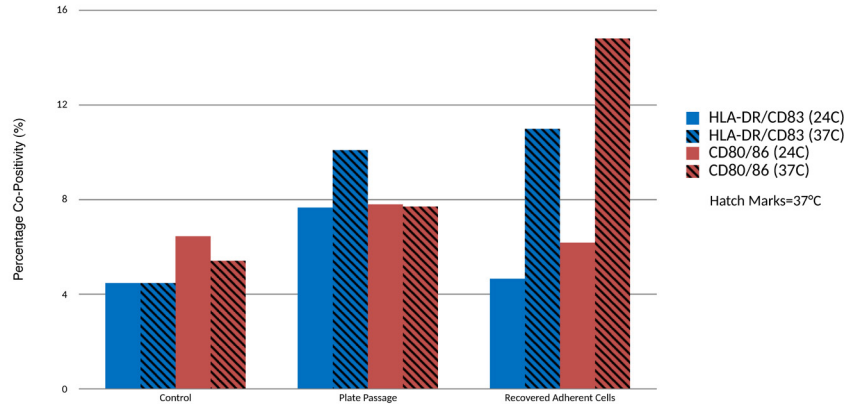
Figure 3a. **Physiologic temperature during plate passage enhances platelet-mediated dendritic cell activation**. Extracorporeal photochemotherapy is currently performed at the clinical bedside without temperature regulation. In this experiment, cells were passed through a platelet pre-coated mini chamber at approximate room temperature (23C) or controlled physiologic temperature (37C), incubated overnight, and phenotyped the next day. The chart shows some activation of classical dendritic cell markers HLA-DR, CD83, CD80, and CD86 at room temperature. At physiologic temperature, there is increased activation of the same dendritic cell markers, particularly in the recovered adherent cells. For example, in the adherent recovered group, HLA-DR and CD83 co-positivity was 4.64% at 23C and 11.0% at 37C. In this same group, CD80 and CD86 co-positivity was 6.17% at 23C and 14.8% at 37C. The adherent recovered cells were collected by increasing fluid shear stress at the end of traditional plate passage. Cells were gated on mono-dendritic cells based first on forward scatter and side scatter properties, next based on CD11c positivity, and then the above markers (HLA-DR and CD83, CD80 and CD86) were plotted on the respective axes. Results shown were from a single donor but representative of 3 experiments from multiple donors which showed similar trends.
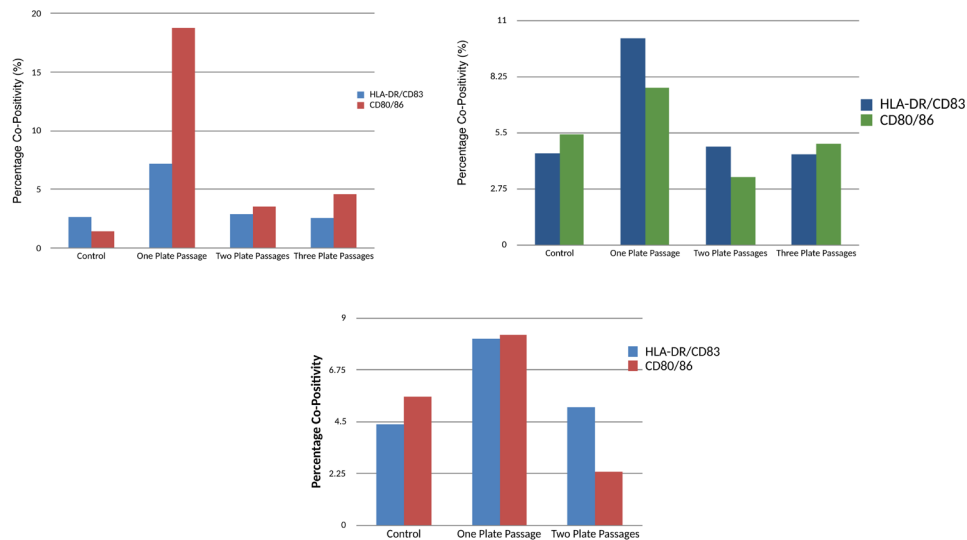
Figure 3b. **Repetitive plate passage does not increase monocyte-to-DC conversion**. PBMC were passaged through the Glycotech flow chamber either one time, as previously indicated, or a total of two or three times under identical flow conditions. In three experiments (from three different human donors), samples of the PBMC removed after each passage indicated that maximum DC differentiation is observed after the first plate passage. This could indicate that flow monocytes are maximally activated rapidly by interactions with multiple platelets during a single passage run, and could also confirm that with longer flow exposure a greater percentage of activated monocytes are firmly adherent to the "platelet lawn" and are lost from the cells exiting the plate chamber. Passage conditions were identical for all three donors except donor number three (bottom) where flow system failure prohibited the final cell passage cycle.

[95]. Platelets have also been confirmed to bind leukocyte Mac-1 and LFA-1 via GPIbα and monocyte CD40 via CD40L, leading some to suggest reclassifying platelets as immune cells [96].

It has been determined that just as platelets play a key role in monocyte-to-DC conversion *in vivo*, they also lie at the heart of the *ex vivo* monocyte-to-DC differentiation observed in ECP (Figure 1). During ECP treatment, plasma fibrinogen coats the walls of the treatment chamber, creating a substrate for platelet adhesion. Platelets encountering this substrate are activated by binding of platelet α2bβ3 and α5β1 integrins to fibrinogen RGD domains. Subsequently, blood monocytes roll, tether, and interact with these bound and activated platelets as they flow through the treatment chamber, causing them to take on phenotypic and functional characteristics of DCs, including cross-presentation of antigen to T cells [16,18]. This phenomenon occurs in a P-selectin/PSGL-1 dependent manner much like the differentiation of monocytes into infDCs during transepithelial migration [18].

OPTIMIZING PHYSIOLOGIC DENDRITIC CELL PRODUCTION BY MODIFYING THE PLATELET-MONOCYTE INTERFACE

Due to ECP's long-mysterious mechanism, advancements in treatment protocol and device design have thus far been nearly exclusively focused on technical metrics and efficiency, such as improving selection of mononuclear cells, reducing red cell contamination, improving the dosing precision of 8-MOP and UVA irradiation, and reducing the treatment's impact on time and labor. However, the developing understanding of how key variables impact the underlying mechanism of ECP presents the opportunity to make hypothesis-driven choices in treatment design, with the potential for substantially improved clinical outcomes. Substantial work is currently being done by our group to characterize the ability to tune ECP's immunological power through these mechanisms.

Platelets, as the apparent central driver of monocyte-to-DC differentiation in ECP, represent perhaps the most obvious variable to investigate. Preliminary experiments to establish the kinetics of the platelet-dependent production of DCs from monocytes were performed using a human flow model in which healthy donor PBMCs were pulsed through a GlycoTech flow chamber that had been coated with autologous donor platelets to form the "platelet lawn" observed in the clinical ECP device. At certain time points (0, 45, 90, and 180 minutes) (Figure 2), samples of PBMC exiting the flow chamber were taken. Additionally, a "shear stress/washout" sample was taken by increasing the flow rate in the chamber to dislodge monocytes that had become adherent to the bound platelets. Following overnight incubation, flow cytometry was used to analyze sample PBMCs for phenotypic markers of monocyte-to-DC differentiation, notably upregulation of HLA-DR, CD80, CD83, and CD86, and downregulation of CD14. Results of this analysis suggest that the strongest platelet-monocyte signaling interactions likely occur by 45 minutes, as subsequent time points show a reduction in DC conversion markers close to levels seen in PBMCs that were never exposed to the flow chamber. Furthermore, monocytes that had become adherent to the platelets coating the flow chamber and therefore required a washout step to be removed from the apparatus demonstrated high levels of DC conversion markers. This indicates that those monocytes that interact most strongly with platelets represent a potential source by which the yield of functional DCs from ECP could be increased, therefore improving the number and potency of the relevant DC populations. Using additional shear stress to remove adherent monocytes from the ECP plate is an active area of investigation and a step which could be easily incorporated into the next generation of ECP devices.

A similar experimental design has also been used to investigate the effect of temperature and plate passage duration on monocyte-to-DC differentiation in ECP-mimicking flow devices. PBMCs were pulsed through the flow chamber and collected as described above, but two flow chambers were run in parallel: one at 37°C and the other at 23°C. Samples collected from both plates were analyzed by flow cytometry for identical markers of DC differentiation (Figure 3a). Monocytes that had been exposed to the flow chamber at physiological temperature (37°C) showed substantially higher levels of DC maturation markers during timepoints of peak activation (45 min and washout) than did those at room temperature (23°C). Next, PBMCs were passaged across the flow chamber plate either a single or multiple times to determine if multiple passages would alter the DC differentiation capacity of device flow. As shown in Figure 3b, maximum monocyte activation is observed following the first passage, indicating platelet-mediated effects occur sufficiently and rapidly upon initial flow over the "platelet lawn". This may also indicate that with increased exposure a larger percentage of platelet-exposed monocytes firmly adhere to the platelet base and are thus lost to the flow-through samples removed at later time points.

These results indicate that temperature and passage duration may be important factors in maximizing the production of DCs in ECP and that redesigned ECP devices could allow cell/device interactions to proceed at 37°C and for fixed, optimized durations (not controlled by hematocrit alone as in current ECP), two more easily-incorporated alterations to currently-utilized ECP protocols.

In the time since these initial experiments were performed in the Glycotech chamber, our group has also developed a more accurate miniaturized ECP device

A

B
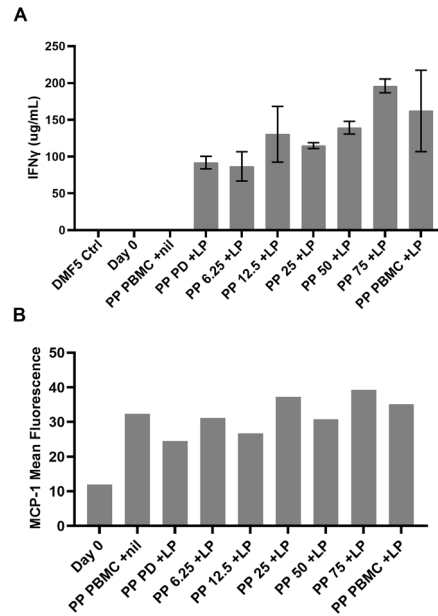
Figure 4. **Dose-dependence of TI-triggered monocyte-to-DC differentiation to platelet density**. **A.** Mean concentration of IFNγ in supernatants of DMF5 T cells co-incubated for three days with equal numbers of plate-passed (PP) or untreated (NP) healthy human donor PBMC loaded with MART-1 long peptide (LP) or no antigen (nil) during overnight incubation. "PD" refers to PBMCs depleted of platelets by anti-CD41 depletion through LD MACS columns (Miltenyi Biotec). By mixing platelet-depleted PBMCs with unaltered PBMCs in different ratios, conditions with a range of platelet densities were created (6.25, 12.5, 25, 50, 75) with 6.25%, 12.5%, 50%, and 75% of unaltered platelet concentration respectively. "Day 0" refers to PBMCs not incubated overnight. "DMF5 Ctrl" refers to a control condition in which DMF5 cells were cultured for 3 days in the absence of PBMCs. IFNγ concentration of supernatants was quantified by ELISA. Data are cumulative over three independent experiments; error bars represent standard deviation. **B.** Flow cytometric analysis of mean fluorescence intensity for intracellularly stained MCP-1 in CD11c+ cells within human PBMCs. PBMCs were treated as described above, but samples for flow cytometric analysis were isolated prior to co-incubation with DMF5 cells.

applicable to both animal and human modeling [17]. With this device, known as the "Transimmune" (TI) chamber or plate, we were able to study the effects of platelet density on the induction of the platelet-monocyte signaling cascade, focusing in particular on the ability of ECP plate-passed monocytes to become functional APCs capable of effective antigen cross-presentation to CD8+ T cells. Using a previously described protocol to assay for antigen-specific stimulation of CD8+ T cells [72], healthy donor PBMCs were passed through TI chambers pre-coated with varying densities of autologous platelets ranging from full platelet counts to full platelet depletion, resulting in differing monocyte-interacting surfaces. Following exposure to a MART-1 long peptide antigen and co-culture with MART-1-specific DMF5 CD8+ T cells, supernatants were collected and analyzed by ELISA for interferon-γ (IFNγ) production as a measure of DC cross-presentation capacity (Figure 4a). IFNγ is produced by DMF5 T cells when effectively stimulated by dendritic APCs that are able to process and cross-present the relevant MART-1 antigen in the context of the relevant

Class I HLA and costimulation; thus, greater concentrations of IFNγ indicate more efficient monocyte-to-DC conversion and increased functional capacity. With this criteria in mind, a trend whereby a small baseline level of monocyte activation is observed in the platelet-depleted conditions, perhaps due to residual platelets present even after selective depletion, while increasing platelet concentrations are associated with increased DC maturation and cross-presentation capacity. This indicates platelet density could significantly affect the functional capacity of ECP-derived immunogenic or tolerigenic DC, and next-generation devices could potentially be "tuned" by platelet density to precisely control DC maturation state and function.

Additionally, flow cytometry was also used to quantify the level of intracellular Monocyte Chemoattractant Protein 1 (MCP-1), a proinflammatory cytokine secreted by stimulated monocytes, in this case produced by plate-passed monocytes following platelet interaction (Figure 4b). A similar trend to that seen in the DMF5 INFγ cross-presentation was noted in the intracellular expres-

sion of MCP-1 by plate-passed monocytes, indicating that the differences in cross-presentation observed were most likely to be due to differential degrees of monocyte-to-DC maturation as a result of exposure to different platelet densities, driven by the platelet P-selectin/monocyte PSGL1 interaction. Taken together, these data indicate that a variety of factors, including temperature, platelet density, and duration of platelet/monocyte interactions can significantly affect the generation and functional capacity of ECP-generated physiological DC.

The potential translation of this *in vitro* flow data into downstream alterations in the ECP device parameters could be undertaken in small clinical trials where a current Therakos clinical device could be altered to include software changes which allowed manual control of PBMC flow rates and duration, as well as device-side temperature control. Each of these parameter changes are feasible with minor alterations to the current system without significant alteration of the CTCL treatment cycle. The effect of these changes could initially be characterized from treated patient samples by *in vitro* flow cytometry and multiplex cytokine analysis as described here. Simultaneously, *in vivo* monitoring of metrics associated with improving clinical course, including lower CD4/CD8 ratio, a higher percentage of monocytes and activated/memory CD8+ T cells, and lower numbers of circulating abnormal T cells and Treg at baseline could be evaluated during each monthly treatment cycle. Initial trials could target ECP "non-responders" who could act as their own historical controls and who represent an ideal population to determine whether device optimization could increase ECP clinical response rates as well as individual response levels.

CONCLUSIONS AND FUTURE DIRECTIONS

As advancements in the mechanistic understanding of ECP continues to unlock its potential application to a wide range of diseases, including solid tumors, autoimmune disorders, and post-stem cell allograft and organ transplants, it is crucial to develop a thorough understanding of the many complex factors that impact its immunological effects and subsequently its effectiveness as a clinical therapy. The critically important work of characterizing the effect these variables have in tuning the immunotherapeutic impact of ECP is currently in progress, proudly spearheaded by our group. Armed with this knowledge, the evolution of ECP in the near term, whether it be in polarizing towards immunization or tolerization to target specific diseases, or in improving the yield, efficacy, and efficiency of DC in clinical ECP devices, will be driven not by empirical measures as in past decades but by increased scientific understanding and careful optimization.

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