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Marker Characterization Of Ecp-Induced Monocyte-Derived Dendritic Cells

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Marker characterization of ECP-induced monocyte-derived dendritic cells

A Thesis Submitted to the
Yale School of Medicine
in Partial Fulfillment of the Requirements for the
Joint Degree of Doctor of Medicine and
Master of Health Sciences

by
Kristina Jing Liu
2013
MARKER CHARACTERIZATION OF ECP-INDUCED MONOCYTE-DERIVED DENDRITIC CELLS

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Extracorporeal photopheresis (ECP) in the treatment of cutaneous T-cell lymphoma, GVHD and autoimmune conditions continues to spur the question of how ECP is capable of inducing immunogenicity and tolerance. We previously demonstrated that ECP treatment leads to large-scale conversion of peripheral blood monocytes into functionally competent leukocytes with dendritic cell phenotypes, which may play key roles in the immunomodulatory capabilities of ECP. To characterize this population of cells on a molecular level, we assessed for differential surface expression of selected gene-products on monocytes after treatment with a model-ECP apparatus. Five gene-products (CXCL16, SIRPa, ICAM1, TNFR1, PLAUR) showed significant increases in surface expression after model-ECP treatment as compared to PBMC (p <0.01 for all). To identify transcription factors (TFs) expressed by ECP-treated monocytes but not peripheral blood monocytes, rtPCR was performed. Interactions with platelets during ECP passage was also assessed by using model-ECP plates coated with low- and high-density platelets. Seven TFs demonstrated increases in mRNA after passage through the model-ECP plate (ΔRQ range 1.35- 6.78). Increased platelet density induced directional changes in expression (RQ < 0.5) for VDR, NFkB2, CDKN1A and BCL3. In summary, passage through the ECP plate apparatus caused activation of novel surface molecules and transcription factors that define and characterize a unique subset of “physiologically-induced” DCs.
Acknowledgements

The past year has been a truly memorable experience. I am very grateful to so many people, without whom I would not have had the opportunity to study a fascinating subject in depth, but also grow personally in a tremendous way.

I’ve had the great fortune of working with the best mentors I could have ever asked for. Dr. Girardi, your scientific acumen and dedication to your laboratory are only matched by your infallible sense of humor. Thank you for taking me under your wing this past year. Dr. Edelson, your words of constant encouragement are what kept me going in the face of challenging experiments. Your have this uncanny ability to make every individual, like me, feel unique and valued, for which I am deeply grateful. Dr. Tigelaar, thank you for the many troubleshooting tips along the way; your genuine commitment to the scientific process is one I aspire to possess in the future. I want to also thank Dr. Jae Choi, who gave me the opportunity to collaborate with him on a great project.

Julie, thank you for teaching a novice like me so patiently, and for showing me that scientific excellence lies within the smallest details. Renata, your knowledge of all things RNA is second-to-none, and I am so touched by all the little things you do (like brining in soymilk when you know I’m lactose intolerant). Eve, I’m grateful for all the time you spent teaching me plate techniques. Inger, Maureen and Sue, you were like my “work mothers,” always kind and helpful without exception, and making sure I was well-fed and well-loved. Kacie, your organization and coordination were invaluable, and your bright smile would always light up my day.
I’d also like to thank Dr. Forrest, Ms. Donna Carranzo and Ms. Mae Geter from the Office of Student Research for their support and help in obtaining funding for this project, made possible by the Doris Duke Charitable Foundation.

Last, but certainly not least, I want to thank my parents, Min and Ling, who are the kindest, most supportive parents I could have ever asked for. Thank you for always being my shelter from the storm and for teaching me that if my heart can believe it, then I can achieve it.
Preface

I conceptualized this thesis as a movie with three side-reels. The over-riding theme of all four is the goal towards the improved treatment of CTCL, as approached from two sides: the study of photopheresis and the analysis of peripheral blood subsets in CTCL. Within the latter, we attempted to harness information from two different, but equally important, cellular compartments: malignant T cells vs. normal immune cells (such as T cells and dendritic cells). From the malignant T cell compartment, we sought to better understand the pathogenesis of CTCL with the goal of discovering novel therapeutic targets. From the dendritic cell side, we investigated aspects of fundamental biology also with the goal of discovering novel immunotherapeutics. By accessing the blood of patients and healthy subjects, much was learned about the impact of these cellular compartments on the immune system, and the potential to harness them to improve the treatment of CTCL. I hope you enjoy the feature presentation.
Molecular characterization of ECP-induced monocyte-derived dendritic cells

I. Introduction
   a. Extracorporeal photopheresis: an immunomodulatory chameleon
   b. The roles of lymphocyte apoptosis, antigen-presenting cells and regulatory T cells
   c. Pathways of T cell co-stimulation
   d. Dendritic cells: key players in adaptive immunity
   e. Effects of ECP on monocyte to dendritic cell maturation
   f. Characterization of ECP-induced monocyte-derived DCs

II. Statement of purpose

III. Methods

IV. Results

V. Discussion

Appendix A: DNA Copy-Number Analysis of Selected Genes in Patients with CTCL

Appendix B: Identification of Novel Genetic Alterations in CTCL via Whole Exome Sequencing

Appendix C: Detection of peripheral blood involvement in CTCL by multi-parametric flow cytometry analysis
I. Introduction

*Extracorporeal photopheresis: an immunomodulatory chameleon*

Extracorporeal photopheresis (ECP) is an immunotherapy initially developed in the 1980’s by Dr. Richard Edelson *et al.* for the treatment of cutaneous T cell lymphoma (CTCL). The usage of ECP has since expanded to include the treatment of chronic and acute GVHD, prophylaxis and treatment of solid-organ transplant and several autoimmune diseases, such as systemic sclerosis. ECP is performed via collection of leukocytes from a patient using a leukopheresis device, which centrifuges whole blood to produce a leukocyte-rich fraction. The buffy coat is then mixed with liquid 8-MOP, a photosensitizing agent, and passed through a thin plastic apparatus that allows exposure to controlled-doses of UVA radiation. The compound 8-MOP is biologically inert in the absence of UVA, and once photoactivated, remains reactive for only microseconds, thereby making ECP an exceptionally safe and directable form of chemotherapy. Due to its efficacy in the treatment of CTCL and its extremely favorable side-effect profile, the US Food and Drug Administration expeditiously approved ECP for the treatment of advanced CTCL in 1988. Since its inception, the efficacy of ECP has been studied in numerous uncontrolled trials for the treatment of mycosis fungoides (MF), the most common variant of CTCL, and Sézary syndrome (SS), a form of CTCL with malignant T cell involvement of the peripheral blood. With high rates of clinical skin improvement, total remission and a remarkably low toxicity profile, ECP has become the recommended first-line therapy for advanced CTCL, particularly in patients with circulating malignant T cells in the peripheral blood.
Despite its efficacy and clinical applicability, the mechanism of action of ECP remains not well elucidated and a heated focus of on-going research. Early hypotheses attributed the effects of ECP to the destruction of malignant lymphocytes after exposure to 8-MOP plus UVA. While this certainly induces apoptosis of cells, it is not sufficient to explain the widespread effect of ECP since only 5-10% of total lymphocytes pass through the treatment apparatus. Furthermore, ECP’s remarkable immunogenic effects in treating malignant diseases must be also explained in the context of tolerogenic effects seen in the treatment of GVHD and numerous autoimmune conditions.

The roles of lymphocyte apoptosis, antigen-presenting cells and regulatory T cells

Treatment of the peripheral blood mononuclear cells (PBMC) with 8-MOP and UVA leads to large scale apoptosis of lymphocytes through the Fas/FasL and Bcl-2 related pathways. This results in the activation of apoptotic proteases leading to the disintegration of lymphocytes and increased apoptotic fragment uptake by antigen presenting cells. ECP-treated monocytes, however, appear largely resistant to apoptosis; they are driven to take on more immature dendritic cell-like phenotypes as well as release cytokines such as IL-10 and TNF-α. Current evidence points to antigen-presenting cells (APC) as playing a major role in the induction of tolerance, as ECP-treated APCs, such as dendritic cells (DC), phagocytose apoptotic lymphoid fragments and present the antigen to T cells without costimulation from a true “danger” signal. Studies have shown that the maturation state of DCs as well as the cytokine milieu greatly influences the
generation of the local immune response. The presence or lack of an inflammatory environment and the presence of mature vs. immature dendritic cells can skew the development towards an immunostimulatory or immunosuppressive state.\textsuperscript{13} Surveillance of the immune system by regulatory T cells (Treg) is not studied in this thesis but deserve mention as another key player in ECP-induced immune modulation. The population of CD4+CD25+ regulatory T cells is increased after ECP along with high expression levels of CD45RO and Foxp3, a finding suggestive of how ECP might dampen autoimmune responses and induce immune tolerance.\textsuperscript{14,15}

\textit{Pathways of $T$ cell co-stimulation}

The complex mechanisms of T cell co-stimulation may also serve to explain how ECP is able to establish central and peripheral self-tolerance for the treatment of GVHD and autoimmunity.\textsuperscript{13} Naïve CD4+ and CD8+ T cells require activation before acquiring effector functions, and this process is stringently regulated to ensure purposeful activation, and requires several signals. The first signal involves the engagement of a major histocompatibility complexes (MHC) on APCs with T cell receptors (TCR) on T cells, which generates a specific signal involving the CD3 complex.\textsuperscript{16} Additional co-stimulatory signals are generated through other cell surface molecules, and lastly, cytokines produced by APCs also influence T cell activation.\textsuperscript{17,18} These three signaling pathways work together to induce T cell activation and the accompanying gene transcription, which leads to clonal expansion and differentiation of antigen-specific T
cells. Conversely, the lack of co-stimulatory molecules or purposeful suppression of these pathways leads to T cell anergy and immunosuppression.

Examples of co-stimulatory pairs involved in T cell activation include CD28:CD80/86, CTLA-4:CD80/CD86, PD-1:PD-L1/2, CD40:CD154 and several others. The classic axis of CD28:CD86 regulated T cell activation is very well characterized in the literature. Conversely, CTLA-4 also binds CD80/CD86, with even higher affinity, and in turn inhibits the T cell response by limiting interactions with CD28. This decreases inflammatory cytokine secretion as well as suppresses clonal T cell expansion, and a CTLA4-Ig fusion protein has been developed specifically to inhibit this pathway of co-stimulation in allograft rejection.19-21 Similar to CTLA4, PD-1 also possesses inhibitory activity and plays an important role in suppressing T cell activation. The PD-1 double-knockout mouse model has the propensity to develop autoimmunity, demonstrating the importance of PD-1 in the maintenance of central and peripheral tolerance. Similarly, blocking antibodies against PDL1 results in increased antigen-specific T cell expansion and differentiation.22-24 LFA-1:ICAM interaction belongs to a family of well-characterized integrin pathways involved in T cell migration, T cell and APC interactions and the provision of co-stimulatory signals. LFA-blocking antibodies used in animal models have shown prolongation in the survival time of several solid organ transplants, demonstrating the importance of ICAM co-stimulation in T cell clonal expansion.25-27 The activities of these co-stimulatory pathways highlight the diversity of molecules involved in all stages of the immune response, as well as the pivotal role of APCs in promoting or suppressing T cell activation/differentiation and cytokine production.16
Within this complex yet specific network of signaling molecules may lie the answer to how ECP is able to enhance or attenuate the immune response.

Dendritic cells: key players in adaptive immunity

The understanding of dendritic cell physiology has evolved tremendously since their first observation in the skin by Paul Langerhans. They are known for their unparalleled activity as “sentinel” cells that bridge the innate and adaptive immune systems. Dendritic cells are not only capable of stimulating naïve T cells in the presence of danger signals, but they also interact with T cells on a continuous basis in the absence of foreign antigen to enforce peripheral T-cell tolerance. In humans, DCs can originate from lymphoid and myeloid lineages. “Classical” DCs (cDCs) are considered to be those derived from myeloid committed CD34+ progenitor cells, and monocyte-derived DCs (mDCs) can also form in the presence of GMCSF and ILF-4 ± TNF α. These DCs, when mature, are known as interstitial DCs, which can activate naïve CD4+ and CD8+ T cells as well as induce differentiation of naïve B cells. DCs that originate from CD34+ cells committed to the lymphoid lineage, on the other hand, become plasmacytoid DCs (pDCs). They are adept at cytokine secretion, especially interferon α/β, although they are also capable of antigen presentation in the presence of inflammation or infection. Studies of innate or acquired DC loss provide insight into the complex roles they play in immunity and tolerance, since T cells receive negative, tolerizing signals from DCs but also require a “baseline” signaling through the TCR from DCs for survival. This push-
and-pull balance of interaction between T cell and DCs may fundamentally be the question at hand when analyzing the seemingly opposite therapeutic responses induced by ECP. Take for example DC-specific deletion of αvβ8 integrin (requisite for TGFβ activity on T cells) or A20 (NFκB pathway negative regulator), both of which can lead to widespread activation of the immune system. Alterations in gene expression either due to experimental manipulation, or due to therapeutic treatment, could alter the complex roles of DC in T cell homeostasis and immune-modulation. The type of immune response elicited is likely quite nuanced, depending on the activation state and class of dendritic cell, their maturation status as well as the cytokine milieu in which these DC-T cell interactions occur. It is these complexities that make dendritic cell immunobiology a particularly worthwhile area of investigation in better understanding the efficacy of ECP, as it applies to both tolerance induction and anti-cancer immunization.

**Effects of ECP on monocyte to dendritic cell maturation**

Previous work performed by the late Dr. Berger identified an interesting phenomenon that occurred after the ECP processing of peripheral blood monocytes: these cells underwent large-scale conversion into leukocytes with dendritic cell phenotypes. This prompted a more in-depth look at these newly formed DCs, which may possess augmented antigen-presenting functions that enhance the anti-tumor response after ECP. The results demonstrated ECP’s ability to rapidly generate functionally competent DCs using monocytes derived from CTCL patients and healthy subjects, without the addition
of cytokines or multi-day culture. Furthermore, the results support the premise that ECP treatment induced significant changes in gene expression, including many genes associated with DC differentiation and function. The number of genes with altered expression after ECP treatment was substantial: 498 upregulated and 631 down-regulated genes were found in common between patients and normal subjects.

Characterization of ECP-induced monocyte-derived DCs using surface molecule expression and transcription factor expression

The study by Berger et al. made available a vast number of candidate genes amongst the upregulated subgroup that may serve to better define and characterize these ECP-induced DCs, as well as shed light on aspects of their functionality. Our collaborator, Dr. Lieping Chen, also provided a list of human transmembrane genes for cross-comparison usage.

DCs can also be divided into multiple subtypes with defining functions and characteristics. Furthermore, states of infection or inflammation can induce additional subsets of DCs from peripheral blood monocytes, which can be quite different functionally and molecularly. While the identification of various DC subsets using surface markers is useful, the redundancy of certain markers on other myeloid cell lineages and the variability of surface expression secondary to numerous internal and external factors can make distinguishing these subsets difficult. Therefore, it is often necessary to make these distinctions using patterns of surface expression rather than a
single marker. Recent studies suggest that the identification of specific DC subsets may require the analysis of surface phenotype, in addition to the identification of lineage-specific transcription factors.\textsuperscript{37-39} In a study by Satpathy \textit{et al.}, a transcription factor (TF) belonging to the BTB-ZF (Broad complex, Tramtrack, Bric-à-brac, and Zinc finger) family, was identified as being specifically expressed in classical DCs.\textsuperscript{37} Furthermore, using this TF, cDCs were distinguishable from all other immune lineages. Given the unique process of producing monocyte-derived DCs using ECP treatment, we wanted to perform \textit{in silico} analysis of ECP-induced upregulated genes in search of potentially novel, defining transcription factors that may enable examination of further functional properties of these cells. Thus, we begin this assessment of unique surface molecule expression and transcription factor expression with the ultimate goal of defining and characterizing ECP-induced monocyte-derived DCs. This project fits nicely within a larger endeavor to better understand the efficacy of ECP, likely mediated in large part through dendritic cells, and will provide important insight into defining a process of developing more “physiologic” DCs for use in many other branches of research.
II. Statement of Purpose

We aimed to utilize a tabletop apparatus, the Glycotech flow chamber, as a model for the ECP UV-exposure plate, to assess the effect of plate-passage on peripheral blood monocytes. Previous work has shown that treatment of monocytes with the ECP plate led to the generation of maturationally-synchronized, functionally-competent leukocytes with dendritic cell phenotype. Our central hypothesis is that the passage of monocytes through the Glycotech chamber induces activation of specific, novel surface molecules that define this population of monocyte-derived dendritic cells. We plan to accomplish this objective of identifying markers of global monocyte activation using flow cytometry to compare the differential expression of selected, surface candidate genes, with the goal of determining whether this differential expression alters the immuno-modulatory effects of these monocyte-derived dendritic cells and contribute to the overall efficacy of ECP.
III. Methods

In silico analysis of differentially expressed DC genes after ECP treatment

Previous analysis of the ECP-induced monocyte transcriptome (performed within our department) revealed activation or suppression of >1000 genes common to ECP-processed monocytes from patients and healthy subjects. Raw data from this analysis was obtained, and samples separated into 1) PBMC obtained from the ECP machine pre-treatment, and 2) post ECP treatment after overnight incubation in a platelet storage bag with RPMI 1640 and 15% AB serum. Fold change in expression was calculated as ratio $= \frac{\text{Average pre-ECP expression}}{\text{Average post-ECP expression}}$ for both health control and patient cohorts. Differential gene expression was considered as $>2$ fold change and a p value of $<0.05$. Only genes with net positive $>2$ fold change (upregulated genes) were chosen for further analysis, resulting in a list of 466 genes.

Using a database of 1950 full-length human transmembrane genes with immunologic and hematopoietic cell surface expression (courtesy of Dr. Lieping Chen, Department of Biological and Biomedical Sciences, Yale University; full table available as supplemental material\textsuperscript{40}), the list of 466 upregulated genes were cross-referenced to produce cell surface, “ECP-activated” genes shared between these two datasets. To further select for genes likely to be unique to ECP-induction, five available expression profiles of human monocyte derived DCs grown in GMCSF and IL-4 were obtained. Lists of upregulated genes between immature dendritic cells (after 3-7 days of culture) and elutriated monocytes were obtained from these expression profiles and individually compared to the list of 87 “ECP-activated” genes to produce genes unique to ECP induction.
An extensive literature search was performed to assess the function of these genes and their relationships to immunologic pathways. Furthermore, the raw data from the original transcriptome analysis was recalculated to show exact numeric value of expression change. Using these two strategies, genes were prioritized based on highest levels of differential expression after ECP treatment and/or interesting gene function to begin in vivo analysis in batches.

Physiologic generation of human monocyte-derived DCs
Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Yale Human Investigational Review Board approved the protocol to obtain blood from volunteers. Aliquots of peripheral blood were procured and stored in 60mL heparin-treated syringes. Mononuclear cells were isolated from the peripheral blood of healthy donors by Ficoll-Paque density gradient centrifugation. The peripheral blood mononuclear cells (PMBC) were re-suspended in RPMI 1640 medium (Gibco) and separated into three experimental groups: 1) Day 0, 2) PBMC Day 1, 3) Plate-passed Day 1. The “Day 0” experimental group denotes 20 x 10^6 cells that are separated and stained on the day of peripheral blood collection. “Plate-passed Day 1” indicates the 80 x 10^6 cells that are re-suspended in 8mL RPMI 1640 and passed through the Glycotech plate apparatus (set-up and illustration below). Following one-way plate passage, these cells are resuspended in RPMI 1640 supplemented with 15% AB serum and cultured for 20 hours in 6-well polystyrene tissue-culture plates at a density of 10^6 cells/mL. To generate cells for comparative phenotypic analysis (“PBMC Day 1), 60 x
10^6 cells are re-suspended in RPMI 1640, allowed to sit in the incubator to simulate temperature conditions for the Plate-Passed experimental group, cultured for 20 hours similar to conditions described above and collected for FACS analysis.

*Glycotech Plate Chamber*

Previous research performed by our group had demonstrated that human monocytes could be efficiently converted to dendritic antigen presenting cells by passage over a platelet-coated plastic surface. This is in contrast to conventional methods of in vitro monocyte-to-DC conversion, which requires the addition of exogenous growth factors. Briefly, a polystyrene surface is coated with autologous platelet-rich plasma and allowed to form an immobilized platelet layer. A Glycotech flow chamber is placed on top of this surface and vacuum-sealed, and 4ml of RPMI 1640 is passed through at 1 ml/min and discarded during the wash step. PMBC (“Plate-Passed” experimental condition) is passed through the chamber at 0.8 ml/min to allow for ample interaction time between monocytes and the platelet coating. Subsequently, 40 ml RPMI is passed through the chamber at 8 ml/min to create high velocity flow to remove any adherent monocytes. The total 48 ml collected during PBMC plate passage and high-velocity wash phases is combined and cultured for 20 hours and harvested for phenotyping analysis using multi-parametric flow cytometry (Figure 1).
Figure 1. Diagram of experimental setup using the Glycotech flow chamber. Peripheral blood monocytes are passed over a platelet-coated polystyrene surface under vacuum seal under specific experimental conditions: PBMC is passed through the chamber at 0.8 ml/min followed by flushing of the chamber with 40ml RPMI at 8 min/ml. All volume after passage is collected for overnight culture. *Graphic depiction courtesy of David Khalil, Yale Department of Dermatology.

FACS analysis

All three experimental groups were analyzed by flow cytometry. In all samples, cells were also paired with matching isotype controls. Monoclonal antibodies specific for monocytes and DC maturation included: CD14 (lipopolysaccharide receptor, monocytes); HLA-DR (human leukocyte antigen DR; class II major histocompatibility complex [MHC] molecule); CD83 (DC marker); and CD80 and CD86 (B7.1 and B7.2 costimulatory molecules). Cells were blocked with 1:100 normal mouse IgG in 5% AB serum for 15 minutes then incubated with selected antibodies targeting prioritized surface molecules for 30min at 4°C (Table 1). Anti-CATB and anti-Neu1 rabbit polyclonal
antibodies required wash and incubation with FITC-conjugated donkey anti-rabbit antibody for an additional 30 min at 4°C. Cells were then washed three times and fixed in 2% paraformaldehyde. To determine the ability of plate passage to induce DC maturation markers, cells were incubated with PE-conjugated anti-CD83, -CD86, FITC-conjugated anti-HLA-DR, -CD80, PCP-conjugated anti-CD11c and APC-conjugated anti-CD14. All flow cytometric data were analyzed using FlowJo software (Tree Star). Monocyte-derived DC populations are identified as CD11c+ cells with appropriate forward and side scatter.

Table 1. Selected antibodies targeting prioritized surface molecules.

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<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Catalog #</th>
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<td>CTSB (rabbit pAb)</td>
<td>Abgent (San Diego, CA)</td>
<td>AP7367c</td>
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<tr>
<td>CXCL16 (anti-human APC-conj. mAb)</td>
<td>R&amp;D Systems (Minneapolis, MN)</td>
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In silico identification of upregulated transcription factors after ECP induction

Using the previously delineated list of 466 upregulated genes from the ECP-induced DC transcriptome, transcription factor genes were identified using the TFCat (http://www.tfcat.ca/index.php) database.

RNA isolation and gene expression analysis

Peripheral blood monocytes from a single donor were separated into three subsets: Sample A = monocytes cultured overnight following passage over plate coated with low density of platelets; Sample B = monocytes cultured overnight following passage over plate coated with high density of platelets; Sample C = monocytes cultured overnight without plate-passage. Total RNA was isolated from each subset using the RNeasy MicroKit (Qiagen, Valencia, CA). cDNA was then prepared from three samples of RNA using ABI High Capacity cDNA Reverse Transcription kit with RNase Inhibitor (ABI, Foster City, CA). Amplification of cDNA was achieved using TaqMan Pre-Amp Mastermix and TaqMan assays specific to the eight transcription factors, all according to manufacturer’s protocol. Real-Time PCR was performed using TaqMan Gene Expression Master Mix with TaqMan assays. Ct values obtained were normalized against housekeeping gene HPRT1 and \( \Delta C_T \) calculated using Sample C as baseline. The expression difference between conditions was calculated using \( RQ = 2^{-\Delta \Delta CT} \). Monocyte processing done by Tyler Durrazzo (YMS Class of 2010); RNA isolation performed by Renata Filler; RNA expression analysis performed by Kristina Liu under the guidance of Renata Filler.
IV. Results

*In silico analysis identifies 66 upregulated transmembrane genes after ECP induction*

Comparison of the database of 466 upregulated genes identified via RNA array against the list of 1950 full-length human transmembrane genes with immunologic and hematopoietic cell surface expression yielded 87 genes in common. Further comparison against five expression profiles of human monocyte derived DCs grown in culture with GMCSF and IL-4 yielded 66 genes unique to ECP induction. Assessment of gene function, known relationship to immunologic pathways, and the comparison of raw transcriptome data prioritized 12 candidate genes for initial testing (Table 2).

**Table 2. ECP-enhanced expression of cell surface/transmembrane genes.** The 12 genes tested during the first round of experiments are shown in bold.

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<tr>
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Plate passage induces dendritic cell phenotype in normal subjects

We confirmed findings from previous investigations that plate-passage can induce a dendritic cell phenotype on processed monocytes from normal subjects\textsuperscript{34}. We analyzed the co-expression of CD83 (a marker of early DC maturation) and HLA-DR (highly expressed in DCs) to assess for evidence of a monocyte to DC transition in six normal subjects. The peripheral blood from these subjects was processed through the Glycotech chamber as a laboratory model of the ECP exposure plate. Twelve separate analyses of peripheral blood samples were run (two blood draws per normal subject on separate dates).

We observed large increases in the percentage of monocytes co-expressing CD83 and HLA-DR in both plate-passed and overnight cultured monocytes (Figure 1A). While overnight culture alone produced an increase of 12.8\% in co-expression of these two surface markers, plate-passage in addition to overnight culture resulted in an additional 5.2\% increase in expression. As shown in Figure 2, the last set of bars indicate the mean ± SD for all 11 blood draws (patient n=6), revealing significant enhancement in CD83/HLA-DR co-expression between plate-passed and overnight culture conditions (p<0.01) and the plate-passed and overnight culture versus day 0 conditions (p<0.0001 for both comparisons). Of note, the repeat blood draw from Subject 5 yielded very different results from the rest of the cohort such that it was deemed an outlier and not incorporated into statistical calculation. The membrane expression of the co-stimulatory molecules CD80 and CD86 were increased after both overnight culture and plate-
passage, as demonstrated by representative flow cytograms; however differences in the expression of these markers did not differ dramatically between overnight culture and plate-passage (Figure 2A). As expected with monocyte to DC transition, surface expression of CD14 is decreased in both plate-passed and overnight culture conditions (Figure 2B). These results confirm previous analyses performed by our laboratory demonstrating that monocytes can be induced to enter the DC differentiation pathway after both overnight culture and plate-passage. More significantly, plate passage is able to induce greater expression of DC maturation markers than overnight culture alone.

Figure 2. The percentage of monocytes induced to become leukocytes with DC phenotype as analyzed by the percentage of cells co-expressing CD83 and HLA-DR. The three experimental conditions indicate: D0= fresh PBMC; Overnight culture = cultured overnight in media without cytokines; Plate = passage through the Glycotech chamber and cultured overnight without cytokines. Dual-color flow cytometry analysis was performed on each experimental condition from 11 blood draws using 6 healthy donors. Monocyte population was gated on CD11c+ population and confirmed using forward and side scatter. Data bars to the right of graph are the mean ± SD. *, P < 0.01, compared with D0; **, P < 0.01, compared with Overnight Culture.
Figure 3. (A) Increase in co-expression of CD80 and CD86 on monocytes after overnight culture and plate-passage. Representative flow cytograms from 3 donors showing markers of maturation at three experimental conditions: D0= fresh PBMC; Overnight culture = culture overnight in media without cytokines; Plate = passage through the Glycotech chamber and cultured overnight without cytokines. Donor 1 is represented in the top 3 graphs, Donor 2 is represented in the middle graphs and Donor 4 is represented in the bottom graphs. Donors 3, 5, and 6 had similar trends of co-stimulatory marker expression (data not shown). Two-color membrane staining using CD80 (FITC) and CD86 (PE) was performed for the identification of surface expression.

(B) Decrease in surface expression of CD14 after overnight culture and plate-passage. Monocytes undergoing the transition to leukocytes with DC phenotype were further assessed for loss of CD14+ surface expression. The three experimental conditions are the same as in Figure 2. Change in CD14+ expression calculated as the difference in mean fluorescent intensity (MFI) between CD14 antibody and isotype control antibody for each subject and condition. Data bars to the right of the graph indicate mean ± SD. *, P < 0.01, compared with D0; **, P >0.05, compared with Overnight Culture.
Induction of selected surface markers in treated monocytes

Monocytes in the plate-passed condition are cultured overnight for 18 hours to allow sufficient time for gene activation, cell maturation and surface marker expression. To assess whether the observed phenotypic changes are a result of monocyte interaction with platelets and the plastic plate surface, we cultured a portion of each patient’s monocytes overnight without plate-passage to eliminate any confounding influences exerted from overnight culture alone. Baseline surface expression is established by staining PBMC on the day of sample collection. Expression of the 12 selected surface markers was assessed using flow cytometry. The monocyte population was gated using forward and side scatter and confirmed by CD11c staining. A representative flow cytometry histogram of one of the healthy subjects tested is shown in Figure 4 to demonstrate the use of ΔMFI (mean fluorescent intensity) to calculate increases in expression across experimental conditions.
Figure 4. Representative flow cytometric histogram comparing a normal subject’s monocyte population at Day 0, after overnight culture, and after plate-passage. Numbers in blue and red denote units of fluorescent intensity for isotype control (APC-IgG1) and PLAUR antibody (APC-PLAUR). $\Delta$MFI = MFI of candidate antibody (red) – MFI of isotype control antibody (blue). Monocyte population gated on CD11c+ population and confirmed using forward and side scatter. 2.67 fold and 6.55 fold increases in expression are seen after overnight culture and plate passage, respectively, as compared to fresh PBMC.

This typical flow cytometric histogram of a normal subject’s monocyte population reveals marked increases in expression after overnight culture and plate passage. This is mostly to demonstrate the way we calculated change in expression using $\Delta$MFI (subtraction of background isotype control fluorescence from fluorescent intensity of antibody staining). Here we see significant increases in $\Delta$MFI after both overnight culture and plate passage, and more interestingly, an increase from overnight culture to plate passed.
Twelve selected genes were tested using the three experimental conditions stated above. Statistically significant changes in expression after plate-passage was identified in five genes: ICAM1, PLAUR, TNFR1, CXCL16 and SIRPa. Three of these five genes, ICAM1, PLAUR and TNFR1, further demonstrated significant differences in expression between the plate-passed and overnight culture conditions. Only one gene, TNFR1, was found to have down-regulation of expression after both treatment conditions (Figure 5). These results are consistent with previous findings of a characteristic gene expression profile induced by ECP treatment. The five highly upregulated genes possess a variety of functions associated with DC adhesion, proliferation and function, further supporting the need for more focused, individual gene analyses (Table 2).
**Figure 5. Surface markers with differential expression after plate-passage.**
Calculation of \( \Delta \text{MFI} \) was performed for the twelve candidate genes across 11 separate trials. Five genes demonstrated significant changes in expression after plate-passage as compared to fresh PBMC. Two genes showed significantly increased expression after plate-passage as compared to overnight culture. Statistical analyses were performed using two-tailed, student’s t-test. Significant p-values (\( p < 0.05 \)) are identified in black; p-values > 0.05 are labeled in green.
Table 3. Candidate genes with statistically significant increases in cell surface expression after plate-passage.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC chemokine ligand 16 (CXCL16)</td>
<td>Receptor for CXCR6 expressed on activated T cells; attracts effector and cytotoxic T cells</td>
</tr>
<tr>
<td>Signal-regulatory protein alpha (SIRPa)</td>
<td>Negative regulator of tyrosine kinase-coupled signaling pathways; overexpression inhibits tumor transformation and enhance tumor apoptosis</td>
</tr>
<tr>
<td>Tumor necrosis family receptor 1 (TNFR1)</td>
<td>Though TNF signaling, mediates immunogenic and immunomodulatory functions; major roles in DC maturation, T cell priming, proliferation and recruitment</td>
</tr>
<tr>
<td>Intercellular adhesion molecule 1 (ICAM1)</td>
<td>Interacts with LFA1, responsible for cell adhesion, rolling and migration</td>
</tr>
<tr>
<td>Plasminogen activator, urokinase receptor (PLAUR)</td>
<td>Urokinase receptor involved in degradation of extracellular matrix proteins, chemotaxis, and adhesion via binding to integrins; crucial in the recruitment of T cells</td>
</tr>
</tbody>
</table>

**Increased expression of 7 transcription factors distinguish ECP-treated monocyte-derived DCs**

To identify transcription factors (TFs) expressed by ECP-treated monocyte-derived DCs but not in untreated peripheral blood monocytes, we began by performing an *in silico* analysis of the RNA array data from Berger et al.\(^{34}\) TFs were identified using cross-comparison against the TFCat (http://www.tfcat.ca/index.php) database and further selected for DC-specific gene expression and roles in immunity through extensive
literature searches. Eight candidate TFs were selected for expression comparisons by RT-PCR.

Table 3. TF genes with upregulated expression on RNA array.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell leukemia/lymphoma 3 (BCL3)</td>
<td>Expressed in bone marrow-derived DCs, substantial role in immune regulation; proto-oncogene candidate, identified in some cases of B-cell leukemia&lt;sup&gt;41,42&lt;/sup&gt;</td>
</tr>
<tr>
<td>Broad complex, Tramtrack, Bric-à-brac, and Zinc finger family 3 (BTBD3)</td>
<td>Function largely uncharacterized; activates promoter of CD56, which is involved in proliferation and anti-apoptosis&lt;sup&gt;43&lt;/sup&gt;</td>
</tr>
<tr>
<td>Broad complex, Tramtrack, Bric-à-brac, and Zinc finger family 4 (BTBD4)</td>
<td>Selectively expressed by classical DCs (cDCs) and their committed progenitors&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td>cAMP responsive element modulator (CREM)</td>
<td>Possible DC maturation marker&lt;sup&gt;44,45&lt;/sup&gt;; involved in multiple intracellular signaling cascades&lt;sup&gt;46&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1A (CDKN1A)</td>
<td>Expression tightly controlled by p53, mediates G1 cell cycle arrest; Crucial for differentiation of peripheral blood monocyte precursors to functional DCs&lt;sup&gt;47,48&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growth arrest and DNA-damage-inducible 45 alpha (GADD45A)</td>
<td>Expression increases following stressful growth arrest; mediates activation of p38/JNK pathway; expressed in late maturation of DC&lt;sup&gt;49,50&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFKB2)</td>
<td>One study shows NFkB2 to be requisite for DC cross-presentation; another shows decreased NFkB2 activity enhances DCs’ ability to induce CD4+ T cell response&lt;sup&gt;51,52&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin D receptor (VDR)</td>
<td>Nuclear hormone receptor; assists DCs in sensing their lipid environment and modulating the immune response&lt;sup&gt;53&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
To assess the effect of ECP treatment on TF expression, and to compare the impact of passage through a plate coated with varying densities of platelets, three sets of RNA samples were analyzed: Sample A = monocytes treated with a model-ECP plate coated with low density platelets, then cultured overnight; Sample B = monocytes treated with a model-ECP plate coated with high density platelets, then cultured overnight; and Sample C = monocytes cultured overnight without treatment. The samples were run in triplet and the relative quantitation, or RQ, was calculated\textsuperscript{54}. The RQ demonstrates the fold change in expression of mRNA sequences in the treatment samples, Sample A and Sample B, relative to the same sequence in the control sample, Sample C.

With the exception of GADD45A, which demonstrated essentially unchanged expression compared to the control (RQ = 1.02 and 1.03 for low- and high-density platelets, respectively), mRNA expression was increased for all seven TFs after passage through the model-ECP plate with high density platelets (Figure 6). Increased platelet density induced directional changes in expression (ΔRQ > 0.5) for VDR, NFkB2, CDKN1A and BCL3. BTBD4, a transcription factor recently identified as being specifically expressed in cDCs,\textsuperscript{37} demonstrated ~3 fold increases in expression after treatment (RQ = 2.89 and 3.14 for low- and high-density platelets, respectively). CREM, an important component of cAMP-mediated signal transduction and possible DC maturation marker, was significantly upregulated with both platelet treatments (RQ = 6.78 and 5.9 for low- and high-density platelets, respectively).
Figure 6. Seven transcription factor mRNA levels show increased expression in plate-passed monocyte-derived DCs vs. untreated monocytes. Sample A (blue) denotes plate-passage over a low-density platelet bed; Sample B (red) denotes plate-passage over a high-density platelet bed. Calculation of relative quantitation (RQ) performed via comparison to the control condition (untreated monocytes). Seven of the eight TFs demonstrated increased mRNA expression after plate-passage over high-density platelet beds. Increased platelet density induced directional changes in the expression of four TFs (ΔRQ > 0.5).
IV. Discussion

Tremendous progress in DC research has been made in the past twenty years, starting with the development of a simple method for generating DCs from peripheral blood monocytes. This method of culturing monocytes for three to six days in the presence of GMCSF and IL-4 results in populations of immature dendritic cells, which can be further induced to mature with the addition of stimulatory cytokines to the media. Results from studies by Berger et al. demonstrating the conversion of blood monocytes from CTCL patients into cells with DC phenotype raised the possibility that functional dendritic cells can be produced on a large scale without the addition of exogenous cytokines. Conceivably, DCs produced through this method would better mimic physiologic signaling. By seeking to define and characterize ECP-induced monocyte-derived DCs using surface markers and unique patterns of TF expression, these cells may one day be preferentially induced to assume immunogenic or immunosuppressive states and used to tailor ECP to the treatment of specific conditions.

The results of this study provided evidence for a phenotypic, monocyte to DC transition after plate-passage, and extended results from our laboratory’s previous findings to validate the use of the bench-top Glycotech flow chamber as an appropriate model for the significantly larger ECP UV-exposure plate. We demonstrated that plate-passage is critical for the induction of DC phenotype as monocytes cultured overnight produced significantly less differentiated leukocytes with lower surface co-expression of CD83 and HLA-DR, and higher surface expression of CD14. Two of the twelve candidate genes
also required passage through the plate for high levels of gene activation as overnight
culture induced comparably lower levels of expression. Our laboratory is currently
exploring the relationship of monocyte-to-platelet interactions as a major factor in ECP-
induced monocyte activation. To assess whether the density of the adherent platelet bed
alters monocyte gene activation, quantitative analysis of TF expression was performed
using Glycotech chambers with low- and high-density platelet beds. Four of eight TFs
(50\%) assessed demonstrated directional changes in expression with platelet density, and
seven of eight TFs (87.5\%) were found to have increased expression after plate-passage
over the high platelet bed. These findings further support our original hypothesis that
plate-passage, as a model for the ECP apparatus, rapidly produces large numbers of
monocyte-derived DCs under more physiologic environments than what previous culture
methods allowed, and these cells possess a very distinct expression pattern that will
enable its delineation, both in phenotype and function, from other DC subsets.

The five cell surface markers that demonstrated increased expression after plate-passage
merit further exploration for their potential roles in DC biology or DC-T cell interaction.
These molecules, as briefly discussed in Table 2, all possess interesting DC or
immunologically related functions. For example, CXCL16, or CXC chemokine ligand
16, is a transmembrane chemokine actively transcribed by myeloid DCs to attract effector
cells with its corresponding receptor, CXCR6.\textsuperscript{56,57} It has the unique function of being
both an endocytic receptor as well as the only known ligand of CXCR6 (CXC chemokine
receptor 6) expressed on activated T cells.\textsuperscript{58,59} Furthermore, CXCR6 expression on
central memory T cells becomes significantly upregulated after interaction with mature
DCs expressing CXCL16, suggesting that this interaction is important in supplementing the effector memory T cell response in states of infection and inflammation.\textsuperscript{59} CXCL16 appears to be one of the initially produced chemokines in the setting of viral infections to attract effectors and cytotoxic T cells to provide a timely response to curb the infection.\textsuperscript{60} It also acts as an inflammatory cytokine in the setting of bacterial infections via regulation of IFN-γ and cell-mediated immunity.\textsuperscript{58,61,62} In addition, membrane-bound CXCL16 plays important roles in antigen presentation, such as facilitating bacterial phagocytosis by professional APCs such as dendritic cells.\textsuperscript{62} Thus, increased CXCL16 expression after treatment with plate-passage may indicate enhanced aptitude of these monocyte-derived DCs in the uptake of various pathogens and antigens as well as attracting T and Natural Killer T (NKT) cells.\textsuperscript{56}

SIRPα, or signal-regulatory protein alpha, is a transmembrane glycoprotein and negative regulator of receptor tyrosine kinase-coupled signaling pathways.\textsuperscript{63} It is expressed predominantly on monocytes, DCs and their precursors. In 1999, Seiffert et al. identified the ligand for human SIRP as CD47, a broadly-expressed plasma membrane protein with myriad cellular functions.\textsuperscript{64} CD47 is associated with αβ3 integrins and regulates integrin function; in the hematopoietic system, this is limited to megakaryocytes and platelets.\textsuperscript{65} Evidence has also shown that CD47 is involved in adhesion to vitronectin, transendothelial migration and costimulation of T-cell activation\textsuperscript{66,67}. Ligation of SIRPα with a binding antibody leads to the inhibition of many leukocyte functions such as phagocytosis, transmigration and TNF production.\textsuperscript{68} In monocytes, SIRPα acts as a
negative regulator of adhesion and recruitment during inflammation, as mediated by β2 integrins. This is suggestive of SIRPα’s role in reducing the migration and attachment of monocytes and other leukocytes in the setting of inflammation. Using glioblastoma cells as an in vitro tumor model, overexpression of SIRPα1 led to inhibition of transformation and enhanced apoptosis following tumor irradiation, as well as reduced tumor cell migration. These findings were thought to result from negative regulation of EGFR-induced PI3-K pathway activation. Thus it may be worth examining the presence of CD47 expression on CTCL cells and their interaction with plate-passed monocyte-derived DCs with enhanced SIRPα expression.

The next upregulated candidate gene, TNFR1, is especially reflective of the multi-faceted nature of ECP. TNFR1, or tumor necrosis family receptor 1, belongs to a well-known family of ligands and receptors that play important roles in inflammation and autoimmunity. Using animal models with dysregulated TNF expression, it has been shown that the TNF/TNFR system possesses immunogenic and pro-inflammatory as well as immunomodulatory and disease-suppressive functions. This is supported by the evidence of TNF-α and its receptors’ pleiotropic roles in DC maturation, T cell priming and proliferation, and T cell recruitment and function. While some studies have shown that TNF-α is essential for T cell activation, others report that TNF-α and TNF receptors can actually suppress T cell immunity. This discrepancy is thought to be a result of the differential timing of TNF-α release during the immune response. TNF-α blocking agents have been used clinically with success in several inflammatory and autoimmune conditions without thorough understanding of the exact molecular level at
which TNF-α inhibitors are interfering with pathogenesis. The results observed in our study showing down-regulation of TNFR1 expression after plate-passage is interesting, and may indicate that these newly produced monocyte-derived DCs are maintained in a fairly immature state, or may be reflective of a finding that is unique to the processing of monocytes from healthy subjects. The combination of plate-passage without a pre-existing antigen to initiate or sustain an immune response may decrease the relevance of TNF-α signaling and result in the down-regulation of its receptors. Further analysis of TNFR1 expression on monocytes isolated from patients with CTCL and GVHD should be performed.

ICAM1, or intercellular adhesion molecule 1, belongs to a class of well-studied cell adhesion molecules belonging to the immunoglobulin superfamily. ICAM1 interacts with its ligand LFA1 (lymphocyte function associated antigen 1), and this pairing is integral to many aspects of normal immune function, including immune cell migration and response. Members of the CAM (cellular adhesion molecule) family are responsible for the adhesion, rolling and migration of numerous cellular subsets, including monocytes, eosinophils, memory T cells and neutrophils. Furthermore, immunogenic stimuli, such as TNF-α, lead to increased cell surface expression of ICAM1, facilitating the recruitment of leukocytes to sites of inflammation. Looking specifically at ICAM1 expression in monocytes and monocyte-derived dendritic cells (cultured in GMCSF/IL-4), studies reflect either slight down-regulation or constant expression suggestive of relative non-importance in monocyte to DC differentiation. The significant increase in expression seen in our plate-passed monocyte-derived DCs as compared to simple
overnight culture suggest an element of plate-specific stimulation of these monocytes that may allow them to more effectively engage, for example, naïve CD8+ T cells and induce their activation and differentiation into potent cytotoxic T cells. It will be important to confirm published results of ICAM1 expression on classical DCs in the same experimental context as our plate-passed monocyte-derived DCs.

PLAUR (plasminogen activator, urokinase receptor), or CD87, is an intriguing molecule involved in adhesion, proteolysis and chemotaxis. When its ligand, urokinase (uPA) binds to PLAUR, plasminogen is converted to proteolytically active plasmin and facilitates extracellular matrix protein degradation. This enzymatic activity allows monocytes and dendritic cells to penetrate collagen barriers during cell-directed migration to the periphery or lymph nodes. In addition, PLAUR can bind vitronectin, an integrin ligand, as well as other integrins at vitronectin-independent sites. It also possesses domains with chemotactic activity; in fact, PLAUR has been found to be requisite for monocyte chemotaxis in in vitro experiments using blocking antibodies and antisense oligonucleotides. Studies have shown that both immature and mature monocyte-derived dendritic cells express low levels of PLAUR. The relevance of PLAUR and uPA in the generation of appropriate immune responses has been tested using homologous recombinant mice lacking uPA. These animals exhibit an immunodeficient phenotype resulting from inadequate recruitment of T cells, and ultimately succumb to uncontrolled infection. In a study examining the role of CD87 in the activation of T cells by DCs, monocyte-derived DCs (after culture with GMCSF and IL-4) were tested in proliferation assays and mixed lymphocyte reaction assays with
CD87-blocking monoclonal antibodies. These antibodies were able to inhibit autologous T cell proliferation as well as block the activation of naïve and memory T cells, highlighting the important role of PLAUR in immune induction.\textsuperscript{91} The significant increase in the expression of PLAUR on plate-passed monocyte-derived DCs as compared to DCs after overnight culture may indicate plate-passage associated enhancement in cell-directed migration as well as T cell stimulation. These five cell surface molecules, along with other candidates in the testing pipeline, will serve to define and characterize this unique subset of ECP-induced monocyte-derived DCs that represent a more physiologic model of DCs \textit{in vivo}.

To complement the search for ECP-induced monocyte-derived DCs surface markers, we extended our \textit{in silico} analysis to transcription factors. Identification of population-specific TFs would expand and confirm categorization based on patterns of surface marker expression. Using gene expression analysis to identify TFs expressed in ECP-induced monocyte-derived DCs, we identified seven TFs with increased expression in monocytes after plate-passage (over platelet beds with high and low density). Using gene expression analysis to identify TFs expressed in ECP-induced monocyte-derived DCs, we identified seven TFs with increased expression in monocytes after plate-passage over high-density platelet beds. BTBD4, a recently identified TF specifically expressed by cDCs, was found to have significantly increased expression after plate-passage.\textsuperscript{39} While the functional role of BTBD4 is yet undetermined, it would be interesting to explore in an animal model, such as the BTBD4 knockout mice, how these cDCs function in the setting of immune challenge, or to assess the role of BTBD4 using functional assays in both
ECP-induced and GMCSF/IL-4 cultured DCs. Four TFs (VDR, NFkB2, CDKN1A and BCL3) demonstrated directional changes in expression with increased platelet bed density, suggestive of possible monocyte-platelet interactions that altered gene expression activation. VDR, or vitamin D receptor, is a nuclear hormone receptor shown to play a role in the differentiation and function of monocyte-derived DCs. The ligand of VDR, 1,25(OH)2D3 (calcitriol), is the active form of vitamin D, and is essential for the regulation of serum calcium and phosphate to maintain normal bone mineralization. Surprisingly, VDR is not just expressed in the gut or musculoskeletal system; instead it is expressed in essentially every tissue in the human body, thereby making vitamin D a key player in many conditions such as autoimmune disease, infections and cancers.

Vitamin D exerts its effect through the binding of nuclear VDR, which regulates downstream gene expression via many molecular pathways. Studies looking at the direct effect of calcitriol on monocytes and DCs have shown that it plays an essential role in DC differentiation and immunostimulatory capacity, through regulation of several genes with immune functions, particularly genes that make up a tolerogenic DC phenotype. Calcitriol treated DCs are especially adept at inducing CD4+CD25+ Tregs to limit the inflammatory response. It would be informative to see whether expression of VDR is similarly enhanced with the plate-passage of monocytes from CTCL and GVHD patients, and whether the upregulation of such an “immunosuppressive” TF is dependent on the internal milieu from which the monocytes are derived.

Recently, the noncanonical NFkB pathway has been implicated in controlling the
immunoregulatory phenotypes in both classical and plasmacytoid DCs. The noncanonical NFkB pathway refers to a pathway that involves the induction of NFkB-inducing kinase (NIK) and its downstream signaling events. Activation of this pathway involves signaling through TNFR family members, such as CD40. In a study specifically assessing the role of NFkB2 in DC function, NFkB2 was found to be crucial in DC function such as the cross-priming of CD8+ T cells. In a separate study looking at RelB, another member of the NFkB family, it was found that RelB is regulated by cytoplasmic NFkB2, which serves as a negative regulator of DC’s ability to express co-stimulatory molecules and induce CD4+ T cell responses. Given the complexity of NFkB2 signaling and its myriad of downstream effects in DC maturation and function, it will be important to assess the functional competency of plate-passed monocyte-derived DCs against GMCSF/IL-4 cultured DCs and their NFkB2 expression to further explore the aforementioned findings.

CDKN1a, a cyclin-dependent kinase inhibitor, is another TF whose expression was significantly elevated after plate-passage. Kramer et al. identified the obligate role of CDKN1a induction in monocyte differentiation. Monocytes induced to enter the DC differentiation pathway under GMCSF/IL-4 culture conditions were treated with CDKN1a antisense nucleotides, which blocked their phenotypic differentiation and ability to stimulate T cells. Furthermore, gene microarray data performed on DC exposed to T cells treated with immunostimulatory cryotreatment demonstrated upregulation of CDKN1a along with other genes involved in DC maturation, such as IL1β and Fas. BCL3 is a well-studied oncogene for hematopoietic malignancies such
as B cell chronic lymphocytic leukemias, although not much is known about its relationship to dendritic cell immunobiology. Last but not least, CREM, a cAMP responsive element modulator, demonstrated the highest fold change in expression after plate-passage compared to untreated monocytes. Reports in recent literature has found CREM to be a possible marker of monocyte-derived DC activation.44,45,101 A comparison of CDKN1a, BCL3 and CREM expression on ECP-induced monocyte-derived DCs to GMCSF/IL-4 cultured DCs will shed further light on the impact of plate-passage on monocyte differentiation. Figure 7 highlights the pertinent surface markers and transcription factors as they contribute to the process of monocyte-to-dendritic-cell differentiation.

Figure 7. Summary of the postulated roles of surface markers and transcription factors involved in development of monocyte-derived dendritic cells. DC = dendritic cell. moDC = monocyte-derived dendritic cell.
A study conducted by Woodhead et al. in 2000 demonstrated that PLAUR plays a functional role in DC activation of T cells using GMCSF/IL-4 cultured DCs. This highlights the importance of assessing the functional competence of ECP-induced DCs in the context of each of our candidate genes, many of which have available blocking antibodies. To determine if plate-passed monocyte-derived DCs are capable of stimulating normal T cell responses to MHC Class I and II antigens or mediating cytotoxicity by targeting HLA on specific lymphocytes, we can employ antigen presenting assays or mix lymphocyte reaction assays. Another major objective is to expand the number of subjects assessed to confirm the universal presence of our candidate genes, as well as look for gene expression in the peripheral blood of patients with CTCL, to assess how a diseased internal milieu or treatment with the actual ECP apparatus alters candidate gene expression. We are continuing to identify additional surface markers from the list of 66 candidate genes using the same methodology described above. Ultimately, we hope these surface markers and TFs will serve to better define and characterize this unique population of ECP-induced monocyte-derived DCs. On a more global scale, this project fits nicely into a larger endeavor to better understand the efficacy of the ECP apparatus, likely mediated in large part through dendritic cells, and have significant implications in defining a process of developing more physiologic DCs for other branches of research.
Appendix A: DNA Copy-Number Analysis of Selected Genes in Patients with CTCL

I. Introduction

Copy-number variations (CNVs) are extensive genomic structural variations that range in size from kilobases to megabases. CNVs can result from deletions, duplications, inversions and translocations, which can lead to deletion of a gene or acquisition of additional copies of a gene. Early studies of genetic alterations in CTCL identified candidate regions containing DNA copy-number variations, some of which potentially correlate with disease progression and prognosis.\textsuperscript{102} Previously, a high-resolution genomic analysis and meta-analysis of patients with leukemic CTCL performed in our laboratory has defined regions of significant copy-number variation to help discern some of the common DNA copy-number alterations characteristic of CTCL.\textsuperscript{103} These regions of copy-number variation can contain a high number of genes; for example, chromosome 17q contains over 300 genes. This current study focuses on select regions of amplification to assess copy-number amplification on a gene-specific level.

II. Statement of Purpose

To validate gene specific copy number amplifications and assess whether these changes occur consistently across a sample population of CTCL patients, with the goal of finding genetic alterations that play a role in pathogenesis.
III. Methods

The top six regions of amplification based on the highest frequency and magnitude of gain from our previously published genomic analysis were selected (Table A1). Within these regions of interest, 381 genes were prioritized using five selection criteria (Figure A1). From this list, six genes fulfilled four of the five selection criteria and twenty-one genes fulfilled three of the five selection criteria. These 27 genes were then further filtered for the availability of targeted therapy to comprise the final list of ten genes: STAT3, BECN1, SKAP1, NGFR, TAC4, PRKAR1a, HN1, GRB2, SPHK1 and UBR4.

Table A1. Significant regions of DNA copy-number amplification described by Lin et al. (JID 2011).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cytoband</th>
<th># of Samples</th>
<th>Genes in Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8q23.3</td>
<td>14</td>
<td>1 gene: CSMD3</td>
</tr>
<tr>
<td>2</td>
<td>17q25.1</td>
<td>8</td>
<td>371 genes</td>
</tr>
<tr>
<td>3</td>
<td>1p36.13</td>
<td>5</td>
<td>1 gene: UBR4</td>
</tr>
<tr>
<td>4</td>
<td>3p11.1</td>
<td>8</td>
<td>5 genes: PROS1, NSUN3, ARL138, DHFRL1, STX19</td>
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<tr>
<td>5</td>
<td>6p21.1</td>
<td>2</td>
<td>1 gene: C6orf223</td>
</tr>
<tr>
<td>6</td>
<td>8p23.3</td>
<td>8</td>
<td>2 genes: DLGAP2, ERICH1</td>
</tr>
</tbody>
</table>
Copy number alterations occurring in these ten genes were assessed by quantitative PCR (TaqMan Copy Number Assays, Applied Biosystems) using DNA prepared from purified malignant T cells from 14 patients. Two sets of controls were used for comparison: pooled DNA from CD4+ T cells of healthy volunteers and purchased pooled human DNA (Promega, Fitchburg, WI). Six genes showing copy number amplification were further tested using DNA from 5 CTCL and 1 T-cell lymphoma cell line.

### IV. Results

As Figure A2 shows, copy number amplification for each selected gene is calculated relative to both sets of controls (N1 = normal volunteers; N2 = Promega pooled human genomic DNA). For the analysis of SKAP1, the copy number amplification calculated is

<table>
<thead>
<tr>
<th>Gene Selection Workflow</th>
<th>Genes = 381</th>
<th>Fulfills 3 of 5 selection criteria</th>
<th>Genes = 27</th>
<th>Individual assessment “plus and minus”</th>
<th>Genes = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>8q23.3</td>
<td>17q25.1</td>
<td>Selection Criteria:</td>
<td>Genes = 27</td>
<td>Assessment:</td>
<td>Genes = 10</td>
</tr>
<tr>
<td>1p36.13</td>
<td>3p11.1</td>
<td>1. Previously found to have CN amp in CTCL</td>
<td></td>
<td>• Functionality &amp; possible pathway</td>
<td></td>
</tr>
<tr>
<td>6p21.1</td>
<td>8p23.3</td>
<td>2. Possesses T-cell specific function</td>
<td></td>
<td>• Known targeted therapy</td>
<td></td>
</tr>
<tr>
<td>Genes = 10</td>
<td></td>
<td>3. Regulates cell cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regions of interest</td>
<td></td>
<td>4. Favors genetic stability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Lin et al. JID 2012 ranked these regions of CN amp</td>
<td></td>
<td>5. Overexpressed in other cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Magnitude</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure A1.** Gene selection workflow used in the prioritization of candidate genes for copy-number analysis.
closely matched between the two controls, and 8 of 14 patients were found to have copy-number amplification >2.5 (Figure A2a). Conversely, Figure A2b shows NGFR, which demonstrated poor correlation between the calculated copy-number amplifications between the two controls.

**Figure A2.** **Representative graph of CNV in SKAP1** (a), a top-ranking candidate gene, showing 43% of patients with >3 gene copies. Copy-number amplifications relative to both controls are comparatively similar. In contrast, there are disparities in copy-number amplifications relative to controls for NGFR (b). Genes exhibiting such differences in calculated CN were excluded from further analysis due to inability to ascertain true number of gene copies.
Of the ten genes, one gene was excluded from further analysis due to lack of amplification, three genes were excluded based on lack of correlation between the two controls used, and six genes were amplified in >30% of patients tested (Table A2). Four genes (SKAP1, PRKAR1a, GRB2 and STAT3) were then further tested for copy-number amplifications using DNA from four CTCL and one T cell lymphoma cell lines. STAT3 was amplified in four of the six cell lines. Amplifications of the other three genes were found in certain cell lines, although the results did not appear gene-specific (Table A3).
Table A2. Summary of gene copy numbers calculated relative to normal (CN=2.00).
N1 indicates pooled normal controls; N2 indicates purchased Promega normal DNA.
Shaded boxes indicate calculated copy number amplification (CN > 2.5).
### Figure A3. Summary of copy number analysis for five CTCL cell lines (SeAx, Sez4, Pno, Hut78, MyLa) and one T cell lymphoma cell line (Jurkat).

N1 indicates pooled normal controls; N2 indicates purchased Promega normal DNA. Shaded boxes indicate calculated copy number amplification (CN >2.5).

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Control DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STAT3</strong></td>
<td><strong>SKAP1</strong></td>
</tr>
<tr>
<td>Cytogenetic Band</td>
<td>17q21.2</td>
</tr>
<tr>
<td>GeneLoc Start</td>
<td>40,465,342</td>
</tr>
<tr>
<td>GeneLoc End</td>
<td>40,540,513</td>
</tr>
<tr>
<td><strong>SeAx</strong> N1</td>
<td>2.66</td>
</tr>
<tr>
<td>N2</td>
<td>2.55</td>
</tr>
<tr>
<td><strong>Sez4</strong> N1</td>
<td>2.64</td>
</tr>
<tr>
<td>N2</td>
<td>2.54</td>
</tr>
<tr>
<td><strong>Pno</strong> N1</td>
<td>2.30</td>
</tr>
<tr>
<td>N2</td>
<td>2.21</td>
</tr>
<tr>
<td><strong>Jurkat</strong> N1</td>
<td>2.08</td>
</tr>
<tr>
<td>N2</td>
<td>2.00</td>
</tr>
<tr>
<td><strong>Hut78</strong> N1</td>
<td>3.85</td>
</tr>
<tr>
<td>N2</td>
<td>3.70</td>
</tr>
<tr>
<td><strong>MyLa</strong> N1</td>
<td>4.45</td>
</tr>
<tr>
<td>N2</td>
<td>4.28</td>
</tr>
</tbody>
</table>

V. Discussion

In recent years, significant research effort has been directed toward understanding the molecular genetic events underlying multiple types of malignancies with the hopes of improving diagnosis and identifying novel therapeutic targets. Studies utilizing comparative genomic hybridization and copy number analysis have unearthed a number of chromosomal abnormalities in CTCL, although with varying results. In this study, we aimed to build upon previous work performed in our laboratory to validate...
gene-specific copy number amplifications to delineate genetic alternations characteristic of CTCL.

Six genes within candidate regions of copy-number amplification were identified via our analysis: SKAP1, PRKAR1a, STAT3, BECN1, GRB2 and HN1. SKAP1, a src kinase associated phosphoprotein, encodes a T-cell adaptor protein that plays a critical role in the T cell receptor “inside-out” signaling. SKAP1 complexes with RapL to bind lymphocyte function-associated antigen 1 (LFA-1), a β2 family integrin, in a pathway that regulates T cell adhesion, motility and interaction time with antigen presenting cells.\textsuperscript{110,111} Furthermore, van Doorn et al. found that SKAP1 resides in a loci of recurrent gain and demonstrated copy-number amplification associated increases in expression in MF.\textsuperscript{112} Another candidate gene, PRKAR1a, is a cAMP-dependent protein kinase regulatory subunit shown to be amplified and overexpressed in analyses by Lin et. al and Caprini et. al.\textsuperscript{103,106} As discussed in Lin et. al, overexpression of PRKAR1a has been found to induce increased expression of IL-2; indeed, it is possible PRKAR1a amplification may result in IL-2 pathway activation, thereby enhancing the proliferation of malignant T cells through the up-regulation of JAK/STAT, P3K/AKT and mitogen-activated protein kinase pathways.\textsuperscript{103,113,114} It is also noteworthy that STAT3, also involved in the IL-2 signaling pathway and whose constitutive activation has been implicated in Sézary syndrome, is another highly prioritized candidate gene.\textsuperscript{115-117}

Several genes were excluded from further analysis using cell line DNA due to disparities in copy number calculation based on the two controls employed. This may have resulted
from differential binding of the chosen primers with purchased versus pooled control DNA. Due to these large differences in calculated copy number, we were unable to accurately assess whether true amplification occurred and will defer further testing of these genes until further analysis. Copy number analysis using CTCL and T cell lymphoma cell lines demonstrated inconsistent amplification in all genes except STAT3, a result consistent with previous reports of amplification and differential expression of STAT3 in Sézary syndrome. Uniform amplification of all six genes was seen in Sez4 and Hut78 cell lines, possibly indicating more widespread genetic alterations within these cell lines that are reflective of potential dissimilarities between patient samples and cells lines, which have undergone multiple passages requiring additional growth factors.

Looking forward, these genes with copy number variations will need confirmation of gene expression changes using quantitative real-time PCR. If a dosage-effect is seen, then expression can be inhibited with siRNA technology to assess the genes’ functions in proliferation or cellular functions of CTCL cells. We have long believed that persistent changes at the genomic level in cancer cells represent a critical target for the study of carcinogenesis. We hope to have contributed to this effort through the study of selected candidate genes in CTCL patients as well as established cell lines, and that this may serve as part of the platform on which functional validation of copy number variation and potential therapeutic ventures can launch.
Appendix B: Identification of Novel Genetic Alterations in CTCL via Whole Exome Sequencing

Recent advances in genome-wide analyses have significantly increased our understanding of the genomic landscape behind human diseases, including cutaneous T-cell lymphoma.\textsuperscript{105,106,118} The human genome is comprised of ~3 billion nucleotides of DNA; however, only a small percentage – 1.5 percent – of those nucleotides are actually translated into proteins that play a functional role in the body. Sequencing of the “exome” therefore arises as an ideal strategy for the identification of genetic variants that underlie human disorders since the vast majority of variants that have effect on disease-related traits encode protein. Furthermore, the cost of whole exome sequencing remains significantly less expensive than whole genome sequencing, making this a novel, robust and cost-effective method for the identification of functional variation responsible for cutaneous T-cell lymphoma.\textsuperscript{119-121}

We hypothesized that there exist single nucleotide variants within the malignant clone of CTCL cells that are: 1) associated with pathogenesis, in particular those leading to the development of malignant or immunosuppressive phenotypes; 2) associated with prognosis and markers for disease staging; and 3) useful for the identification of potential novel targets for therapeutic intervention. To test this hypothesis, we selected for phenotypically similar patients with clinically severe Sézary syndrome. Patients enrolled include patients actively being treated in the photopheresis unit, past patients of photopheresis (currently receiving chemotherapy at Smilow Cancer Center) or past patients whose samples have been frozen down for previous studies conducted in our
Malignant T lymphocytes were enriched from the peripheral blood of these patients using: 1) Vβ-specific antibody if available, or 2) negative selection with either CD7 or CD26 antibodies as determined based on phenotypic data. Cell aliquots are removed during the enrichment process to assess for cell viability, to verify the purity of the enriched populations, as well as confirm certain phenotypic features of the cell populations (Figure B1). CD4+ T cells from healthy donors are also isolated via positive selection using CD4+ magnetic beads for use as the control.

Figure B1. Purification of malignant T cells using negative selection. Peripheral blood samples of CTCL patients are enriched for the malignant T lymphocyte population using negative selection with CD7 and CD26 antibodies (as determined by flow cytometric phenotypic data in this patient). Cell aliquots are removed to assess for viability using forward and side scatter (data not shown). Compared to the unfractionated population (a) with 12.4% CD3+CD4+ population, the enriched fraction is 93.5% CD3+CD4+ (C) as well as >99% CD7-CD26- (b).

Genomic DNA was purified from 18 patients and two healthy donors. Samples of sufficiently high purity were submitted for whole exome sequencing, and the identification of variants is currently underway with results available pending completion.
Appendix C: Detection of peripheral blood involvement in CTCL by multi-parametric flow cytometry analysis

Identification and quantification of Sézary cells can be monitored through analysis of the peripheral blood, e.g. flow cytometry, including T-cell receptor variable region beta (TCR-Vβ). At Yale we use multiple T cell markers for the diagnosis and tracking of disease burden in patients with leukemic CTCL. We sought to explore the utility of a multi-parametric flow cytometry strategy in the evaluation of tumor burden in CTCL by conducting a prospective case-series of 13 patients referred to the Yale Photopheresis Clinic over 18 months. Flow cytometry analysis was performed using multiple TCR markers, including: CD2, CD3, CD4, CD5, CD7, CD8, CD25, CD26, CD45, CD45RA, CD45RO (Yale Laboratory Medicine) and TCR-Vβ panel (ARUP Laboratories, Salt Lake City, UT).

Flow cytometry consistently revealed a phenotypically abnormal T cell population with altered expression of lymphocyte markers, including CD3, CD4, CD7 and CD26. Nine of the thirteen patients had loss of CD7, twelve of thirteen patients had loss CD26 and eight of thirteen patients had alterations in additional markers (Table C1, Figure C1).
### Table C1. T cell markers used at Yale to identify the neoplastic population in CTCL patients.

Patients 1-7 have expanded TCR Vβ found on ARUP panel; patients 8-13 do not have an identifiable TCR Vβ. Notably, 100% of patients’ neoplastic populations were CD5+ and CD8- (data not shown). Loss of CD7, a common phenotype observed in circulating CTCL cells, was only observed in 64% of patients. 93% of patients had partial or complete loss of CD26. Other significant phenotypic changes include alterations in CD2, CD3, CD4, CD25 and CD45.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4/CD8</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
<th>CD7</th>
<th>CD4+7-%</th>
<th>CD25</th>
<th>CD26</th>
<th>CD4+26-%</th>
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<tbody>
<tr>
<td>1</td>
<td>3.6</td>
<td>+</td>
<td>dim</td>
<td>dim</td>
<td>+</td>
<td>16.4</td>
<td>+</td>
<td>-</td>
<td>50.2</td>
<td>RO dim</td>
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<tr>
<td>2</td>
<td>26.8</td>
<td>-</td>
<td>dim</td>
<td>dim</td>
<td>dim</td>
<td>69.8</td>
<td>-</td>
<td>-</td>
<td>71.4</td>
<td>RO +</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>dim</td>
<td>47.8</td>
<td>dim</td>
<td>-</td>
<td>62.6</td>
<td>RA+/RO dim</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>dim</td>
<td>dim</td>
<td>7.6</td>
<td>-</td>
<td>-</td>
<td>28.7</td>
<td>RO +</td>
</tr>
<tr>
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<td>dim</td>
<td>+</td>
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<td>-</td>
<td>dim</td>
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<td>-</td>
<td>65.9</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>15.8</td>
<td>-</td>
<td>-</td>
<td>17.7</td>
<td>RO+</td>
</tr>
</tbody>
</table>
Figure C1. Initial flow cytometry analysis on Patient 1. All plots shown are derived from the lymphocyte gate. ~10% of total circulating cells (30% of lymphocytes) are CD3dim+ CD4dim+ CD8− CD2+ CD5+ CD7+ CD26− T cells, consistent with involvement of peripheral blood by the patient's known T-cell lymphoproliferative disease (Flow cytograms generated by Yale Laboratory Medicine).

The abnormal population appeared to be highly enriched for the neoplastic T cell clone. In seven of thirteen patients with detectable TCR-Vβ, enrichment of TCR-Vβ in the abnormal population had a mean of 88% (range 66-95%) (Table C2, Figure C2).
Table C2. TCR- Vβ and blood involvement characteristics of Vβ positive patients.

Lymphocytes with atypical phenotype refer to the population delineated by markers shown in Table 1. Vβ panel of antibodies (ARUP) were used to assess relative prevalence of T cells expressing each of these markers, specifically within the atypical populations. In patients with low tumor burdens, such as patient 1, the percentage of Vβ in total lymphocytes is 23% vs. in 91% in the atypical population, showing that this population is highly enriched for the neoplastic clone.

<table>
<thead>
<tr>
<th>Patient</th>
<th>TCR Vβ</th>
<th>% of Lymphocytes w/ Atypical Phenotype</th>
<th>% Vβ in Atypical Population</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>25</td>
<td>91</td>
<td>1.19-4.60</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>67</td>
<td>85</td>
<td>0.47-2.91</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>45</td>
<td>95</td>
<td>1.08-1.93</td>
</tr>
<tr>
<td>4</td>
<td>13.2</td>
<td>30</td>
<td>93</td>
<td>1.31-6.55</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>50</td>
<td>94</td>
<td>1.19-4.60</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>71</td>
<td>66</td>
<td>4.16-8.91</td>
</tr>
<tr>
<td>7</td>
<td>7.2</td>
<td>78</td>
<td>92</td>
<td>0.02-3.12</td>
</tr>
</tbody>
</table>

Figure C2. Immunophenotypic analysis of the peripheral blood specimen from patient 1 performed by ARUP laboratories. Abnormal population (CD3dim, CD4dim, CD8-) is 25% of lymphocytes, and of that population, 91% express TCR Vβ 20. This finding meets the criteria for T-cell monoclonality. The other T cell subsets (bright CD3+, CD4+) show levels of TCR- Vβ expression close to or within normal range.
In patients without the presence of an obvious TCR-Vβ clone, we have nevertheless been able to identify the presence of a phenotypically abnormal population with alterations in multiple similar T cell markers (Table C1). While the markers were variably lost from patient to patient, within each individual the phenotypically abnormal lymphocyte population retained consistent immunophenotype. For example, the abnormal population in Patient 2 was consistently CD3dim, CD4dim, CD7- and CD26- over 12 months (data not shown). The use of CD3, CD4, CD7 and CD26 facilitated the identification of the abnormal population in two patients who did not meet criteria using CD7 and CD26 alone based on ISCL criteria.\textsuperscript{122}

Leukemic CTCL can mimic a number of other erythrodermic conditions clinically, including severe psoriasis, atopic dermatitis, GVHD and pityriasis rubra pilaris. Diagnosis can be facilitated by the identification of specific peripheral blood findings, such as an increased CD4/CD8 ratio, aberrant expression or loss of CD26 and CD7, as well as T cell clonality as determined by Vβ testing.\textsuperscript{123-126} However, due to only moderate sensitivity and specificity offered by the commonly used markers, the diagnosis Sézary syndrome using any single marker remains unsatisfactory.\textsuperscript{123,124,127} The addition of T cell receptor Vβ chain antibodies, especially when analyzed on gated T cells with an aberrant immunophenotype, improved detection sensitivity for the neoplastic clone as well as for the monitoring of tumor burden; however T cell clonality can occur under reactive, nonmalignant conditions and patients can have neoplastic proliferation without an identifiable Vβ chain.\textsuperscript{124,128-130} Therefore, the accurate diagnosis of Sézary syndrome likely relies upon the usage of multiple markers on flow cytometry, and our results reflect
added benefits of assessing for aberrant CD2, CD3, CD4, CD5 and CD45RA/RO expression, particularly in patients with low tumor burden.\textsuperscript{131,132}

Limitations of this study include the small sample size, sampling from a single academic center, as well as the lack of extension to clinical status and disease prognosis. This project is being extended to involve larger numbers of patients with lengthier follow-up, and data on clinical status and treatment response will be collected towards the goal of establishing more sensitive criteria for the diagnosis and monitoring of tumor burden.
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