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Mechanistic insights into extracorporeal photochemotherapy: Efficient induction of monocyte-to-dendritic cell maturation

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Abstract

Extracorporeal photochemotherapy (ECP) is a widely used immunotherapy for cutaneous T cell lymphoma, as well as immunomodulation of graft-versus-host disease (GVHD) and transplanted organ rejection. ECP's mechanism encompasses large-scale physiologic platelet induction of dendritic cells (DCs). The normal bidirectional immunologic talents of DCs likely contribute heavily to ECP's capacity to immunize against tumor antigens, while also suppressing transplant immunopathology. Our understanding of how ECP physiologically induces monocyte-to-DC maturation can enhance the treatment's potency, potentially broaden its use to other cancers and autoimmune disorders and tailor its application to individual patients' diseases. ECP's next decade is filled with promise.

Keywords

Photopheresis; Dendritic cells; Antigen-presenting cells; Cancer; Tolerance

1. Introduction

The Journal's commemoration of this 25th anniversary of the worldwide clinical introduction "Extracorporeal Photochemotherapy" (ECP) offers a special opportunity to reflect on its therapeutic record, as well as the rich promise of its futuristic evolution. ECP's expeditious 1988 FDA approval for cutaneous T cell lymphoma (CTCL) followed our first clinical report [1] of its efficacy by only one year, since advanced CTCL was at that time regularly fatal. When, soon thereafter, the CD8 T cell dependency of its anti-CTCL effects was discovered [2], ECP was identified as the first approved cellular immunotherapy for any cancer.

Since the next FDA-approval for a cellular immunotherapy for a cancer was issued more than two decades later, it was clearly a treatment ahead of its scientific time. Over the intervening years, six prominent features have distinguished it from other immunologic interventions: (1) its beneficial effects as both an immuno-stimulator in a cancer and an immune-modulator in the transplant setting; (2) its unusually advantageous safety profile for

a potent therapy; (3) its large scale physiologic induction of antigen presenting cells (APCs), including dendritic cells (DCs); (4) its remarkable clinical specificity for pathogenic T cells; (5) its capacity to extracorporeally sequester and modify processed leukocytes; and (6) its enormous accrual of clinical experience.

ECP's therapeutic impact on tens of thousands of patients, afflicted with cutaneous T cell lymphoma (CTCL) lymphoma, organ transplant rejection and graft-versushost disease (GVHD), has been thrilling to us all. Sharing that contribution with so many extraordinarily committed and innovative physicians worldwide has been my career highlight.

The story is now in an advanced second stage that offers great amplification of ECP's accomplishments, as the scientific principles underlying its efficacy rapidly crystallize and can now direct the next set of developments. When, in 1982, we first witnessed the initial astonishing response in one my own leukemic CTCL patients, we recognized that ECP had unexpectedly immunized that patient against, and actually eliminated, his malignant T cells.

In the first report of ECP's systemic efficacy, we suggested that, because scientific knowledge existing at that time could not originally explain ECP's clinical effects, its underlying mechanism must rely on a previously unrecognized therapeutic principle [1]. We also understood that discovery of that principle would be a challenge that would take the measure of many synergistic researchers and would await a much improved understanding of fundamental immunology itself. The opportunity to weave this perspective, with scenes experienced and lessons learned from that odyssey, offers me a rare chance to tie the storyline that has led to this celebration with predictions of where that immunologic positioning system can now take us.

2. ECP's origin

ECP grew out of my Columbia University research program, which was based on the belief that accruing advances in fundamental T cell biology could be translated into improved treatments for devastating and life-threatening leukemic CTCL. By the conclusion of my fellowship at the National Cancer Institute, we had already shown that CTCL, as a clonal proliferation of malignant skin-homing T cells, is an ideal cancer model for introduction of new immunotherapies [3]. As a neoplastic amplification of skinhoming clonal CD4 T cells, CTCL is readily assessed, both clinically and biologically, since CTCL cells accumulate in the two most accessible tissues, skin and blood. Since its clonotypic T cell receptor (TCR) proteins provide tumorspecific antigens, potential to generate selective anti-CTCL immune clinical responses enticed us.

Because CTCL's clinical manifestations directly reflect the features of the dominant subclone, we initially attempted to exploit that biology to develop effective therapies, leading to multiple transient successes. For example, we found that the leukemic stage of CTCL, typically causing total skin infiltration by malignant T cells ("erythroderma"), could be ameliorated by intensive repetitive leukapheresis [4]. The migratory equilibrium between CTCL cells in the blood and skin compartments was so fluid that aggressive leukaphoretic removal of malignant cells, performed essentially on a daily basis over a month, from the blood of a highly leukemic patient, led to parallel diminution of the cutaneous infiltrates and

marked reduction of the CTCL cell blood counts. However, those improvements were disappointingly temporary, as new CTCL cell production continued, unimpeded by any immunologic defense. Similarly, we successfully reduced the systemic burden of malignant T cells through intravenous administration of anti-thymocyte globulin [5], but malignant T cell subclones resistant to the anti-T cell antibodies rapidly became dominant and lethal. These early interventions convinced my colleagues and me that, while individual T cell directed therapies had reportable clinical effects, they were merely temporary. As the physicians of these patients, we were not impressed with short-term clinical responses. We needed to find a way to more selectively and persistently suppress, and even eliminate, the malignant CD4 T cells, while leaving the normal T cell compartment, and immunocompetence, largely intact.

With this ambitious goal in mind, we incorporated the phototoxic chemical pyrene into liposomes coated with anti-CD4 monoclonal antibodies [6]. We reasoned that, following CTCL cell binding of these antibody-delivered poison packets, long-wave ultraviolet energy (UVA) activation of the photo-excitable pyrene would selectively kill the malignant T cells. Before administering these liposomes to patients, we fortuitously first tried to enhance the likelihood of lasting clinical impact by extracorporeally reducing the body's load of CTCL cells by a procedure we now call "ECP".

To accomplish this cytoreductive preparative step, we conceived a methodology to improve upon the efficiency of leukapheretic preliminary depletion of the body burden of CTCL cells. As indicated above, we already knew that leukemic CTCL cells are in migratory equilibrium with tissue-localized CTCL cells. So, we reasoned that combination of leukapheresis with a different photoactive drug, 8-methoxypsoralen (8-MOP), might markedly enhance the depletion of the body's burden of CTCL cells. That drug naturally occurs in nature in a variety of plant products, including lime and parsnip, is biologically inert unless photoactivated, remains activated for only millionths of a second and, if not bound to nucleic acids and certain proteins, is excreted in virtual entirety within one day. So, 8-MOP is essentially a powerful DNA-active chemotherapeutic agent, which can be turned on by a light switch, directly impacting those cells in the light's path and nowhere else.

We showed that UVA activated 8-MOP (PUVA) cross-links pyrimidine bases of DNA of sister strands, thereby locking shut life-sustaining genes and causing apoptosis of the extracorporeally targeted lymphocytes. By developing monoclonal antibodies specific for these DNA-photoadducts [7], we were able to exquisitely titrate their number and show that, as few as three such cross-links per million DNA base pairs could, with unique gentleness, cause universal lymphocyte apoptosis, while largely sparing the similarly exposed monocytes (6). We now know that this unique capacity to titrate 8-MOP binding to precisely the correct point to open a window between lymphocyte apoptosis and monocyte survival is key to ECP's clinical success, as will be mentioned later in this perspective.

Our intention had been to first demonstrate the safety of this tumor depletion method in five of my own carefully selected leukemic CTCL patients. We therefore started very conservatively with monthly administrations and planned to increase the frequency to daily treatments, until the tumor burden could be maximally reduced, only after initially

demonstrating safety. We were quite concerned with the safety of this novel malignant cell depletion method, since it relied on the reticuloendothelial filtration system for removal of the large bolus of intravenously returned dying lymphocytes. We were wary of the possibility of causing “tumor-lysis syndrome”, precipitated by a systemically overwhelming release of catabolic products of decomposing cancer cells, resulting in hyperkalemia, hyperuricemia, hyperphosphatemia, hypocalcemia, acute uric acid nephropathy, acute kidney failure and commonly death. Fortunately, because of the exceptionally gradual apoptosis caused by 8-MOP, those adverse sequelae have not been encountered by a treatment now known to be remarkably safe.

Yet, it is the originally unintended immunotherapeutic consequences of our device design that we now understand are responsible for ECP’s immunologic potency. It is PUVA’s remarkably gentle apoptotic effect that facilitates uptake of intact malignant cells still expressing abundant tumor antigens by APCs. It is the large surface area of the UVA exposure chamber that amplifies the stimulation of processed monocytes by adherent platelets to enter the DC maturational pathway. It is the maturational truncation of the most heavily UVA-exposed passaged monocytes that freezes one subpopulation of resulting DC in an immature tolerogenic state. And it is the shielding from the UVA light, by red blood cells, of those passaged monocytes in the middle of the flowing stream that permits their descendant DCs to mature to an immunogenic anti-cancer state.

To our astonishment, the very first patient, after only three monthly treatment cycles (treatment on two successive days at four-week intervals), and involving exposure of merely an estimated three percent of the body’s burden of CTCL cells, entered a persistent complete remission. He remained CTCL-free for the next two decades, before dying from an unrelated cause. We were amazed that, despite the exceptionally abnormal karyotype of CTCL cells, and their involvement of his blood and peripheral lymph nodes, he responded so fully. Shortly thereafter, the same complete response was noted in a second similar CTCL patient. These two then-mysteriously-based clinical responses exceeded by far, both in specificity for the malignant clones and in duration, anything we could have reasonably anticipated from any of the passive (antibody-mediated) immunotherapy approaches we had envisioned. It was immediately clear to us that we should replace the more limited plan of testing the potency of our CD4-directed pyrene liposomes and focus entirely on investigating the clinical effects and scientific core of what is now known as “ECP”.

The prevalent immunologic dogma in 1982, when those two clinical responses were observed, was that cellular immunity does not naturally protect against cancer, a conclusion based on the observation that athymic, i.e. T cell deficient mice, were not predisposed to multiple malignancies. DC biology was then in its absolute infancy; clonal T cell receptors had been hypothesized but were just being discovered; and CTCL cells were not known to express tumor-specific antigens. There was no escaping the conclusion that, through the photochemical alteration and intravenous return of a tiny fraction of the body’s total malignant T cell population, we had somehow immuno-protected our patients against their advanced CTCL, even though that concept was in conflict with conventional scientific wisdom. We reasoned that, if our conclusion was correct, two secondary concepts must also be accurate. First, CTCL cells must bear tumor specific antigens (against which ECP

somehow selectively immunized the afflicted patients). Second, we had by chance landed on a basic therapeutic principle that would inspire us and other research groups to become search parties for next thirty years and beyond.

As ECP has become a primary therapy for leukemic CTCL, the cumulative worldwide experience has confirmed and extended those early observations, serving as a proof of the potency of anti-cancer cellular immunity, at least in the CTCL context.

It is instructive that, as abundant worldwide clinical experience accrued from ECP treatments, two quite different UVA exposure chambers yielded quite similar clinical results, both in CTCL patients and transplant recipients. The “American” [8] system is composed of two rigid parallel plastic plates encasing a 1 mm blood film, flowing at up to 100 cc/min. The “French” [9,10] system is structurally substantially different, since its UVA exposure chamber is a compressible blood bank bag which, rather than being a conduit through blood flows, is gently shaken. The finding that both types of devices appear to lead to similar clinical results suggested that the dose–response curves relating ECP’s intrinsic variables (i.e. UVA exposure, flow rates and intra-device cellular interactions) to efficacy is forgiving. Indeed, the recently detailed recommendations of the Italian Society of Hemapheresis and Cell Manipulation make no distinction between the two quite different ECP devices [11].

3. ECP’s specificity and bidirectional T cell effects

The early CTCL experience pointed convincingly to ECP’s generation of a clinically potent tumor-specific cytotoxic T cell response. A majority of leukemic erythrodermic CTCL patients with intact CD8 T cell compartments responded significantly to ECP, and in certain cases completely [2]. In stark contrast, those patients starting ECP therapy with depleted normal CD8 T cell populations, due either to the intrinsic features of the disease or to prior chemotherapy, almost invariably responded poorly or not at all. This finding confirmed and strengthened our initial impression that ECP was stimulating anti-CTCL CD8 T cell-mediated responses in the immunocompetent patients.

Since CD8 T cells were already known to recognize antigenic peptides in the context of antigen-presenting cell (APC), including DC, class I major histocompatibility complexes (MHC), these findings pointed to other critically important answers [12]. First, CTCL cells naturally display tumor clone-specific antigens, in the context of MHC Class I, permitting them to be selectively targeted by anti-CTCL CD8 T cells. Second, dormant anti-CTCL CD8 T cells exist and can be reactivated in CTCL patients with advancing disease. Third, APC’s, including DCs, can process those CTCL antigens and present them to CD8 T cells.

During the same period, clinical results revealed that ECP, conducted in principally the same manner, could effectively suppress dangerously potent auto-aggressive T cell mediated diseases, especially rejection of transplanted hearts [13] and post-allotransplant GVHD [14]. At initial consideration, it was quite surprising that the same treatment could stimulate a potent anti-CTCL CD8 T cell response, while suppressing powerfully deleterious CD8 T (and probably also CD4) cell responses in the transplantation setting. The solving of that part of the mystery, as discussed later in this perspective, would require more than a decade

of scientific advances, including awaiting discovery that ECP transforms processed monocytes into APCs that can be either up- or down-regulatory in an antigen specific manner.

4. Framing the mechanistic question

To explain ECP's efficacy, any unifying mechanistic schema must account for its two singular clinical effects: (1) potent specific targeting of CTCL clones and pathogenic T cells mediating GVHD and organ transplant rejection; while (2) avoiding the general immunosuppression that typifies most T cell directed therapies. It has been clear, from ECP's outset, that the key trigger to ECP's efficacy must reside in the UVA-exposure system, since ECP differs from simple leukapheresis solely by the intravenous return of leukocytes treated in that chamber. I.e., the leukapheresis first step in ECP is not, by itself, immunotherapeutic in CTCL [4].

A pivotal role for antigen presenting cells (APCs) in ECP's generation of specific T cell immunity to pathogenic clones has also been clear, just as it is in T cell responses against any antigen. The primary unknown was the location and identity of those APCs, i.e. are they tissue localized cells which process and present antigens originating from ECP-induced apoptotic lymphocytes or are they APCs themselves induced from monocytes in the ECP apparatus?

Another lingering question in cancer immunology in general had been: how do tumor antigens access the class I MHC APC presentation system, as that is required for antigen-specific CD8 T cell stimulation. That question was answered in 1998 by the discovery that DCs have that capability, referred to as "cross presentation" [15]. DCs are especially efficient APCs and are commonly derived from blood monocytes. Since DCs are primarily tissue-based cells and comprise an extremely minor (>0.1% of blood borne leukocytes) intravascular population, immediately following report of that seminal discovery, we hypothesized that a prominent element of ECP's immunotherapeutic efficacy might be the UVA chamber's direction of processed monocytes into the DC maturational pathway. It should also be noted at this point that, while DC cross-presentation capacity is now established, it is not clear that DCs are the only APCs with that talent.

Although intravenous return of PUVA-induced apoptotic lymphocytes is a convenient source of cellular antigens, we recognized that these damaged cells could not provide the pivotal immunotherapeutic influence underlying ECP's effects. It is also well established that skin-directed PUVA, a commonly used treatment for the skin-localized phase of CTCL, does not lead to resolution of non-irradiated CTCL skin lesions, sanctuary sites, like shielded skin folds and hair covered regions. Since such skin-directed PUVA therapy produces substantial apoptosis of CTCL cells infiltrating the skin, and since such apoptotic malignant cells are then filtered by draining lymph nodes, it has long been clear to us that merely increasing, via PUVA exposure of CTCL cells, the body's burden of apoptotic CTCL cells is insufficient, by itself, to explain the ECP-induced anti-CTCL immune response.

So, instead, we sought direct evidence of the candidacy of ECP-processed monocytes as the primary trigger underlying ECP's successes. We established, long ago, that monocytes are far more resistant than lymphocytes to the apoptotic effects of PUVA.

Later in this perspective, I will summarize direct human evidence supporting a mechanistic role for ECP-induced DCs. I will also describe the growing understanding of precise molecular pathways how ECP physiologically signals processed monocytes to mature into APCs, including DCs. To place those findings in the context of selected relevant experimental animal data, I will first highlight selected relevant experimental animal data.

5. Experimental animal studies

Although, at this writing, nearly 900 peer review original papers and reviews relating to ECP have been published, only a small fraction report efforts to identify the mechanistic roles of individual variables in reductionist animal systems. That paucity likely reflects a realization that the absence of an animal equivalent to the ECP flow system limits the relevance of such scientific inquiry.

In the conclusion of this perspective, I will indicate that, now that centrally critical variables controlling ECP's conversion of monocytes to APCs have been identified, it is now possible to create a device that is scalable from mouse to man. At this moment, we can still distill some important insights from at least three sets of mouse experiments, while keeping in mind that they are not truly models of ECP.

In the first decade following ECP's clinical introduction, our own group, initially at Columbia University and later at Yale University, did intensively search for mechanistic leads in a number of mouse systems. Two of those animal systems provided clues [16,17] that, in the context of current understanding, now become even more significant.

The first of these animal models revealed the potency and specificity of the anti-cancer effect [16]. We utilized a rapidly lethal T lymphoma line (2B4.11), provided by colleagues at the NIH. 2B4.11 resulted from fusion of a cytochrome C specific T cell line produced in normal B10a mice with a malignantly transformed thymoma line, referred to as BW5147, developed from AKR/j cells [18]. The resulting tetraploid hybridoma line had two important features: tumor-specific antigenic peptides, derived from the clone specific T cell receptor of the normal parental line, and unrestricted cancerous growth programmed from the thymoma parental line. After subcutaneous inoculation of 2B4.11 malignant cells into B10a/AKR hybrid mice, with which the 2B4.11 line was histocompatible, the tumor cells quickly grew into a lethal lymphoma that killed all inoculated mice within one month. Another malignant hybridoma, differing in its production using a normal cell line with TCR receptors for conalbumin, rather than cytochrome C, but being equally malignant in the same mice, was used as an immuno-specificity control. The experimental system was designed to permit us to test the possibility that PUVA-treated 2B4.11 malignant T cells could immunologically protect selectively against 2B4.11 tumor and not the control tumor.

The test mice were selectively immuno-protected against the lethality of the 2B4.11 clone. Importantly, the gentle apoptosis induced by PUVA pretreatment of the malignant cells was

the most potent method of making the apoptotic cancer cells immunogenic. The most effective immuno-protection was produced via intravenous infusion of DCs, preloaded with apoptotic UVA-8-MOP-treated 2B4.11 cells. In short, DC's loaded with PUVA-treated malignant cells were the key ingredients in the protective anti-cancer vaccine, reminiscent of the leukemic CTCL responses to ECP.

The second set of experiments, also conducted by our group, investigated the capacity of PUVA-treated normal effector T cells to inhibit rejection of a full thickness skin allograft across a complete histocompatibility barrier [17]. Black CBA/j (H2k) skin, after transplantation to white Balb/c (H2d), was completely rejected, without remnant, within two weeks. Anti-skin allograft T cells then markedly expanded the spleens of the rejecting Balb/c recipients. These spleens were removed from sacrificed mice; the splenic T cells were then PUVA-treated; and the apoptotic T cells were intravenously introduced into fresh syngeneic Balb/c mice, in an effort to "vaccinate ala ECP" these animals against the T cell clones capable of mediating rejection of subsequently transplanted fresh Balb/c full thickness skin.

After this ECP-like infusion of 8-MOP/UVA-treated leukocytes, newly transplanted skin now survived the full six weeks of observation, despite being completely histoincompatible with the recipient. Since simultaneously transplanted skin from a third incompatible strain underwent accelerated complete rejection within two weeks, clearly the ECP mouse analogue approach tolerized the mice to the allografts.

The definitive distillation from these skin allotransplant experiments is the potency of the tolerogenic procedure, across complete histocompatibility barriers. If this phenomenon can be now be repeated with an ECP device scaled to mouse size, while completely reflecting the properties of a human-sized device, it might now enable experimental stem cell transplants across haplotype mismatches in first-degree relatives. At the very least, such a relevant mouse system will permit clarifying animal experiments not previously possible.

The research group of Thomas Schwarz confirmed the critical role of PUVA-exposed monocytes in the induction of antigen-specific tolerance [19]. They found, in a mouse model of contact sensitivity to the hapten dinitrofluoro-benzene (DNFB), that intravenous return of a mixture of PUVA-treated monocytes and PUVA-treated anti-DNFB T cells produced specific tolerance to the hapten. This effect was completely precluded by depletion of the monocytes from the reinfusion suspension, proving that PUVA-treated monocytes mediated the tolerance.

6. ECP: manufacturer of dendritic antigen presenting cells

With so many clues pointing toward the possibility that ECP may be a DC-based therapy, and since DCs commonly derive from monocytes circulating in blood, we directly investigated whether the ECP chamber expedites conversion of monocytes to DCs and activate APCs. A majority of ECP-plate-processed monocytes, whether from ECP-treated patients or normal subjects, when examined for 18 h *in vitro*, acquired phenotypic and functional properties of activated APC, including features typical of monocyte-derived immature DCs [20].

That same study revealed that ECP's induction of monocyte-to-DC maturation resulted from activation and transcription of genes encoding DC differentiation markers and functional proteins. Full genome analysis of the ECP-induced monocyte transcriptome identified a highly distinctive gene activation signature involving enhanced transcription of nearly 500 genes. Of these, approximately 70 of the activated genes encode cell membrane-embedded proteins, including several known to be initiating points for signal transcription pathways, suggesting that the monocyte-to-DC maturation was initiated by bioactive membrane interactions in the ECP chamber. The rapidity with which the processed monocytes acquired properties of activated APC, especially DCs, without addition of the supra-physiologic levels of growth factors commonly used *in vitro* to mature DC from monocytes, suggested that ECP was efficiently pushing monocytes towards DC maturation in a physiologic manner. These intriguing findings ultimately led to our novel identification of a central role for platelet P-selectin, as discussed below.

Since normally DCs comprise such a miniscule fraction of blood leukocytes, return to the patient of these induced DC-like APC must tremendously amplify antigen-presenting capacity, especially of those antigens distinguishing co-ECP-processed expanded blood lymphocyte populations. To enhance the uptake of these apoptotic lymphocytes by the incipient DC, we incorporated a first major variation in the ECP process. Overnight incubation of incipient apoptotic lymphocytes with incipient DC, preliminary to the return of the ECP processed cells, increased the efficiency of tumor antigen internalization and processing. In this manner, we were able to improve ECP's clinical and laboratory efficacy in CTCL patients who had previously failed to respond to ECP [21].

Previously persistent aspects of the mechanistic puzzle suddenly began to appear logically resolvable. For example, the question of how ECP both stimulates and suppresses immune reactions seemed likely to reflect the maturity of the prevalent DC populations. Low levels of surface co-stimulatory surface molecules, especially CD80 and CD86, typify immature DCs. The low density of this stimulatory membrane proteins is known to enhance DC tolerogenicity [22]. In contrast, mature DCs, typified by higher levels of the surface co-stimulatory molecules, preferentially stimulate positive responses by T cells whose TCR recognize the displayed antigens. Therefore, depending on their maturity, DC can either be up-regulatory or down-regulatory of antigen-specific T cell responses. Essentially, DCs are potent antigen-specific master switches.

We therefore commenced to determine whether ECP's capacity to be up-regulatory in the context of CTCL or down-regulatory in the context of transplanted organ rejection and GVHD might be explicable on the basis of simultaneous production of both stimulatory and suppressive DC populations. We realized that, during passage through the UVA exposure plate, individual monocyte exposure to UVA-activated 8-MOP was not uniform, but rather conformed to a Gaussian distribution. We postulated that those monocytes receiving minimal exposures are capable of fully mature into immune-stimulatory long living DC. Those monocytes receiving higher exposures experience truncated maturation, become tolerizing immature DC, and have an 8-MOP influenced shorter survival.

It has been broadly recognized that ECP's reversal of acute GVHD and organ transplant rejection commonly occurs significantly faster than its immuno-therapeutic impact on CTCL. This observation suggested to us that, following ECP, suppressive short-lived maturationally truncated DC might dominate early, while stimulatory longer-lived mature DC would be expected to dominate later.

How does passage through the ECP plate influence monocytes to differentiate into DC? To answer this question, we designed a miniaturized chamber model of the ECP flow system that permitted direct visualization and analysis of the roles of plasma proteins and cellular components in this phenomenon. We discovered that DC differentiation occurs through transient engagement of flowing monocytes with device-adherent activated platelets and their ligands (Fig 1). Since entry of chamber-passaged monocytes into the DC differentiation pathway did not depend on the supraphysiologic concentrations of cytokines involved in conventional laboratory generation of DC from monocytes, we conclude that ECP's induction of DC is physiologically based.

In laboratory studies nearing completion, our results identify the following mechanistic sequence, by which platelets drive maturation of monocytes to dendritic cells (Fig 1). First, monomeric plasma fibrinogen coats the flow chamber plastic surface of the flow chamber. Second, via their α IIb β 3 receptor, resting passaged platelets adhere to the fibrinogen layered on the plastic surface. Third, platelets, activated by adherence to immobilized fibrinogen, rapidly express preformed P-selectin. Fourth, passaged monocytes now interact with platelet P-selectin via their own PSGL-1, leading to partial monocyte activation and concomitant integrin receptor conformational changes. Fifth, the monocytes secondarily bind additional platelet ligands, notably including those RGD domains. Sixth, the monocytes are influenced to enter the DC maturational pathway. Via this sequence, all intrinsic to ECP treatments, platelets drive the conversion of blood monocytes to DC, which importantly occurs under physiologic conditions and likely reflects a natural *in vivo* pathway perhaps typical of real life DC differentiation.

In the vast majority of laboratory protocols that induce DC for clinical studies, blood monocytes are exposed to supra-physiologic doses of cytokines and growth factors (e.g. GCM-CSF and IL-4, followed by a second maturational step involving TNF- α). ECP-generated DC differ from the conventionally produced ones used in most anti-cancer immunization protocols by not requiring artificially high levels of growth factors. Introduction of laboratory-produced DC produced under conditions that cannot be reproduced *in vivo* may be at least one reason why clinical trials of such artificially produced DC have commonly yielded disappointing results. The ECP method, as we now appreciate, has long been producing and reinfusing large numbers of DC, which are then exposed to PUVA-injured cancer cells in CTCL and autoreactive apoptotic T cells in the transplant scenario. ECP's clinical efficacy and advantageous safety profile likely reflect its being a significantly more physiologic method of producing and loading DC from monocytes.

Are the DCs that are produced, and antigen-loaded, by ECP identical to those conventionally produced in response to supra-pharmacologic cocktails? Even if ongoing detailed comparisons of phenotype and function indicate major overlaps between these two sets of

DC, their being derived in entirely different manners is a fundamental distinction. Since, at least in the laboratory, ECP-processed monocytes do not begin to display a DC phenotype until they internalize particulate antigen during short-term culture, a process we refer to as “Transimmunization” to reflect transfer of antigen the DC, we recommend that these special APCs be distinguished as “DC^T”. It will be important to continually compare their function to that of conventionally produced DC. But there will always be major differences between the two populations, since DC^T are produced in a more physiologic manner and are associated with a treatment with a strong track record of clinical success.

In this context, the ECP story may become even more compelling in the near-term future, as the evolution of the immunotherapy can now evolve to new forms, with broadened clinical applications, now driven by scientific insights, rather than empiricism. Along with international colleagues, we have developed a modified device, enabling capacity to individualize “tuning” of multiple controlling ECP variables, ranging from density of adherent platelets, to adjusted flow rate, to control of UVA lights, to facilitation of DC processing of apoptotic cells. This device, which we are currently studying, is scalable from mouse to man, ensuring that animal experiments can, for the first time, accurately mimic human treatments, with respect to all conditions. Time will tell whether a treatment which began as a form light-activated chemotherapy and evolved to a widely used immunotherapy for one cancer, GVHD and organ transplant rejection may be the forerunner of immunotherapies for a broad spectrum of cancers and immunologic diseases.

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Fig. 1. Platelet driven monocyte-to-dendritic cell maturation

Physiologic interactions between ECP-processed monocytes and ECP-activated platelets induce the monocytes to commence differentiation into DC. Firm binding of passaged platelets, via three receptor-ligand interactions, activate the platelets to instantaneously transport preformed P-selectin and fibronectin to their surface. (A) After plasma fibrinogen coats the UVA transparent plastic surface of the flow chamber, passaged platelets (via their $\alpha_{IIb}\beta_3$ chain) bind to the γ -chain of immobilized plastic-fibrinogen. (B) Through their $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ receptors, platelets bind to repetitive RGD tripeptides of plastic-immobilized fibrinogen. (C) P-selectin on now activated platelets transiently binds PSGL-1 on the surface of passaged monocytes, activating the monocytes and resulting in integrin receptor conformational changes. (D) Partially-activated monocytes, via their reconfigured integrin receptors, adhere to additional platelet-expressed ligands, including RGD domains of platelet fibronectin. (E) via these activation signals, monocytes are induced to enter the DC maturational pathway. PUVA, while not involved in any of these interactions, contributes to monocyte differentiation, through secondary uptake of PUVA-induced apoptotic lymphocytes, which are far more sensitive to PUVA than are monocytes.