2-23-2009

Genotypic Confirmation of Transimmunization-Induced Dendritic Cell Maturation

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

by

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2008

GENOTYPIC CONFIRMATION OF TRANSIMMUNIZATION-INDUCED DENDRITIC CELL MATURATION

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Transimmunization (TI), a novel modification of the widely used immunotherapy extracorporeal photopheresis (ECP), induces conversion of processed monocytes into cells expressing phenotypic and functional features of dendritic antigen presenting cells (DC). To further characterize TI-induced DC, we analyzed differential gene expression in the monocyte/DC population after TI treatment. Because ECP, the therapy upon which TI is based, has the unique capacity to induce both anti-cancer immune responses in cutaneous T cell lymphoma (CTCL) patients and tolerogenic responses in graft-versus-host disease (GVHD), we studied TI-induced gene expression changes in both of these patient populations as well as in healthy normal control individuals with the goal of fully characterizing the gene expression profile(s) induced by TI. Peripheral blood leukocytes from 6 patients (3 patients with CTCL and 3 patients with GVHD) were procured prior to ECP, immediately after ECP, and following TI processing, and were then enriched for monocytes/DC. RNA was extracted and gene expression compared using Affymetrix total human genome microarrays to analyze 39,000 genes. Differential gene expression was considered as a $\geq$ 2-fold change and P-value $\leq$0.05. TI induced significant up-regulation of genes associated with DC maturation including: DC-LAMP, CD80, CD40, and Decysin. In addition, TI induced down-regulation of monocyte genes such as CD33 and CD36. These changes in gene expression were seen in both CTCL and GVHD patients, suggesting that TI is capable of mediating DC differentiation regardless of disease process. However, some genes (e.g. IL-19, Tryptophan 2,3-dioxygenase) were differentially expressed after TI only in GVHD patients, while others, (e.g. heat-shock proteins 70, 27, and 40) were differentially expressed only in CTCL patients. Our microarray findings were confirmed by quantitative real-time PCR on patient samples as well as on samples from healthy normal controls that underwent the TI procedure. Analysis of the microarray data using GeneGo pathway analysis software demonstrated that the chemokines and adhesion signaling pathway was significantly involved in the mechanism of both ECP and TI, suggesting a crucial role for cell adhesion in these therapies. Taken together, our gene expression and pathway data suggest that TI activates specific signaling cascades that lead to activation and up-regulation of mature DC genes. Our results support the use of TI as a method of generating mature dendritic cells for immunotherapy.

Acknowledgements

First and foremost, I would like to thank my advisors Dr. Carole Berger and Dr. Richard Edelson. Words can simply not express how grateful I am for all you have done for me and how fortunate I feel to have been a part of your research program. Dr. Edelson, your enthusiasm and energy are contagious, and your ability to inspire others is truly remarkable. I hope one day to be able to inspire in others even the smallest amount of hope and purpose you have inspired in me. Your insightful comments, constant encouragement, and wonderfully entertaining stories and advice about science, medicine, and life have been a true highlight of this year. It has been an experience I will carry with me for the rest of my life and one that I am confident will shape my future career.

The person most responsible for my accomplishments and personal growth throughout my time in lab is Dr. Carole Berger. I am so grateful I have had the opportunity to interact with such a wonderful mentor, outstanding scientist, and truly caring person on a daily basis for the past year and a half. Your guidance and dedication are the reason I have been able to accomplish the work presented here. You are a superb mentor and also a wonderful friend- any time I had a question or idea your door was open and you were willing to help. Thank you for all of your fantastic advice, about science and also about life. I am so grateful for all you have done for me- you have truly been the best advisor a student could ask for.

A huge thanks to fellow lab members Dr. Gabe Vasquez, Nianci Wang, and Zimei Zhang. Thank you for all of your help and also for your excellent camaraderie and companionship- you have made my experience in lab such a positive and enjoyable one.

Thank you to Dr. Tigelaar and Dr. Girardi for their scientific guidance and advice. Also thank you for agreeing to be on my thesis committee and for your help with this document. I have the utmost respect for both of you as careful and thoughtful scientists and I am extremely grateful for your input on this work.

Thank you to all of the Yale Photopheresis patients who donated samples of their photopheresis treatments so that we could accomplish this project. Your sense of hope has been energizing and truly humbling. My proudest accomplishment would be if this work would ultimately benefit you and others with your disease.

I would also like to acknowledge research support from the NIH T32 Clinical Research Fellowship and the New York Cardiac Center. These funds, along with additional funding for laboratory supplies and microarray experiments from the Yale SPORE, made this project possible.

Last but certainly not least, thank you to my family and friends. Thank you Hristos for your endless encouragement and support. Thank you to Claire and Natalie, the best sisters in the entire world, for always being able to make me laugh and for your countless encouraging words- you are the best! Thank you to my parents for being a constant source of encouragement, moral support, and above all, inspiration.

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Introduction

Extracorporeal photopheresis (ECP) is a novel immunomodulatory therapy that was initially developed to treat cutaneous T cell lymphoma (CTCL). This therapy holds the distinction of being the first FDA-approved selective immunotherapy for cancer, having obtained an expedited approval in 1988 because of its impressive clinical efficacy. Over the past 20 years ECP has been administered over 250,000 times at roughly 150 treatment centers throughout the USA and Europe (1). Not only is this therapy effective, it also has an excellent safety profile with no limiting toxicity and remarkably few adverse reactions (2). Since the inception of ECP in the early 1980s, its success has been driven by clinical results. The initial report on ECP, published in the *New England Journal of Medicine* in 1987, described the effects of ECP treatment on 37 patients with otherwise resistant CTCL. 27 of these patients responded to ECP, with an average 64% decrease in cutaneous involvement (3). Two patients with severe erythrodermic CTCL experienced complete remissions in response to ECP, a provocative result because spontaneous remissions do not occur in this type of CTCL, indicating the remissions were due to ECP (4).

The rich history of the development of ECP starts with the curiosity of Dr. Richard Edelson, a dermatologist then at Columbia University. Dr. Edelson played a large role in the research characterizing CTCL, a clonal expansion of skin-associated CD4+ helper T cells (5). In early stages of the disease, the malignant cells are confined to the epidermis in patches and plaques (5). In more aggressive cases of CTCL the

malignant CD4+ cells can spread throughout the skin and also to the blood, causing diffuse erythroderma and severe leukemic disease (6). A common treatment for plaque-stage CTCL is PUVA therapy (P, psoralen and UVA, ultraviolet light). In this therapy, orally administered 8-methoxypsoralen (8-MOP) is photoactivated by exposure of diseased skin to ultraviolet A. When activated, 8-MOP is transformed to a transiently excited state, in which it is capable of covalently cross-linking DNA (7). Importantly, in its biologically inert state 8-MOP is completely safe and is excreted from the body in 24 hours, making it a unique chemotherapeutic agent. Dr. Edelson saw that PUVA therapy offered plaque-stage CTCL patients an effective treatment with few side effects, and he speculated whether a variation of this therapy could somehow be used to treat CTCL patients with more severe disease, especially those in whom the malignant CD4+ cells had spread to the blood. At the time, patients with the erythrodermic and leukemic phase of CTCL were commonly resistant to chemotherapy and had a survival of less than 30 months (3). In some patients, intensive leukapheresis (removal of blood leukocytes) was effective in slowing the disease course (3). Armed with the knowledge of the effectiveness and safety of PUVA and the experience in using leukapheresis, Dr. Edelson and colleagues conceived a completely novel therapy, which they termed Extracorporeal Photopheresis. This therapy involved administering oral 8-methoxypsoralen to the patient and then performing a leukapheresis step, collecting the patient's white blood cells and plasma. The 8-MOP treated white blood cells and plasma were then passed through thin plastic tubing sandwiched between UVA lights, activating the 8-MOP. After treating the patient's leukocytes with 8-MOP + UVA, the treated cells were returned back to the patient. The original goal of ECP was simply to induce cell death in

the treated malignant CD4+ cells. However, Edelson and colleagues observed that this therapy induced a much greater anti-cancer response than simply causing apoptosis of treated cells. Since only 5% of malignant cells pass through the ECP apparatus during each treatment procedure, the fact that ECP induced full remissions in a subset of patients and partial remissions in others (with an average 64% decrease in cutaneous involvement) implied that ECP was capable of inducing a systemic anticancer response. Edelson and colleagues set out to learn more about the mysterious mechanism of ECP, asking how a therapy that only treats a fraction of malignant cells produces such profound clinical effects.

There were several important clinical observations and scientific findings that lead to an understanding of the cellular mechanism of ECP. First, it was observed that the best responders to ECP were patients that had a relatively intact immune system, reflected as a near normal CD4:CD8 ratio, before beginning therapy (8). These patients had robust numbers of CD8+ cytotoxic T lymphocytes, the cell type capable of recognizing cellular antigen in the context of MHC class I and then inducing death in target cells (9). At the time ECP was introduced, scientists had recently discovered that CD8+ T cells could recognize tumor antigens and cause tumor cell death (9). Since an intact CD8+ T cell compartment seemed critical for a successful clinical response to ECP, Dr. Edelson and colleagues became interested in how ECP affected CD8+ T cells.

The crucial piece of information came from studies examining apoptotic cell death after ECP. Yoo and colleagues found that ECP induced apoptosis in all peripheral

blood T cell subpopulations, including the malignant CD4+ T cells. However, the monocyte population did not undergo apoptosis after ECP (10). In fact, instead of dying, the monocytes (normally responsible for surveying the cellular environment and ingesting potential antigenic material) were observed phagocytosing the ECP-treated apoptotic cells. Monocytes may serve as precursors to dendritic cells, or antigen presenting cells, which play a crucial role in the immune system. Dendritic cells (DC) display antigen in the context of MHC molecules for recognition by lymphocytes (9). Fascinated that monocytes were resistant to ECP-mediated apoptosis, Berger and colleagues set out to characterize ECP-treated monocytes. Their results showed that ECP treatment activated monocytes and initiated their differentiation into DC (11). These ECP-induced DC actively phagocytose apoptotic lymphocytes, process cellular antigen, and present tumor-specific CTCL antigens on MHC molecules (12). When CTCL antigens are presented in the context of MHC I molecules, they can be recognized by cytotoxic CD8+ T cells, and these cells are capable of mediating an anti-tumor immune response (13). The fact that patients with a relatively normal CD8+ T cell compartment respond best to therapy supports the idea that ECP-induced DC are stimulating a CD8+ T cell response against CTCL cells.

An important link between ECP-induced apoptosis and initiation of an anti-tumor CD8+ T cell response is likely the presentation of tumor antigen by dendritic cells (11). The fact that ECP induces monocyte to DC differentiation is extremely important and makes ECP a powerful immunotherapy. In considering how monocyte activation occurs during ECP, one hypothesis has been that the passage of monocytes through the thin (1

mm thick) plastic UVA exposure plate induces DC differentiation. Monocytes are known to adhere to plastic, activating cell adhesion molecules and downstream signaling cascades (14). The flow turbulence and sheering forces in the ECP apparatus likely create a situation where the monocytes repetitively adhere and disadhere to the plastic surface, amplifying cellular signals and leading to monocyte activation (12).

As researchers worked to uncover the mechanism underlying ECP, the clinical use of this therapy dramatically accelerated. The initial report published in 1987 by Edelson and colleagues demonstrated a 64% response rate and inspired many clinicians to use ECP to treat refractory CTCL. Data published on more than 500 CTCL patients receiving ECP at centers around the world confirmed the impressive initial clinical findings of Edelson, with an average overall response rate of 62% and an average 20% complete response rate (4). In addition, a follow-up study in which ECP was compared with historical controls demonstrated that ECP increased survival time from 30 months to more than 66 months (15).

Since CTCL is characterized by a clonal population of T cells and ECP appeared to have selective effects on this pathogenic T cell population, clinicians began to evaluate the use of ECP to treat other T cell mediated diseases (4). ECP has been tested in a variety of nonmalignant immune-mediated diseases, such as systemic sclerosis, pemphigus vulgaris, rheumatoid arthritis, psoriatic arthritis, systemic lupus erthematosus, and to a lesser extent atopic dermatitis and inflammatory bowel disease (4). In addition, ECP has been used to treat acute and chronic organ transplant rejection and graft versus

host disease (GVHD) after allogeneic bone marrow transplantation. In a review of 184 cases of chronic GVHD treated with ECP, the response rates were as follows: skin (75% response), liver (66% response), lung (25% response), gut (18% response) and mucous membranes (68% response) (16). The response rates for acute GVHD were 58% in skin and 40% in liver (16). In the majority of patients with GVHD, concurrent immunosuppression could be reduced during ECP and no increase in opportunistic infections was reported (16). A trial testing ECP for the prevention of rejection in cardiac transplantation, published in the New England Journal of Medicine, found that ECP significantly decreased the risk of cardiac rejection without increasing the incidence of infection (17). ECP has subsequently been used to treat lung and renal transplant rejection in a small number of patients and has been reported to be of benefit (18, 19). The fact that ECP has a dose-sparing effect on concurrent immunosuppressive therapy is important for GVHD and organ transplant rejection patients because it may spare these patients, who often are on multiple immunosuppressive agents, some of the adverse effects associated with long-term immunosuppression. The growing acceptance of ECP for transplant rejection is evidenced by the use of ECP as part of the anti-rejection regimen in the recent high-profile French face transplant (20). This patient received treatment with the French version of ECP, which uses a slightly different apparatus than the Therakos machine used here in the United States. In France, a "two independent steps" technique is used, in which the first step consists of mononuclear cell collection by a continuous blood cell separator, and then irradiation of collected cells is performed using an independent machine (21).

The fact ECP appears to be able to generate selective immunosuppression in various autoimmune and alloimmune disorders has generated interest among researchers, particularly because in CTCL patients this same therapy appears to generate a selective anti-tumor immune response (22). How could ECP generate tolerance in some patients and an anti-tumor immune response in others? The answer may lie in the ability of ECP to initiate monocyte to dendritic cell differentiation, for the simple reason that dendritic cells control T cell responses. DC are capable of mediating such diverse, contradictory functions as anti-tumor immune responses and tolerance depending on the signals they send to T cells as they present antigen (23). It is thought that DC can "decode" environmental signals, and depending on the presence or absence of certain inflammatory or "danger" signals present at the time they are capturing and processing antigen, develop into either DC capable of stimulating T cell responses or DC that induce tolerance (23). With this in mind, it is important to mention that in ECP therapy the environment in which newly differentiating DC capture antigen (apoptotic cells) is in the patient. Because 8-MOP + UVA induces a gradual apoptosis, the lymphocytes do not die during the ECP procedure itself (which takes about 3 hours), but instead undergo apoptosis after they are reinfused into the patient (11). The presence or absence of various forms of inflammatory stimuli in the patient may therefore play a crucial role in whether the induced DC are immunogenic or tolerogenic. In addition, because the disease processes and immune state of CTCL and GVHD patients are so different, it is possible that the environment from which the DC precursors come may influence their subsequent differentiation.

Focusing in on the success of ECP in treating CTCL patients in combination with the new scientific knowledge about ECP inducing monocyte to DC differentiation, a group of researchers led by Drs. Edelson and Berger set out to logically modify the existing ECP procedure in order to improve its efficacy as an anti-cancer treatment. This modification of ECP involves overnight incubation of the treated cells, allowing for more efficient cell-to-cell contact and processing of the apoptotic malignant T cells by newly formed DC, further driving monocyte to DC differentiation and increasing the yield of DC produced by the procedure (11). Cells undergo the ECP procedure and are then incubated overnight and returned to the patient the next day. This new methodology, called Transimmunization (TI) to denote the transfer of immunogens from tumor cells to antigen-presenting cells (DC), has a significant therapeutic impact. In a phase I trial, CTCL patients that had previously failed ECP sustained clinical responses after TI treatment (24). TI is potentially a more potent anti-cancer immunotherapy because the activated monocytes are more fully differentiated into DC when they are reinfused into patients, and such DC are more potent antigen presenting cells (23).

The maturity state of DC used for immunotherapy is thought to play an important role in therapeutic efficacy. It has been hypothesized that whether DC will induce immunity or tolerance largely depends on the degree of maturation they reach (25). In the presence of inflammatory signals DC will become mature and stimulate the immune system, whereas in the absence of such signals they will remain in an immature state and induce tolerance (25). Therefore, many current approaches for generating DC for cancer immunotherapy include the addition of maturation stimuli. However, the risk of inducing

exogenous maturation stimuli is that excessive stimulation during ex vivo cell manipulations may exhaust DC and also reduce their capacity to capture antigen (26). Obtaining the appropriate culture conditions for DC maturation is difficult- the amount and timing of the maturation stimulus must be exact in order to generate effective DC (25). In fact, this is just one of the many challenges facing researchers who are attempting to manufacture DC-based anti-tumor immunotherapies. There are many variables in the design and production of DC vaccines, including selecting the type of tumor antigen(s) for DC loading, determining the DC culture and maturation steps (e.g. the cytokine cocktail used to achieve maturation and length of culture, typically 1-2 weeks), deciding on the use of co-stimulatory molecules for DC activation ex vivo, and choosing methods to monitor the DC potency (27). Despite careful manipulation of these variables, to date there has been limited success using DC-tumor vaccination, with the majority of tumor vaccines having inconclusive efficacy results (27).

Compared to other procedures for generating DC for tumor immunotherapy, TI is efficient and straightforward. In TI, apoptotic tumor cells supply the full array of tumor antigens, eliminating the need to identify and select specific tumor antigens to load onto DC. The culture conditions are simple in TI: the ECP-treated cells are incubated overnight without the addition of exogenous factors. Other methods for generating DC involve culture for at least 5-7 days in high concentrations of exogenous cytokines. It does not appear necessary to use exogenous factors or co-stimulatory molecules for DC activation in TI. Instead, the DC are activated in a physiologic manner in which the attachment to plastic and subsequent activation of cell surface receptors may replicate

signaling events that occur in vivo as monocytes emigrate from the blood into peripheral tissues, which involves adhesion and activation of surface receptors such as chemokine receptors and integrins (28). This is followed by incubation with an additional maturation stimulus in the form of stressed, apoptotic tumor cells which provide "danger signals" and enhance DC immunostimulatory function (29). TI-induced DC are particularly appealing because they can be generated rapidly (in 24 hours). TI holds the potential to be a widely used immunotherapy, and the full characterization of TI-induced DC is essential to building upon its efficacy and clinical use.

In addition to characterizing TI-induced DC at the cellular level, elucidating the underlying mechanism of DC activation and differentiation is important for guiding modifications that may increase treatment efficacy, and also to determine if the therapy might be applicable to other cancers or immunologic diseases. One method that is widely used to study cellular mechanisms, including the mechanism of DC differentiation, is expression microarray technology (30). Microarray technology is a powerful means for systematically and extensively assessing entire transcriptomes, providing clues as to what cellular processes and signaling pathways are involved (24). We used Affymetrix expression microarray technology to gain insight into the mechanism of TI. Affymetrix GeneChips are one of the best established microarray platforms, and are known to generate highly reproducible results (25). Affymetrix microarrays consist of small DNA fragments bound to a glass surface. These fragments are short (25-mer) oligonucleotides, and there are up to 20 perfect match oligos for each gene, as well as an equal number of corresponding mismatch oligos. By extracting and labeling RNA from samples and

hybridizing this RNA to gene expression arrays, the amount of labeled RNA can be monitored at the site of each oligo, enabling whole-genome analysis of gene expression.

Our microarray analysis of differential gene expression after ECP and TI demonstrates that TI induces a gene expression profile consistent with mature DC. Importantly, this monocyte to DC conversion is not just seen in CTCL patient samples, but also in samples from patients with graft-versus-host disease and from healthy normal control samples, indicating that the conversion into mature DC is generalizable and not unique to CTCL patients. TI is safe, clinically practical, and has a distinct advantage over ECP in that it produces greater numbers of DC that are further differentiated down the pathway to mature DC. In addition, TI has an advantage over other widely used DC maturation protocols in that it rapidly induces mature, tumor-loaded DC without the addition of exogenous cytokines. TI has a proven track record in terms of safety, and it may provide an answer to many of the problems with other forms of DC immunotherapy. Our hope is that understanding the mechanism of TI will lead to an optimization and broadening of the scope of this therapy.

Statement of purpose

Our purpose was to characterize the mechanism by which Transimmunization, an immunotherapy wherein leukocytes treated with extracorporeal photopheresis are incubated overnight, induces dendritic cell differentiation and regulates immune responses through the analysis of differential gene expression. Using expression microarray technology, we performed genome wide analysis of samples taken from patients undergoing these immunotherapies. Previous work has shown that monocytes treated with Transimmunization express phenotypic and functional characteristics of activated dendritic cells. Therefore, we hypothesized that Transimmunization would induce differential expression of genes involved in monocyte to dendritic cell differentiation. Identification of the gene expression signature induced by this therapy may elucidate the mechanism(s) of Transimmunization-induced dendritic cell activation through the identification of key signaling pathways or novel genes.

Materials and Methods

Patient samples

Peripheral blood leukocytes from patients undergoing ECP using the Uvar XTS Photopheresis System (Therakos, Exton, Pa., USA) were obtained under the guidelines of the Yale Human Investigational Review Board. Informed consent was provided according to the Declaration of Helsinki. Samples (10 ml) were procured from 1) a leukocyte enriched leukapheresis sample taken from the photopheresis apparatus collection bag immediately before 8-MOP is added; and 2) an ECP specimen taken immediately post UVA exposure. This ECP sample was divided and half of the sample was cultured overnight, mimicking transimmunization. For a select group of patients we also took a 5 ml sample of untreated whole blood, drawn directly from the patient prior to treatment. It should be mentioned that the leukocyte enriched leukapheresis sample taken from patients undergoing treatment is drawn from the Uvar XTS Photopheresis System, and by the nature of this system the leukapheresis flows through the plastic exposure plate first before flowing into the collection bag. Therefore, this sample does not meet the classical definition of a leukapheresis. However, because it has only undergone transient exposure to the plastic treatment plate and has not been treated with 8-MOP + UVA, it will still be referred to as leukapheresis (or leuk) throughout this document.

Overnight culture of patient samples

To mimic transimmunization, the peripheral blood mononuclear cells (PBMCs) were isolated from the ECP sample by centrifugation over a Ficoll-Hypaque gradient. Half of the total lymphocytes isolated from the ECP sample buffy layer were washed 2x with 10

ml Hank's balanced salt solution (HBSS, Gibco, Carlsbad, CA) + 1% Penicillin/Streptomycin (Pen/Strep, Gibco, Carlsbad, CA) and then resuspended in RPMI-1640 medium (Gibco, Carlsbad, CA), supplemented with 1% Pen/Strep and 15% pooled normal AB serum (Gemini Bio-Products, West Sacramento, CA). Cells were plated in 6-well polystryene tissue culture plates (BD Falcon, Franklin Lakes, NJ) and were cultured at 37°C in 5% CO2 at a density of 5 x 10^6 cells/ml. Following overnight culture (20 hours) cells were harvested using a cell scraper and were washed 1x with 10 ml of RPMI-1640 + 1% Pen/Strep before undergoing enrichment for the monocyte population. For all of the patient samples, I performed the sample preparation and set up the cell cultures.

Normal control samples

A 5 ml sample of untreated whole blood was obtained immediately prior to the leukapheresis procedure. Peripheral blood leukocytes from healthy normal control donors were isolated by leukapheresis using the Baxter CS-3000 apparatus. A portion of the leukapheresis was set aside for RNA isolation. The majority of the leukapheresis was treated with a bench-top ECP apparatus consisting of a bag containing the leukapheresis sample, a bag with normal saline, a pump, plastic tubing, UVA light box containing a plastic plate, and plastic collection bag. The plastic plate used in this apparatus is identical to the plate used in the Therakos machine. This system was designed to mimic the photopheresis system used to treat patient samples, however it has the advantage of being an open system, thereby providing access to samples of treated cells at different time points. This open bench-top apparatus therefore allows for more detailed

mechanistic studies. Samples of leukocytes that had been treated with 8-MOP and were undergoing ECP were taken at the following time points: 1 hour UVA exposure, 2 hours UVA exposure, and 3 hours UVA exposure. These samples were divided and half of the sample buffy layer was cultured overnight in RPMI-1640 medium + 1% Pen/Strep, 7.5% pooled normal AB serum and 7.5% autologous serum (total 15% serum), in plastic tissue culture plates at 37°C, 5% CO2. The remaining leukocytes that had been subjected to the entire 3 hour period of UVA radiation were cultured overnight in a 1 liter platelet storage bag (PL-2410, Baxter), as is used for transimmunization. This bag is made of a plastizer that does not diffuse but makes the bag pliable, strong enough to withstand centrifugation and gas diffusible. The cells can be resuspended by manipulating the soft bag, limiting cell loss due to sticking. After overnight culture in the bag, the PBMCs were isolated by centrifugation over a Ficoll-Hypaque gradient. A portion of these transimmunization-treated cells were taken for RNA isolation. For all normal control samples, the sample acquisition, treatment with the bench-top ECP apparatus, and cell cultures were performed by Dr. Carole Berger with the assistance of Nianci Wang.

Cell isolation and enrichment for monocyte population

Peripheral blood mononuclear cells (PBMCs) were isolated from patient and normal control samples by centrifugation over a Ficoll-Hypaque gradient. Cells were washed 2x with RPMI 1640 (Gibco, Gaithersburg, MD) containing 10% AB serum, 1% Pen/strep, and 2 mM EDTA. Subsequently PBMCs underwent magnetic bead depletion of the non-monocyte populations. To achieve enrichment of the monocyte population, samples were either depleted of the CD4+ fraction or depleted of the CD4+, CD8+, and CD19+

fractions for a more rigorous isolation of the monocyte population. For patient samples that were depleted of the CD4+ fraction, the PBMCs were incubated with 20 µl MACS α-human CD4 microbeads (Miltenyi Biotech, Auburn, CA) per 1 x 10^7 cells and then passed over a column in a magnetic field, according to the manufacturer's instructions. For some of the patient and normal control samples, the CD4+, CD8+, and CD19+ cell fractions were removed from DCs by incubation with a mixture of CD4, CD8, and CD19 antibody conjugated to magnetic beads (Miltenyi Biotech, Auburn, CA) using 20 µl per 1 x 10^7 cells of each antibody followed by passage over a column in a magnetic field, according to the manufacturer's instructions. I performed the monocyte enrichment on all patient samples and Dr. Carole Berger performed the monocyte enrichment on normal control samples.

RNA isolation and microarray hybridization

Total RNA was isolated using RNeasy Mini Kit columns with on-column DNase I treatment as described by the manufacturer (Qiagen, Hilden, Germany). RNA yield and purity were measured using the NanoDrop ND-1000 Spectrophotometer and the Agilent 2100 Bioanalyzer. An oligo- dT primer containing the promoter sequence of the bacteriophage T7 RNA polymerase was then used to synthesize double-stranded cDNA from 1 ug of total RNA by the One-Cycle cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA). The cDNA was subsequently cleaned up by the GeneChip Sample Cleanup Module (Affymetrix Inc.). Next the cDNA was in vitro transcribed into biotin-labeled anti-sense mRNA (referred to as cRNA), using the oligo- dT primer and biotinylated ribonucleotides contained in the GeneChip IVT Labeling Kit (Affymetrix Inc.). Clean-up

of the biotinylated cRNA was performed using the Sample Cleanup Module. The biotinylated cRNA is then fragmented to a size of 35-200 bases by incubating at 94º C for 35 minutes in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). The chemically fragmented biotinylated cRNAs are then added to hybridization buffer (100 mM MES, 1M [Na], 20 mM EDTA, 0.01% Tween 20, herring sperm DNA (0.1 mg/ml; Promega), fragmented cRNA (0.05μg/μl)). Acetylated BSA (0.5 mg/ml, Invitrogen) and four control bacterial and phage cRNA (1.5 pM BioB, 5 pM Bio C, 25 pM BioD, and 100 pM Cre) samples were also included in the hybridization buffer to serve as internal controls for hybridization efficiency. The hybridization buffer was placed on Affymetrix HG U133 Plus 2.0 human chips and the arrays were hybridized for 16 h at 45°C in a rotisserie oven (GeneChip Hybridization Oven 640, Affymetrix Inc). After hybridization, arrays were washed and stained with streptavidin-phycoerythrin, a fluorescent molecule that binds to biotin. Because biotinylated cRNAs were hybridized to the arrays, the streptavidin-phycoerythrin binds the hybridized cRNA. After the first staining with streptavidin-phycoerythrin, a standard signal amplification step was performed that employs anti-streptavidin antibody (goat) and biotinylated goat IgG antibody. After this step the array is again incubated with streptavidin-phycoerythrin, providing an amplified level of fluorescence. The washes and staining were carried out used the Affymetrix Fluidics Station 450 according to the following protocol: arrays were washed for 30 cycles with non-stringent Wash Buffer A (Affymetrix Inc), and then 18 cycles with stringent Wash Buffer B (Affymetrix Inc). Arrays were stained with Streptavidin-Phycoerythrin Solution (2X Stain Buffer, 50 mg/mL BSA, 1 mg/mL streptavidin phycoerythrin) for 10 min at 25°C, 5 minutes at

35°C, then again for 10 min at 25°C. Arrays were then washed for 30 cycles with Wash Buffer A. Next, arrays were incubated with the Anti-Streptavidin Antibody solution to amplify the signal. The Antibody Solution (2X Stain Buffer, 50 mg/mL BSA, 10 mg/mL Goat anti-streptavidin, 0.5 mg/mL biotinylated Goat IgG) was incubated for 10 min at 25°C, 5 minutes at 35°C, then again for 10 min at 25°C. The arrays were then stained with the Streptavidin Phycoerythrin Solution for a second time for 10 min at 25°C, 5 minutes at 35°C, then again for 10 min at 25°C. Finally, a wash with 45 cycles of Wash Buffer A was performed. The array was then scanned on an Affymetrix GeneChip Scanner 3000, a confocal scanner that measures the fluorescence intensity emitted by the labeled cRNA bound to the probe arrays. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array. I performed the RNA isolation on all samples, with the exception of several samples from normal control individuals that were isolated by Zimei Zhang. The Affymetrix Microarray Facility at the Yale University W. M. Keck Foundation Biotechnology Resource Laboratory performed the measurements of RNA yield and purity, the synthesis, labeling, and hybridization of cRNA to the Affymetrix GeneChip, and the labeling and scanning of the GeneChip.

Data Analysis

Raw data without normalization generated from Affymetrix GeneChip® Operating Software Version 1.2 (GCOS 1.2) (Affymetrix Inc.) were analyzed using GeneSpring software 7.2 (Agilent Technologies-Silicon Genetics, Redwood City, CA). Data was normalized using Robust Multi-Array (RMA) normalization. Only those probe sets with a minimal fold change of 2.0 combined with an average signal intensity of 500 or higher in

either the leukapheresis or treated samples were included in the analysis. Comparisons were made between leukapheresis samples and ECP-treated samples, and also between leukapheresis samples and TI-treated samples. Differential gene expression was considered as a $\geq$ 2-fold change and P-value $\leq$0.05. Dr. Aiping Lin, a biostatistician at the Keck Biotechnology Resource who specializes in microarray analysis, performed the normalization and analysis of the raw microarray data. To identify pathways relevant to ECP and TI-induced DC, we used MetaCore Software (GeneGo Inc, St Joseph, MI), which is a curated database containing approximately 500 canonical signaling and metabolic pathways. This pathway data was generated using a stringent method recommended by Dr. Aiping Lin. Because the gene expression data from the 3 CTCL and 3 GVHD patients was extremely similar, Dr. Lin recommended we combine this data to look at gene expression changes as a result of TI across the patient groups. This strengthened the statistical significance of our data, because the sample size was N=6, instead of N=3 when the patient groups were analyzed separately. To analyze significantly involved pathways as a result of treatment, combined gene lists for ECP versus leukapheresis and TI versus leukapheresis consisting of all genes with a fold-change of $\geq$ 2 and P-value $\leq$0.05 were up-loaded onto the MetaCore Software. Using MetaCore algorithms, a list of the most significantly involved pathways was generated, including P-values representing the likelihood that a given pathway is affected by ECP or TI treatment. Dr. Gabe Vasquez performed the pathway analysis.

Quantitative real-time polymerase chain reaction: Validation of microarray data

To validate alterations in gene expression on the microarray, changes in the expression of selected genes were confirmed in aliquots of the same RNA samples used for the microarray using quantitative real-time PCR. First, RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Each 20 µl reaction contained: 1 µg RNA, 2.0 µl 10X RT Buffer, 0.8 µl dNTP Mix, 2.0 µl RT Random Primers, 1.0 µl MultiScribe Reverse Transcriptase, 1.0 µl RNase inhibitor, and nuclease-free H2O to make a 20 µl reaction volume. Reverse transcription was carried out in a 96-well thermocycler (MJ Research PTC-200) in the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec. For quantitative real-time PCR, each 25 µl reaction contained: 200 ng cDNA, 12.5 µl TaqMan Universal PCR MasterMix, 1.25 µl TaqMan probe, and nuclease-free H2O to make a 25 µl reaction. All quantitative PCR reactions were performed as singleplex (using one probe per reaction) and in triplicate. TaqMan real-time PCR was used to detect transcripts of DC-LAMP, CCR7, Indoleamine, and CD14. Primers and probes for each sequence were obtained as Inventoried Taqman Gene Expression Assays and are listed as Gene Symbol: Assay ID (Applied Biosystems, Foster City, CA: TaqMan FAM-MGB probes: DC-LAMP: Hs00180880_m1, CCR7: Hs00171054_m1, Indoleamine: Hs00158032_m1, CD14: Hs00169122_g1). HPRT1was used as reference gene. Quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR system with the following temperature profile: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 seconds and 60°C for 60 seconds. Quantification of transcripts was made by the comparative computed tomography

method. I performed all of the quantitative real-time PCR for validation of the microarray data.

Quantitative real-time polymerase chain reaction: Validation of target genes in patient and normal control samples

For a select group of patients and for all normal controls, we compared gene expression changes after treatment to a baseline expression level in untreated, whole blood drawn directly from the patient. Because the monocyte-enriched cell population we isolated from whole blood contained few cells, we had very small quantities of cDNA. The Applied Biosystems TaqMan® PreAmp Master Mix Kit was used to increase the quantity of cDNA for gene expression studies in all samples. Before running the preamplification reaction, the TaqMan Assays of interest (containing primers and probes) including endogenous control HPRT-1 were pooled together, as per the manufacturer's instructions with all assays at a final concentration of 0.2X in 1X Tris–EDTA (TE) buffer. Each preamplification reaction contained 25 µl TaqMan PreAmp MasterMix, 12.5 µl pooled assay mix, and up to 250 ng cDNA + nuclease free water to make 12.5 µl for a total reaction volume of 50 µl. The preamplification reaction was performed in a 96-well thermocycler (MJ Research PTC-200) in the following conditions: 95°C for 10 min, and 95°C for 15s followed by 60°C for 4 min for 14 cycles. In order to perform TaqMan real-time PCR, the preamplified products were diluted 1:20 using TE buffer. The gene expression reaction was performed in 25 µl reactions containing 12.5 µl Gene Expression Master Mix, 1.25 µl TaqMan Gene Expression Assay, 6.25 µl preamplified cDNA product, and 5 µl nuclease-free water. All reactions were singleplex and were performed

in triplicate. The following Inventoried TaqMan Gene Expression Assays (FAM-MGB probes, listed as Gene Symbol: Assay ID) were used: DC-LAMP: Hs00180880_m1, CD80: Hs00175478_m1, CD40: Hs00386848_m1, CD14: Hs00169122_g1, CD86: Hs00199349_m1, GPNMB: Hs00159620_m1. HPRT1was used as reference gene. Quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR system using the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 seconds and 60°C for 60 seconds. Quantification of transcripts was made by the comparative computed tomography method. I and Zimei Zhang performed the preamplification and quantitative real-time PCR on patient and normal control samples.

Results

Transcriptional response induced by ECP and TI

Samples from 3 CTCL and 3 GVHD patients undergoing ECP were depleted of CD4+ T cells and then hybridized to Affymetrix U133 plus 2.0 gene chips. For each patient, an array measuring RNA transcript levels at the end of the leukapheresis phase of ECP (immediately before 8-MOP is added) was used as a baseline array, and fold-change in gene expression was determined as a change in RNA transcript from this baseline. For all 6 patients, RNA transcript levels were then assessed after ECP treatment on Day 0 and after ECP + overnight incubation (TI). After ECP treatment, the number of genes that were significantly differentially expressed (≥ 2 fold change, $P \leq 0.05$) in the samples from 3 CTCL patients was 206 (202 up-regulated genes, 4 down-regulated genes). The transcriptional response induced by TI was much greater than the response induced by ECP. For the 3 CTCL patients, following TI there were 4553 genes significantly differentially expressed (2868 up-regulated genes, 1685 down-regulated genes). For the 3 GVHD patients there were 72 genes significantly up or down regulated (63 up-regulated, 9 down-regulated) after ECP. Post-TI treatment there were 3966 genes significantly differentially expressed (2191 up-regulated, 1775 down-regulated) in GVHD patients.

Most up and down regulated genes

The most differentially expressed genes in each group are listed in Tables 1-4. Table 1 lists the top 50 up-regulated genes in CTCL patients after TI. Table 2 lists the top 50 up-

regulated genes in GVHD patients after TI. In comparing these lists, it is clear that many of the same genes are highly up-regulated after TI in both CTCL and GVHD patients. In fact, 37 of the 50 genes (74%) on each list are the same (Tables 1 and 2).

CTCL 50 most up-regulated genes

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
NM_002510	Glycoprotein nmb	Negative regulation of cell proliferation	2393.02	6.9E-04
BG166705	chemokine (C-X-C motif) ligand 5	Chemotaxis	747.97	2.6E-04
L27624	Tissue factor pathway inhibitor 2	Blood coagulation	482.34	1.2E-03
N30257	Solute carrier family 16, member 10	Transport	270.57	5.1E-04
NM_006843	Serine dehydratase	gluconeogenesis	265.44	3.3E-02
NM_014331	Solute carrier family 7, member 11	Protein complex assembly	258.16	7.3E-05
NM_002030	Formyl peptide receptor-like 2	Chemotaxis	251.45	1.1E-03
AB000889	Phosphatidic acid phosphatase type 2B	Lipid metabolism	201.10	1.1E-04
AA977975	Vitelline membrane outer layer 1 homolog	---	198.83	1.9E-04
NM_002993	chemokine (C-X-C motif) ligand 6	Inflammatory response	197.41	1.6E-04
NM_014479	ADAM-like, decysin 1	Integrin-mediated signaling pathway	190.32	1.1E-02
BG170541	Met proto-oncogene (hepatocyte growth factor receptor)	Protein amino acid phosphorylation	180.15	4.1E-06
M34455	Indoleamine-pyrrole 2,3 dioxygenase	Tryptophan catabolism	174.70	5.2E-04
BG236136	Unknown (Similar to Immune-responsive protein 1 in mouse)	---	147.49	2.8E-03
NM_004591	Chemokine (C-C motif) ligand 20	Inflammatory response	144.76	1.9E-02
M83248	Osteopontin	Cell adhesion	139.27	3.3E-04
AF003934	Growth differentiation factor 15	TGF beta receptor signaling pathway	128.77	1.2E-03
NM_003020	Secretogranin V (7B2 protein)	Protein folding	117.07	1.9E-02
AI608902	Unknown (transcibed locus)	---	107.35	3.5E-05
BF513121	Integrin, beta 8	Cell adhesion	100.45	2.7E-05
U38321	matrix metallopeptidase 19	Peptidoglycan metabolism	87.39	2.3E-03
AB051513	Zinc finger CCCH-type containing 12C	---	86.35	5.2E-04
NM_003122	Serine peptidase inhibitor, Kazal type 1	---	84.82	1.4E-03
M86849	Connexin 26	Cell-cell signaling	79.29	4.0E-04
NM_004403	Deafness, autosomal dominant 5	Sensory perception of sound	77.58	5.9E-04
AL577322	Syndecan 2	---	66.12	1.5E-03
D55696	Legumain	Proteolysis	63.56	1.7E-02
AK026883	G protein-coupled receptor 157	Signal transduction	60.20	3.4E-05
NM_001442	Fatty acid binding protein 4, adipocyte	Transport	60.05	1.5E-02
NM_014398	Lysosomal-associated membrane protein	---	59.83	5.1E-03
AL389942	Unknown (hypothetical protein LOC285628)	---	58.24	1.8E-03
AI659800	Chromosome 13 open reading frame 31	---	56.38	4.5E-04
AJ276888	Transformed 3T3 cell p53 binding protein	Regulation of cell cycle	51.61	3.5E-04
NM_004110	Ferredoxin reductase	Electron transport	49.69	3.8E-04
NM_000064	Complement component 3	Inflammatory response	49.08	3.6E-04
BF511231	Tissue factor pathway inhibitor 2	Blood coagulation	44.88	7.9E-05
BE566894	Leukotriene B4 12-	Leukotriene metabolism	43.12	3.1E-05
NM_006850	Interleukin 24	Apoptosis	42.52	5.9E-04
BC042665	CD80 molecule	Immune response	41.89	7.8E-04
AB042557	Phosphodiesterase 4D interacting protein	Protein biosynthesis	40.93	3.3E-03

Table 1. 50 most up-regulated genes after TI in CTCL patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

CTCL 50 most up-regulated genes continued

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
AI343467	CDNA FLJ11041 fis, clone PLACE1004405	---	40.20	5.5E-03
NM_000600	Interleukin 6	Immune response	40.09	4.9E-03
NM_013231	Fibronectin leucine rich transmembrane protein 2	Cell adhesion	39.84	1.4E-03
NM_004994	Matrix metallopeptidase 9	Peptidoglycan metabolism	38.02	1.4E-03
L03203	Peripheral myelin protein 22	Synaptic transmission	38.00	1.5E-04
AK024680	Neuropilin 2	Cell adhesion	37.90	1.9E-05
AU149305	Matrix metallopeptidase 14	Peptidoglycan metabolism	35.88	4.3E-04
AB033025	KIAA1199	Sensory perception of sound	34.76	2.6E-06
AI422414	Carboxypeptidase M	Proteolysis	33.34	1.0E-04
AF228422	Chromosome 15 open reading frame 48	---	32.91	1.2E-02

Table 1 cont. 50 most up-regulated genes after TI in CTCL patients continued
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

GVHD 50 most up-regulated genes

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
NM_002510	Glycoprotein nmb	Negative regulation of cell proliferation	1527.98	4.5E-04
M83248	Osteopontin	Cell adhesion	677.58	1.4E-03
BG166705	Chemokine (C-X-C motif) ligand 5	Chemotaxis	661.29	1.5E-03
AI984980	Chemokine (C-C motif) ligand 8	Inflammatory response	436.37	1.9E-02
AA488687	Solute carrier family 7, member 11	Protein complex assembly	399.79	2.3E-04
NM_002993	Chemokine (C-X-C motif) ligand 6	Inflammatory response	257.84	1.8E-02
NM_006843	Serine dehydratase	gluconeogenesis	255.07	4.0E-02
BF513121	Integrin, beta 8	Cell adhesion	245.91	2.3E-03
BG170541	Met proto-oncogene (hepatocyte growth factor receptor)	Protein amino acid phosphorylation	221.23	1.0E-03
N30257	Solute carrier family 16, member 10	Transport	218.39	1.2E-04
AI343467	CDNA FLJ11041 fis, clone PLACE1004405	---	197.53	4.1E-02
AB000889	Phosphatidic acid phosphatase type 2B	Lipid metabolism	184.59	2.2E-05
NM_004591	Chemokine (C-C motif) ligand 20	Inflammatory response	182.82	1.4E-02
U38321	Matrix metallopeptidase 19	Peptidoglycan metabolism	175.69	1.3E-04
NM_002030	Formyl peptide receptor-like 2	Chemotaxis	158.98	2.6E-03
BG236136	Unknown (Similar to Immune-responsive protein 1 in mouse)	---	153.14	4.6E-05
M13436	Inhibin, beta A	Induction of apoptosis	144.90	2.9E-02
NM_003122	Serine peptidase inhibitor, Kazal type 1	---	127.75	8.4E-06
NM_004403	Deafness, autosomal dominant 5	Sensory perception of sound	124.38	4.4E-04
NM_014398	Lysosomal-associated membrane protein	---	109.17	4.8E-04
AA977975	Vitelline membrane outer layer 1 homolog	---	108.49	8.4E-04
AF003934	Growth differentiation factor 15	TGF beta receptor signaling pathway	103.26	9.2E-04
AB051513	Zinc finger CCCH-type containing 12C	---	91.82	1.0E-04
AF228422	Chromosome 15 open reading frame 48	---	87.67	6.6E-03
AI608902	Unknown (transcibed locus)	---	70.22	3.6E-03
M86849	Connexin 26	Cell-cell signaling	68.52	1.3E-03
BF511231	Tissue factor pathway inhibitor	Blood coagulation	68.50	1.4E-03
AA031832	Hypothetical gene supported by BC008048	---	67.30	1.0E-04
NM_014479	ADAM-like, decysin 1	Integrin-mediated signaling pathway	64.97	5.3E-04
NM_001511	chemokine (C-X-C motif) ligand 1	Chemotaxis	61.75	4.8E-03
M34455	Indoleamine-pyrrole 2,3 dioxygenase	Tryptophan catabolism	55.52	2.8E-03
AK026883	G protein-coupled receptor 157	Signal transduction	53.91	2.4E-03
AL389942	Unknown (hypothetical protein LOC285628)	---	51.66	9.4E-03
NM_002658	Plasminogen activator, urokinase	Proteolysis	46.26	2.8E-03
NM_005114	Heparan sulfate 3-O-sulfotransferase 1	---	45.87	6.3E-03
NM_007115	tumor necrosis factor, alpha-induced protein 6	Inflammatory response	45.41	8.2E-03
AU149305	Matrix metallopeptidase 14	Peptidoglycan metabolism	44.18	4.1E-03

Table 2. 50 most up-regulated genes after TI in GVHD patients

Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

GVHD 50 most up-regulated genes continued

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
BC035749	Chromosome 13 open reading frame 31	---	43.64	4.9E-05
AI859620	Interleukin 4 induced 1	Electron transport	42.13	3.8E-03
L03203	Peripheral myelin protein 22	Synaptic transmission	41.24	3.0E-02
BC042665	CD80 molecule	Immune response	40.00	2.2E-04
AL577322	Syndecan 2	---	38.62	4.8E-03
D55696	Legumain	Proteolysis	37.49	2.0E-03
NM_013410	Adenylate kinase 3-like 1	Nucleic acid metabolism	36.30	4.9E-03
NM_002090	Chemokine (C-X-C motif) ligand 3	Chemotaxis	35.89	1.2E-03
AK024680	Neuropilin 2	Cell adhesion	35.89	8.1E-04
NM_022359	Phosphodiesterase 4D interacting protein	Protein biosynthesis	34.45	3.4E-04
AI827972	Methyltransferase like 7B	---	33.12	2.4E-02
BC016631	Chromosome 7 open reading frame 10	Metabolism	32.47	1.5E-02
AF035776	Oxidised low density lipoprotein receptor 1	Inflammatory response	32.26	1.2E-02

Table 2 cont. 50 most up-regulated genes after TI in GVHD patients continued
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

Tables 3 and 4 list the 50 most down-regulated genes in CTCL and GVHD patients, respectively. Again, we see that many (66%) of the most down-regulated genes are the same on both lists. Looking at the gene ontology biological process in each of these groups, it appears that there are many genes involved in the inflammatory immune response, cell adhesion, and regulation of apoptosis that are highly up-regulated, while genes involved in transcription and DNA replication are down-regulated (Tables 1-4).

CTCL 50 most down-regulated genes

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
NM_000110	Dihydropyrimidine dehydrogenase	De novo pyrimidine base biosynthesis	-113.82	1.1E-04
NM_000129	Coagulation factor XIII, A1 polypeptide	Blood coagulation /	-76.66	6.1E-04
AI052659	Complement component (3b/4b) receptor 1	Complement activation	-67.83	6.9E-04
AA650281	STEAP family member 4	Electron transport	-66.16	1.5E-03
NM_018460	Rho GTPase activating protein 15	Regulation of cell shape	-61.97	3.5E-04
BF032500	Chromosome 20 open reading frame 133	---	-61.39	2.7E-04
NM_007309	Diaphanous homolog 2 (Drosophila)	Cytokinesis	-55.75	8.3E-06
NM_013378	Pre-B lymphocyte gene 3	---	-53.46	7.4E-04
NM_017935	B-cell scaffold protein with ankyrin repeats 1	---	-47.90	1.1E-03
U90339	Adenosine kinase	Purine ribonucleoside salvage	-47.06	3.5E-04
AA677057	Fc receptor-like 1	---	-46.29	2.6E-03
AI654161	TBC1 domain family, member 5	---	-46.03	7.6E-04
NM_013959	Neuregulin 1	Nervous system development	-45.41	3.7E-04
AA029441	Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	Regulation of cell growth	-45.35	1.7E-03
AA528080	Hypothetical protein LOC283070	---	-42.75	1.1E-04
BC003574	T-cell leukemia/lymphoma 1A	Development	-41.93	4.4E-03
NM_002738	Protein kinase C, beta 1	Protein amino acid phosphorylation	-41.00	1.9E-03
AL833097	MRNA; cDNA DKFZp313E1515	---	-40.61	1.7E-03
AI733027	Retinol binding protein 7, cellular	Transport	-39.65	7.9E-05
AW003297	Ral GEF with PH domain and SH3 binding motif 2	GTPase mediated signal transduction	-35.68	1.3E-03
NM_022445	Thiamin pyrophosphokinase 1	Thiamin metabolism	-33.13	1.7E-03
AI927701	Chromosome 5 open reading frame 21	---	-33.09	6.8E-04
AW085505	AF4/FMR2 family, member 3	Regulation of transcription	-32.60	5.0E-03
BC036784	RasGEF domain family, member 1B	GTPase mediated signal transduction	-32.55	3.0E-03
AW130600	MRNA; cDNA DKFZp564O0862	---	-31.10	2.2E-03
NM_006567	Phenylalanine-tRNA synthetase 2	Protein biosynthesis	-28.82	1.0E-04
AB051486	Exocyst complex component 4	Vesicle docking during exocytosis	-28.76	5.6E-05
AW080339	Brain and reproductive organ-expressed (TNFRSF1A modulator)	Response to DNA damage stimulus	-28.40	3.8E-04
NM_017784	Oxysterol binding protein-like 10	Lipid transport	-28.03	2.2E-04
NM_002003	Ficolin (collagen/fibrinogen domain containing) 1	Phosphate transport	-27.29	4.1E-04
AL049593	Phospholipase C, beta 1	Regulation of cell cycle	-26.92	2.8E-04
NM_001500	GDP-mannose 4,6-dehydratase	Leukocyte adhesion	-26.88	2.4E-03
AI052003	Vacuolar protein sorting 13B	---	-25.74	2.3E-04
NM_007053	CD160 molecule	Cellular defense response	-24.93	7.0E-04
N39230	Transcribed locus	---	-24.88	3.9E-04
BF221852	LIM domain containing preferred translocation partner in lipoma	Cell adhesion	-24.70	1.4E-04

Table 3. 50 most down-regulated genes after TI in CTCL patients

Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

CTCL 50 most down-regulated genes continued

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
AI244661	Membrane protein, palmitoylated 7	---	-24.47	7.0E-04
AA020010	Kruppel-like factor 12	Regulation of transcription	-23.87	7.1E-04
AF094700	Fas (TNFRSF6) associated factor 1	Apoptosis	-23.23	9.6E-05
NM_024829	Hypothetical protein FLJ22662	---	-23.14	2.3E-03
BF112093	CDNA FLJ30652 fis	---	-22.39	6.0E-04
W60686	LPS-responsive vesicle trafficking, beach and anchor containing	Regulation of DNA replication	-22.26	3.9E-04
BC001886	Ribonucleotide reductase M2 polypeptide	DNA replication	-21.11	2.0E-03
AL137430	---	---	-20.08	7.8E-05
BC001252	Dymeclin	---	-19.80	1.7E-03
R62453	RAB37, member RAS oncogene family	Regulation of transcription	-19.79	3.3E-04
BC005912	Receptor for Fc fragment of IgE, high affinity I, alpha polypeptide	Immune response	-19.26	1.3E-03
AW205790	Sec1 family domain containing 2	Vesicle docking during exocytosis	-18.94	5.8E-05
NM_002758	Mitogen-activated protein kinase kinase 6	DNA damage induced protein phosphorylation	-18.77	4.8E-05
NM_024820	DENN/MADD domain containing 1A	---	-18.42	1.3E-04

Table 3 cont. 50 most down-regulated genes after TI in CTCL patients continued
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

GVHD 50 most down-regulated genes

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
NM_000110	Dihydropyrimidine dehydrogenase	De novo pyrimidine base biosynthesis	-455.61	6.6E-10
NM_018460	Rho GTPase activating protein 15	Regulation of cell shape	-115.21	4.7E-05
NM_007309	Diaphanous homolog 2 (Drosophila)	Cytokinesis	-76.05	6.8E-08
NM_022445	Thiamin pyrophosphokinase 1	Thiamin metabolism	-71.70	5.1E-05
AI300084	TBC1 domain family, member 5	---	-66.88	5.0E-06
AI052659	Complement component (3b/4b) receptor 1	Complement activation	-63.33	1.5E-05
U90339	Adenosine kinase	Purine ribonucleoside salvage	-58.81	4.7E-03
AL049593	Phospholipase C, beta 1	Regulation of cell cycle	-55.33	3.9E-03
NM_006567	Phenylalanine-tRNA synthetase 2	Protein biosynthesis	-54.17	3.9E-06
BC001886	Ribonucleotide reductase M2 polypeptide	DNA replication	-53.32	2.4E-02
AI052003	Vacuolar protein sorting 13B	---	-50.81	7.1E-05
AA020010	Kruppel-like factor 12	Regulation of transcription	-46.84	1.2E-03
AL833097	MRNA; cDNA DKFZp313E1515	---	-46.07	8.4E-04
AF094700	Fas (TNFRSF6) associated factor 1	Apoptosis	-45.67	4.1E-05
AI927701	Chromosome 5 open reading frame 21	---	-42.43	1.7E-04
N66570	Utrophin (homologous to dystrophin)	Muscle contraction	-42.28	3.5E-04
NM_007124	---	---	-40.30	2.5E-04
AI961231	Thymus high mobility group box protein TOX	Regulation of transcription	-39.07	8.3E-04
NM_000129	Coagulation factor XIII, A1 polypeptide	Blood coagulation	-38.39	1.9E-03
AI659561	LPS-responsive vesicle trafficking, beach and anchor containing	Regulation of DNA replication	-36.94	9.8E-04
NM_013959	Neuregulin 1	Nervous system development	-36.92	3.9E-02
AA045175	Membrane-spanning 4-domains, subfamily A, member 6A	Signal transduction	-36.58	1.1E-02
NM_002738	Protein kinase C, beta 1	Protein amino acid phosphorylation	-35.76	3.4E-05
AI244661	Membrane protein, palmitoylated 7	---	-35.54	2.9E-04
NM_018318	Coiled-coil domain containing 91	Protein transport	-35.30	2.2E-05
NM_003726	Src family associated phosphoprotein 1	Immune response	-34.05	1.8E-03
AI420817	CDNA FLJ45742 fis	---	-32.50	6.0E-04
NM_001500	GDP-mannose 4,6-dehydratase	Leukocyte adhesion	-32.47	1.4E-04
NM_007053	CD160 molecule	Cellular defense response	-32.04	9.9E-03
N63244	Tubulin, beta 1	Microtubule-based movement	-31.27	4.0E-03
NM_013233	Serine threonine kinase 39	Protein amino acid phosphorylation	-30.46	9.1E-04
AW276572	SET binding factor 2	Signal transduction	-30.08	1.3E-04
AI498747	V-set and transmembrane domain containing 1	---	-28.78	4.3E-03
AA029441	Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	Regulation of cell growth	-28.63	8.1E-04

Table 4. 50 most down-regulated genes after TI in GVHD patients

Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

GVHD 50 most down-regulated genes continued

Accession No. (Genbank)	Description	Function (Gene Ontology Biological Process)	Fold change (TI/Leuk)	P-value
AI569476	Mastermind-like 3 (Drosophila)	Regulation of transcription	-28.00	1.5E-03
BG200452	B-cell scaffold protein with ankyrin repeats 1	---	-27.47	1.3E-02
AB051486	Exocyst complex component 4	Vesicle docking during exocytosis	-27.28	4.3E-06
NM_002108	Histidine ammonia-lyase	Histidine catabolism	-26.96	2.3E-03
NM_024820	DENN/MADD domain containing 1A	---	-26.85	7.8E-05
BF112093	CDNA FLJ30652 fis	---	-26.49	9.4E-05
BC001252	Dymeclin	---	-26.49	1.2E-05
AW130600	MRNA; cDNA DKFZp564O0862	---	-26.37	4.5E-03
U20350	chemokine (C-X3-C motif) receptor 1	Chemotaxis	-26.33	9.5E-03
R62453	RAB37, member RAS oncogene family	Regulation of transcription	-26.27	8.5E-04
BF739885	Hypothetical protein LOC284262	---	-26.24	9.5E-04
X82240	T-cell leukemia/lymphoma 1A	Development	-26.01	1.5E-02
NM_022746	MOCO sulphurase C-terminal domain containing 1	---	-25.59	2.1E-03
BE222668	solute carrier family 9, member 9	Ion transport	-25.47	3.3E-04
NM_031311	Carboxypeptidase, vitellogenic-like	Proteolysis	-25.13	2.7E-03
AA528080	Hypothetical protein LOC283070	---	-24.24	6.2E-04

Table 4 cont. 50 most down-regulated genes after TI in GVHD patients continued
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data was analyzed using GeneSpring software to identify differentially expressed genes, P-values are listed.

TI induces a similar gene expression profile in patients with CTCL and GVHD

Dr. Aiping Lin, a biostatistician specializing in microarray analysis, analyzed the entire data set containing all of the genes that were differentially expressed after TI (both up and down regulated) in CTCL and GVHD patients. Dr. Lin first performed a cluster analysis of our entire data set. Cluster analysis is a technique for viewing distinct patterns of gene expression within a dataset (33). Using the hierarchical clustering technique, which assigns a similarity score to all gene pairs and calculates the Pearson's correlation coefficient for the pair, Dr. Lin was able to view the data as a gene list organized into a dendrogram, or tree-diagram. Per Dr. Lin, this analysis showed that our expression data clustered based on TI treatment and not disease process (personal

communication, Dr. Aiping Lin, data not shown). Because of this result, Dr. Lin created a gene list divided into 3 sections: 1) genes similarly changed after TI in both CTCL and GVHD patients, 2) genes that were only up or down regulated in CTCL patients, and 3) genes that were only up or down regulated in GVHD patients. Analysis of this gene list showed that of the 4650 total genes that were significantly up or down regulated after TI, 2798 (60%) were changed in a similar manner (same direction of regulation, similar fold change in gene expression) in both CTCL and GVHD patients. 1124 (24%) of up or down regulated genes were unique to CTCL patients and 728 (16%) were unique to GVHD patients. This data is represented graphically in Figure 1.

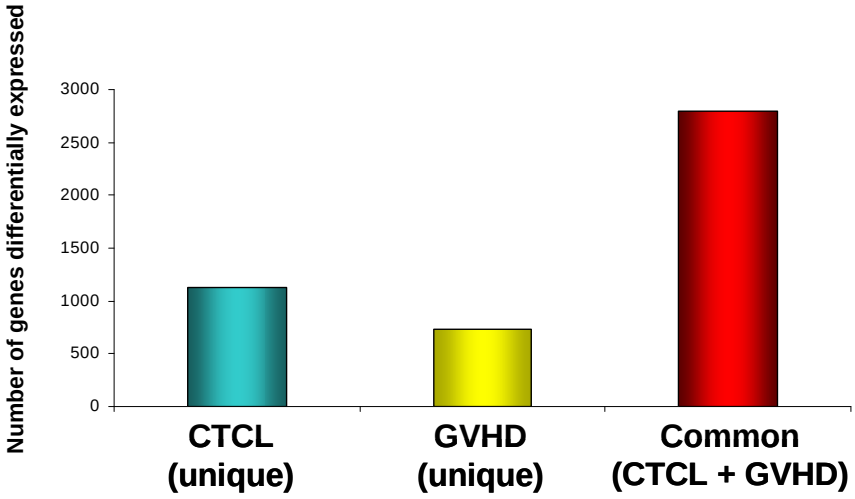

Figure 1: CTCL and GVHD patients differentially express more genes in common after TI than genes uniquely expressed by one patient group.
Number of genes significantly differentially expressed (greater than or equal to 2 fold change, P less than or equal to 0.05) after TI in common between 3 CTCL patients and 3 GVHD patients was 2798 (60%). There were 1124 genes significantly up or down regulated only in CTCL patients (24%) and 728 genes up or down regulated only in GVHD patients (16%) following TI. This suggests that TI treatment has a similar effect on gene expression regardless of disease process.

Because the majority of changes in gene expression were similar between the two patient groups, Dr. Lin suggested we combine the gene expression data from CTCL and GVHD

patients. Therefore, for our main statistical and pathway analysis, we used this combined data set.

TI induces a gene expression profile consistent with mature DC

TI caused significant up-regulation of genes associated with DC maturation (Table 5). The fold changes listed in Tables 5-9 represent combined data (CTCL and GVHD), and are listed as fold change after TI as compared to a baseline gene expression level in the leukapheresis (Leuk) sample, followed by the P value indicating the significance of the change in gene expression. Up-regulation of DC-LAMP (74.05 fold, P = 8.6E-07), a novel lysosome-associated membrane glycoprotein and marker specific for mature DC that is up-regulated during DC activation and differentiation (34) was observed after TI. CD80, a DC costimulatory signal for T cell activation, proliferation and cytokine production (35) that is expressed by DC (36)was up-regulated 40.98 fold, P=1.3E-08 after TI. CD86, another co-stimulatory molecule important for T cell activation (35), was found to have no change in gene expression after TI when using this data set from combined CTCL and GVHD patients. Up-regulation of CD40, which is expressed on mature DC and plays a role in DC survival and cytokine secretion (37) was up-regulated 2.33 fold, P=3.5E-03 after TI. CCR7, a chemokine receptor that drives migration to lymphoid organs (38) has been shown to be up-regulated in LPS-stimulated mature DC (39) and was up-regulated after TI 2.53 fold, P = 2.6E-02. Decysin, a disintegrin/ metalloprotease similar to bacterial proteinase which is up-regulated in LPS matured DC (40) was up-regulated after TI 116.74 fold, P=4.0E-06. In addition, there was massive up-regulation (1904.31 fold, P=2.3E-08) of glycoprotein GPNMB, a gene recently

discovered to be up-regulated in mature DC (41). The mouse homolog of GPNMB is also named dendritic cell-associated heparin sulfate proteoglycan-dependent integrin ligand (DC-HIL) and plays an important role in DC adhesion (42). Consistent with DC maturation, monocyte-associated genes including CD33, a sialoadhesin adhesion molecule expressed on monocytes (43) that is down-regulated in mature DC (30), was down-regulated after TI -2.54 fold, P=4.3E-05. CD36, a scavenger receptor (44) down-regulated during the transition from immature DC to mature DC (30), was down-regulated -4.17 fold, P=5.7E-03 after TI. Finally, the receptor for IgG Fc fragment Ia (or CD64), which is involved in capture of antibody-opsonized antigen (45) was down-regulated -7.01 fold, P=3.7E-04 after TI. CD64 expression on DC is known to be lower than expression of CD64 on monocytes (46).

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL + GVHD	
			TI/Leuk	P-value
	DC maturity genes			
NM_014398	DC-LAMP	Lysosomal protein involved in antigen processing	74.05	8.6E-07
BC042665	CD80	Co-stimulatory molecule	40.98	1.3E-08
NM_006889	CD86	Co-stimulatory molecule	No change	-
NM_001250	CD40	Involved in CD survival, cytokine secretion, and tumoricidal activity	2.33	3.5E-03
NM_001838	CCR7	Involved in migration, chemotaxis	2.53	2.6E-02
NM_014479	Decysin	Expressed in LPS matured DC	116.74	4.0E-06
NM_002510	Glycoprotein NMB (GPNMB)	Up-regulated in mature DC, transmembrane glycoprotein	1904.31	2.3E-08
	Monocyte genes			
NM_001772	CD33	Cell surface protein expressed on circulating monocytes	-2.54	4.3E-05
M98399	CD36	Impairs DC maturation, receptor for apoptotic cells	-4.17	5.7E-03
X14355	Receptor for IgG Fc fragment Ia	Down-regulated in early stages of DC maturation	-7.01	3.7E-04

Table 5. Up-regulation of specific DC markers and down regulation of monocyte markers after TI

Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. Because of the similar patterns in gene expression we were able to combine the CTCL + GVHD data sets. The fold changes in this table represent combined data from all 6 patients. P-values are listed.

TI induces increased expression of inflammatory cytokines and chemokines

Among the TI-induced genes were those relevant to inflammatory immune responses, including several genes encoding inflammatory cytokines and chemokines (Table 6). Interleukin 6, a pro-inflammatory cytokine that is up-regulated in infection as well as in autoimmune diseases such are rheumatoid arthritis and Crohn's disease (47), was up-regulated after TI (49.36 fold, P=1.7E-03). Interferon gamma, a pro-inflammatory cytokine that enhances DC maturation (48) was not changed in this combined CTCL and GVHD data set. Interleukin 1-alpha, a potent inflammatory mediator (49), was up-regulated 69.84 fold after TI, P=4.4E-03. A closely related inflammatory cytokine, Interleukin 1-beta (49), was up-regulated 3.01 fold after TI, although the P-value did not reach significance. Chemokines and their receptors play an important role in DC function (50) and several inflammatory chemokines were up-regulated after TI, such as CCL22 (danger signal, 18.05 fold, P=1.4E-08), CXCL5 (produced in response to stimulation with IL1-beta or TNF alpha, 708.25 fold, P=6.4E-08), CXCL6 (224.88 fold, P=8.1E-06), and CCL20 (156.38 fold, P=1.2E-04). The chemokine receptor CCR5, which is involved in DC migration, was also induced by TI (6.51 fold, P=6.5E-04) (50). TI induced expression of receptors for inflammatory cytokines as well: IL1R1, which leads to NFκB activation (23), was up-regulated 20.12 fold, P=1.4E-05, and IL7R, a receptor that mediates lymphopoiesis (51), was induced 4.13 fold P=1.7E-03.

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL + GVHD	
	Inflammatory cytokines and chemokines		**TI/Leuk**	**P-value**
NM_000600	Interleukin 6	Up-regulated during bacterial and viral infections	49.36	1.7E-03
M29383	Interferon gamma	Inflammatory cytokine	No change	-
M15329	Interleukin 1-alpha	Potent inflammatory mediator	69.84	4.4E-03
NM_000576	Interleukin 1-beta	Enhances DC maturation	3.01	1.3E-01
NM_002990	CC chemokine ligand 22 (CCL22)	Danger signal	18.05	1.4E-08
BG166705	CXC chemokine ligand 5 (CXCL5)	Inflammatory cytokine, produced in response to IL1 or TNFalpha	708.25	6.4E-08
NM_002993	CXC chemokine ligand 6 (CXCL6)	Inflammatory cytokine	224.88	8.1E-06
NM_004591	CC chemokine ligand 20 (CCL20)	Inflammatory cytokine	156.38	1.2E-04
	Inflammatory cytokine and chemokine receptors			
NM_000877	Interleukin 1 receptor, type 1 (IL1R1)	Leads to NFkB activation	20.12	1.4E-05
NM_002185	Interleukin 7 receptor	Mediates lymphopoiesis	4.13	1.7E-03
NM_000579	CC chemokine receptor 5 (CCR5)	Involved in DC migration	6.51	6.5E-04

Table 6. Up-regulation of Inflammatory cytokines and chemokines after TI
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. Because of the similar patterns in gene expression we were able to combine the CTCL + GVHD data sets. The fold changes in this table represent combined data from all 6 patients. P-values are listed.

TI induced changes in genes involved in antigen presentation

Table 7 lists the expression of genes involved in antigen presentation on MHC class I, such as the MHC class I mRNA fragment 3.8-1 was up-regulated after TI (2.01 fold, P=9.7E-04), as well as expression of MHC class I-related gene, which interacts with MHC class I molecules (4.88 fold, P=1.4E-06) (52). However, expression of MHC class II genes was down-regulated after TI: MHC class II DO beta (-6.20 fold, P=9.6E-03), MHC class II DP alpha 1 (-6.18 fold, P=6.7E-03), MHC class II DR beta 1 (-5.51 fold,

P=1.3E-02), MHC class II DMB (-4.77 fold, P=1.5E-04), MHC class II DO alpha (-4.08 fold, P=4.1E-02), MHC class II DP beta 1 (-3.38 fold, P=5.1E-03), MHC class II DQ beta 1 (-3.33 fold, P= 3.5E-02), and MHC class II DM alpha (-3.04 fold, P=7.7E-04). Also, the MHC class II transactivator, which controls expression of MHC class II genes, was down-regulated after TI (-3.39, P=2.6E-03). In the field of DC biology, it is well established that the expression of MHC class II related genes is decreased in mature DC, and the up-regulation of MHC class II protein at the cell surface is due to major changes in the intracellular transport of MHC class II molecules, so the down-regulation of these genes is consistent with a mature DC gene expression pattern (23).

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL + GVHD	
	Antigen presentation		**TI/Leuk**	**P-value**
AF031469	Major histocompatibility complex, class I-related (MR1)	Interacts with MHC Class I molecules	4.88	1.4E-06
AI346483	MHC class I mRNA fragment 3.8-1	Involved in MHC Class I antigen presentation	2.01	9.7E-04
NM_002120	Major histocompatibility complex, class II, DO beta (HLA-DOB)	Involved in MHC Class II antigen presentation	-6.20	9.6E-03
AI128225	Major histocompatibility complex, class II, DP alpha 1 (HLA-DPA1)	Involved in MHC Class II antigen presentation	-6.18	6.7E-03
AA807056	Major histocompatibility complex, class II, DR beta 1 (HLA-DRB1)	Involved in MHC Class II antigen presentation	-5.51	1.3E-02
NM_002118	Major histocompatibility complex, class II, DM beta (HLA-DMB)	Involved in MHC Class II antigen presentation	-4.77	1.5E-04
AL581873	Major histocompatibility complex, class II, DO alpha (HLA-DOA)	Involved in MHC Class II antigen presentation	-4.08	4.1E-02
NM_002121	Major histocompatibility complex, class II, DP beta 1 (HLA-DPB1)	Involved in MHC Class II antigen presentation	-3.38	5.1E-03
M32577	Major histocompatibility complex, class II, DQ beta 1 (HLA-DQB1)	Involved in MHC Class II antigen presentation	-3.33	3.5E-02
X76775	Major histocompatibility complex, class II, DM alpha (HLA-DMA)	Involved in MHC Class II antigen presentation	-3.04	7.7E-04
NM_000246	Class II, major histocompatibility complex, transactivator (CIITA)	Controls expression of MHC class II genes	-3.39	2.6E-03

Table 7. Differential expression of genes involved in antigen presentation
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. Because of the similar patterns in gene expression we were able to combine the CTCL + GVHD data sets. The fold changes in this table represent combined data from all 6 patients. P-values are listed.

TI induced genes involved in immunosuppression

TI also produced an increase in expression of genes associated with immunosuppression or tolerance (Table 8). Indoleamine, an enzyme involved in the catabolism of tryptophan, is known to be expressed by DC and promotes tolerance through the suppression of T cell responses and expansion of T regulatory cells (53, 54). Indoleamine was up-regulated after TI 84.69 fold, P=3.9E-07. Another closely related immunosuppressive gene, kynurenine, whose expression is thought to induce tolerogenic DC (55), was up-regulated 3.79 fold, P=2.9E-06. IL-10, an immunosuppressive cytokine that modulates DC maturation and favors the differentiation of tolerogenic DC (56) was up-regulated 11.27 fold, P=9.8E-06.

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL + GVHD	
	Immunosupressive genes		**TI/Leuk**	**P-value**
M34455	Indoleamine	Suppresses T cell response, promotes tolerance	84.69	3.9E-07
AI074145	Kynurenine	Expression induces tolerogenic DC	3.79	2.9E-06
NM_000572	Interleukin 10	Induces DC "exhaustion", transiently inhibits mature DC	11.27	9.8E-06

Table 8. Up-regulation of Immunosuppressive genes after TI.
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. Because of the similar patterns in gene expression we were able to combine the CTCL + GVHD data sets. The fold changes in this table represent combined data from all 6 patients. P-values are listed.

TI induces genes involved in cell adhesion

After TI treatment, there was significant up-regulation of genes involved in cell adhesion (Table 9). Adhesion molecules play an important role in the function of DC, allowing for cell migration and interaction with T lymphocytes (57) Increased expression of certain adhesion molecules, such as integrins and matrix metalloproteases, is induced upon DC maturation, allowing DC to disengage from the extracellular matrix, cross basement membranes, and travel to draining lymph nodes to activate T cells (58). Several members of the integrin family were up-regulated after TI, including integrin beta 8 (144.84 fold, P=1.7E-07), which activates TGFbeta (59), integrin alpha 5 (component of fibronectin receptor, 3.32 fold, P=1.2E-05) (60), and integrin alpha V (component of vitronectin receptor 5.57 fold, P=1.2E-05) (61). In addition, TI increased transcript levels of osteopontin (304.27 fold, P=1.7E-07), which regulates cytokine production in dendritic cells and induces DC with a Th1-promoting phenotype (62). Delta-1 catenin, a protein which is linked to the cytopasmic domain of transmembrane cadherin proteins (63), was also up-regulated after TI (4.84 fold, P=6.5E-04). In addition, ICAM1, which plays a role in DC interaction with T cells (64) was induced (3.78 fold, P=3.1E-03). Several matrix metalloproteases (MMP) were up-regulated after TI, including: MMP1 (101.58 fold, P=6.4E-03), which modulates sustained integrin mediated signals (65); MMP9 (34.58 fold, P=7.0E-05), which is more highly expressed in mature DC and regulates DC migration (66); as well as MMP14 (39.92 fold, P=4.3E-07) and MMP19 (117.04 fold, P=6.9E-08), which play roles in the degradation of extracellular matrix and cell migration (67).

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL + GVHD	
	Cell adhesion molecules		**TI/Leuk**	**P-value**
BF513121	Integrin beta 8	Activates TGFbeta	148.84	1.7E-07
NM_002205	Integrin alpha 5	Component of fibronectin receptor	3.32	1.2E-05
AI093579	Integrin alpha V	Component of vitronectin receptor	5.57	1.2E-05
M83248	Osteopontin	Regulates cytokine production in dendritic cells	304.27	1.7E-07
NM_001331	Delta-1 catenin	Linked to cytoplasmic domain of transmembrane cadherin proteins	4.84	6.5E-04
NM_000201	ICAM1	Mediates DC interaction with T cells	3.78	3.1E-03
	Matrix metalloproteases			
NM_002421	MMP1	Modulates sustained integrin mediated signals	101.58	6.4E-03
NM_004994	MMP9	Mature DC express more MMP9 than immature DC, plays role in migration	34.58	7.0E-05
AU149305	MMP14	Degradation of extracellular matrix, cell migration	39.92	4.2E-07
U38321	MMP19	Degradation of extracellular matrix, cell migration	117.04	6.9E-08

Table 9. Up-regulation of genes involved in cell adhesion after TI.
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. Because of the similar patterns in gene expression we were able to combine the CTCL + GVHD data sets. The fold changes in this table represent combined data from all 6 patients. P-values are listed.

Analysis of genes unique to CTCL and GVHD

Despite the similarities in the majority of genes, the population of genes that is differentially regulated after TI is also of interest, particularly because this therapy induces different clinical responses CTCL and GVHD patients. Therefore, we also analyzed the 40% of the genes that were unique to CTCL and GVHD patients and found important differences. Since these genes are unique to CTCL patients and were unchanged in GVHD patients, analysis of these different genes may provide us with insight into how TI stimulates anti-tumor immune responses.

In CTCL patients, there was significant up-regulation of many stress related genes that promote inflammatory or immunostimulatory responses (Table 10). The aryl-

hydrocarbon receptor nuclear translocator 2, which is a transcription factor increased during cellular stress (68), was up-regulated in CTCL patients 23.44 fold, P=1.1E-02. Many heat shock proteins (HSPs) were up-regulated after TI only in CTCL patients. HSPs are "danger signals" that play an important role in DC differentiation, particularly in the generation of immunostimulatory DC (69). HSP70-2 (8.75 fold, P=2.2E-02), is expressed in response to stress stimuli and free radials (70). HSP27 (4.49 fold, P=5.3E-03) and HSP40 (4.09 fold, P=6.2E-03) are protective and inhibit oxidative stress induced damage (71). Stress-induced-phosphoprotein 1, a protein that modulates interactions between HSP70 and HSP90 (72), was up-regulated 3.47 fold, P=2.3E-02 after TI. Gene expression of HSP70, a protein that stimulates generation of antigen-specific cytotoxic T cell responses when bound to endogenous peptides presented on MHC class I (73), was up-regulated 2.75 fold, P=3.6E-03. Activator of HSP90, which stimulates the intrinsic activity of HSP90 (74), was up-regulated 2.72 fold, P=2.8E-02. Heat-shock transcription factor 2, responsible for activating HSP genes in response to stress (75), was induced 2.21 fold in CTCL patients, P=4.7E-04. Heat-shock associated protein 1, which binds HSP27, and HSP0, a stress-induced chaperone, were up-regulated 2.10 fold and 2.09 fold, respectively, although P-values did not reach significance (76).

Accession no. (Genbank)	Description	Function	Fold change after TI CTCL TI/Leuk	CTCL P-value	GVHD TI/Leuk	GVHD P-value
	Stress related genes					
NM_014862	Aryl-hydrocarbon receptor nuclear translocator 2	Transcription factor increased during hypoxia	23.44	1.1E-02	No Change	-
NM_005346	Heat-shock protein 70-2	Expressed in response to stress stimuli, free radicals	8.75	2.2E-02	No Change	-
NM_001540	Heat-shock protein 27	Inhibits oxidative stress induced damage, augments AKT activation	4.49	5.3E-03	No Change	-
BC031044	Heat-shock protein 40, subfamily A, member 4	Confers resistance to oxidative stress	4.09	6.2E-03	No Change	-
AL553320	Stress-induced-phosphoprotein 1	Hsp70/Hsp90 organizing protein	3.47	2.3E-02	No Change	-
BC002526	Heat-shock protein 70	Involved in stress response	2.75	3.6E-03	No Change	-
NM_012111	Activator of Hsp90 (AHSA1)	Stimulates intrinsic activity of Hsp90	2.72	2.8E-02	No Change	-
M65217	Heat-shock transcription factor 2	Activates heat-shock response genes under stress	2.21	4.7E-04	No Change	-
NM_024610	Heat-shock associated protein 1	Binds to Hsp27, regulates stress response	2.10	NS	No Change	-
NM_002157	Heat-shock protein 10	Stress-induced chaperone	2.09	NS	No Change	-

Table 10. Stress related genes only up-regulated in CTCL patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. P-values are listed. NS signifies "non-significant" and is listed in place of a P-value for genes where P is greater than 0.05.

In CTCL patients, there were many DC-related genes that were induced after TI (Table 11). CD205, or DC- SIGN, a DC-specific C-type lectin that mediates DC adhesion with T cells (77), was up-regulated 5.57 fold, P=2.5E-02 in CTCL patients after TI. Thymic stromal lymphopoietin receptor, a transmembrane protein expressed in activated DC (78), was up-regulated 5.04 fold, P=9.7E-03. A gene important for antigen processing in DC, cathepsin V (79), was induced 3.78 fold, P=1.1E-02. Fas ligand, which mediates direct killing of cancer cells by DC (80), was up-regulated 2.56 fold, although the P value did not reach significance. CD163, a scavenger receptor (81), was non-significantly up-regulated 2.27 fold. CD86, an important co-stimulatory molecule expressed on DC (35), was up-regulated only in CTCL patients, 2.14 fold, although P-

value did not reach significance. Two DC-associated proteins involved in the ability of DC to stimulate CD4 T cells were down-regulated: dendritic cell-associated lectin, -2.22 fold, P not significant, and CD83, -2.80 fold, P=4.0E-03 (82, 83). Also down-regulated in CTCL patients after TI were several toll-like receptors (TLRs).

Accession no. (Genbank)	Description	Function	Fold change after TI			
			CTCL		GVHD	
			TI/Leuk	P-value	TI/Leuk	P-value
	DC related genes					
AF290886	CD209 (DC-SIGN)	Novel DC-specific C-type lectin, mediates adhesion with T cells	5.57	2.5E-02	No Change	-
NM_022148	Thymic stromal lymphopoietin receptor (CRLF2)	Transmembrane protein expressed by activated DC	5.04	9.7E-03	No Change	-
AF070448	Cathepsin L2	Encodes cathepsin V, involved in antigen presentation	3.78	1.1E-02	No Change	-
D38122	Fas ligand	Involved in direct kiling by DC	2.56	NS	No Change	-
NM_004244	CD163 molecule	Scavenger receptor	2.27	NS	No Change	-
NM_006889	CD86 molecule	Co-stimulatory molecule expressed on DC	2.14	NS	No Change	-
AW237307	Dendritic cell-associated lectin-1	Expressed on DC, interacts with CD4 T cells	-2.22	NS	No Change	-
NM_004233	CD83 molecule	Expressed on DC, involved in CD4 T cell generation	-2.80	4.0E-03	No Change	-
	Toll-like receptors					
AF245702	Toll-like receptor 7	Down-regulated in mature DC, Pro-inflammatory signaling	-9.00	1.2E-02	No Change	-
AW665250	Toll-like receptor 10	Down-reg in mature DC, activates transcritpion through MyD88	-4.41	1.0E-03	No Change	-
NM_003266	Toll-like receptor 4	Down-reg in mature DC, Receptor for LPS	-2.70	NS	No Change	-
	Disease related					
NM_005032	Plastin 3 (T isoform)	Overexpressed in PBMCs of CTCL patients	4.90	NS	No Change	-
	Cell survivial/apoptosis					
NM_002775	HtrA serine peptidase 1	Tumor suppressor, promotes cell death	31.29	6.2E-03	No Change	-
NM_022121	PERP, TP53 apoptosis effector	Plays role in cellular adhesion and survival	9.45	1.1E-03	No Change	-
AI912351	Nucleolar protein 3	Inhibits apoptosis	3.73	4.2E-03	No Change	-
AF229253	Apoptosis inhibitor 5	Antiapoptosis	2.86	NS	No Change	-
	Cell adhesion					
NM_013231	Fibronectin leucine rich transmembrane protein 2	Cell adhesion receptor signaling	39.84	1.4E-03	No Change	-
NM_018204	Cytoskeleton associated protein 2	Involved in cytoskeleton and cytokinesis	3.34	2.0E-03	No Change	-
	NK cell related genes					
AF276292	NK cell Ig-like receptor 4	Receptor on natural killer cells	3.83	NS	No Change	-

Table 11. Genes differentially expressed only in CTCL patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. P-values are listed. NS signifies “non-significant” and is listed in place of a P-value for genes where P is greater than 0.05.

TLRs are known to be down-regulated in mature DC as compared to immature DC (84). TLR7 was down-regulated -9.00 fold, P=1.2E-02; TLR10 -4.41 fold, P=1.1E-03; and TLR4, a receptor for LPS (84), was down-regulated -2.70 fold, although P-value did not reach significance.

A gene specifically related to CTCL and known to be over-expressed in peripheral blood mononuclear cells of CTCL patients, plastin-T (85), was up-regulated only in CTCL patients after TI 4.90 fold, P-value non-significant (Table 11). Genes mediating cell survival and apoptosis specific to CTCL patients included: HtrA serine peptidase 1, a tumor suppressor and promoter of cell death (86), up-regulated 31.29 fold, P=6.2E-03; PERP, a protein that plays a role in both cell adhesion and survival (87), 9.45 fold, P=1.1E-03. Also two inhibitors of apoptosis, nucleolar protein 3 (88) and apoptosis inhibitor 5 (89), were up-regulated 3.73 fold, P=4.2E-03 and 2.86 fold, P non-significant, respectively. Genes involved in cell adhesion, such as the fibronectin leucine rich transmembrane protein 2, which plays a role in cell adhesion receptor signaling (39.84 fold, P=1.4E-03), and cytoskeleton associated protein 2, involved in the cytoskeleton and cytokinesis (3.34 fold, P=2.0E-03) were up-regulated in CTCL patients after TI (90, 91). Finally, a receptor found on natural killer T cells (NK cells), the NK cell Ig-like receptor 4 (92), was up-regulated after TI 3.83 fold, although P-value did not reach significance.

Accession no. (Genbank)	Description	Function	Fold change after TI			
			CTCL		GVHD	
			TI/Leuk	P-value	TI/Leuk	P-value
	DC related genes					
AF290886	CD209 (DC-SIGN)	Novel DC-specific C-type lectin, mediates adhesion with T cells	5.57	2.5E-02	No Change	-
NM_022148	Thymic stromal lymphopoietin receptor (CRLF2)	Transmembrane protein expressed by activated DC	5.04	9.7E-03	No Change	-
AF070448	Cathepsin L2	Encodes cathepsin V, involved in antigen presentation	3.78	1.1E-02	No Change	-
D38122	Fas ligand	Involved in direct kiling by DC	2.56	NS	No Change	-
NM_004244	CD163 molecule	Scavenger receptor	2.27	NS	No Change	-
NM_006889	CD86 molecule	Co-stimulatory molecule expressed on DC	2.14	NS	No Change	-
AW237307	Dendritic cell-associated lectin-1	Expressed on DC, interacts with CD4 T cells	-2.22	NS	No Change	-
NM_004233	CD83 molecule	Expressed on DC, involved in CD4 T cell generation	-2.80	4.0E-03	No Change	-
	Toll-like receptors					
AF245702	Toll-like receptor 7	Down-regulated in mature DC, Pro-inflammatory signaling	-9.00	1.2E-02	No Change	-
AW665250	Toll-like receptor 10	Down-reg in mature DC, activates transcritpion through MyD88	-4.41	1.0E-03	No Change	-
NM_003266	Toll-like receptor 4	Down-reg in mature DC, Receptor for LPS	-2.70	NS	No Change	-
	Disease related					
NM_005032	Plastin 3 (T isoform)	Overexpressed in PBMCs of CTCL patients	4.90	NS	No Change	-
	Cell survivial/apoptosis					
NM_002775	HtrA serine peptidase 1	Tumor suppressor, promotes cell death	31.29	6.2E-03	No Change	-
NM_022121	PERP, TP53 apoptosis effector	Plays role in cellular adhesion and survival	9.45	1.1E-03	No Change	-
AI912351	Nucleolar protein 3	Inhibits apoptosis	3.73	4.2E-03	No Change	-
AF229253	Apoptosis inhibitor 5	Antiapoptosis	2.86	NS	No Change	-
	Cell adhesion					
NM_013231	Fibronectin leucine rich transmembrane protein 2	Cell adhesion receptor signaling	39.84	1.4E-03	No Change	-
NM_018204	Cytoskeleton associated protein 2	Involved in cytoskeleton and cytokinesis	3.34	2.0E-03	No Change	-
	NK cell related genes					
AF276292	NK cell Ig-like receptor 4	Receptor on natural killer cells	3.83	NS	No Change	-

Table 11. Genes differentially expressed only in CTCL patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. P-values are listed. NS signifies “non-significant” and is listed in place of a P-value for genes where P is greater than 0.05.

There was also a group of genes that were only differentially expressed in GVHD patients (Table 12 and 13). Analysis of these GVHD specific genes revealed the following genes of interest. Various immune response mediators were specifically up-regulated in GVHD patients after TI (Table 12). Interleukin 19, a transcriptional activator of the immunosuppressive cytokine IL-10 (93), was up-regulated 50.88 fold, P-value non-significant after TI. CXCL11, an interferon-gamma inducible T cell chemoattractant (94), was non-significantly up-regulated after TI 26.14 fold. In addition, an enzyme closely related to indoleamine named tryptophan 2,3-dioxygenase (TDO2), which is also a rate-limiting enzyme in the catabolism of tryptophan (95), was up-regulated 22.53 in GVHD patients after TI, although P-value did not reach significance. A member of the TNF receptor superfamily (member 11a), which functions as an activator of NFkB (96), was up-regulated 9.00 fold, P=3.8E-02. A transcription factor that plays a negative regulatory role in TLR signaling pathways, called activating transcription factor 3 (97), was up-regulated 4.74 fold, P=2.0E-02. TNFalpha-induced protein 2, which is induced by inflammatory mediators such as TNF, IL-1B, and LPS (98), was up-regulated 3.29 fold, P=5.8E-03. Two pro-inflammatory cytokines were regulated in opposite directions in GVHD and CTCL patients. Interleukin-8 was up-regulated in GVHD patients 3.03 fold, P=3.1E-02 and was down-regulated in CTCL patients -2.31 fold, P-value non-significant. Interferon-gamma, on the other hand, was down-regulated in GVHD patients (-4.52 fold, P non-significant) and up-regulated in CTCL (3.14 fold, P non-significant). A component of complement (C1q binding protein), which inhibits TLR0induced IL-12 production (99), was down-regulated after TI -2.63 fold, P=4.8E-02.

Accession no. (Genbank)	Description	Function	Fold change after TI			
			CTCL		GVHD	
			TI/Leuk	P-value	TI/Leuk	P-value
	Immune response mediators					
NM_013371	Interleukin 19	Transcriptional activator of IL-10	No Change	-	50.88	NS
AF002985	CXCL11	IFN-gamma inducible T cell chemoattractant	No Change	-	26.14	NS
NM_005651	Tryptophan 2,3-dioxygenase (TDO2)	Rate-limiting enzyme in tryptophan catabolism, closely related to IDO	No Change	-	22.53	NS
AW026379	TNF receptor superfamily, member 11a	Activator of NFkB	No Change	-	9.00	3.8E-02
AB066566	Activating transcription factor 3	Negative regulatory transcription factor in TLR pathways	No Change	-	4.74	2.0E-02
NM_006291	TNFalpha-induced protein 2	Induced by TNF, IL-1B, LPS	No Change	-	3.29	5.8E-03
AF043337	Interleukin 8	Pro-inflammatory cytokine	-2.31	NS	3.03	3.1E-02
M29383	Interferon gamma	Pro-inflammatory, activates NFkB	3.14	NS	-4.52	NS
L04636	Complement component 1, q subcomponent binding protein	Inhibits TLR4-induced IL-12 production	No change	-	-2.63	4.8E-02

Table 12. Immune response genes differentially expressed in GVHD patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. P-values are listed. NS signifies "non-significant" and is listed in place of a P-value for genes where P is greater than 0.05.

In GVHD patients there was differential expression of many DC-related genes (Table 13). Fibronectin, which has been shown to be up-regulated in DC induced by GM-CSF and IL-4 in other microarray studies (100), was up-regulated in our study 92.08 fold, although P-value did not reach significance. Tapasin, a protein involved in MHC class I antigen presentation (101), was up-regulated in GVHD patients 3.86 fold, P=3.2E-02 after TI. CD40, a molecule involved in DC survival and cytokine secretion (37), was up-regulated 3.81 fold, P=1.5E-02. Rel-B, which binds NfKB and plays a major role in NFkB signaling (102), was up-regulated 3.27 fold, P=1.5E-04. FLT3, which is induced on DC maturation (103), binds FLT3L, which is used to mature DC (104). FLT3 was down-regulated in GVHD patients after TI, -9.09 fold, P=4.0E-03. Several genes

involved in cell survival and apoptosis were differentially expressed in GVHD patients. The programmed cell death 6 receptor repressor, which is part of the apoptotic machinery controlled by the T cell receptor (105), was up-regulated 28.45 fold, P=1.6E-02. Tax1 binding protein, which inhibits TNF-induced apoptosis (106), was up-regulated 2.61 fold, P=2.0E-02. BCL2-associated athanogene, which enhances the anti-apoptotic effects of BCL2, a protein involved in DC survival (107), was up-regulated 2.33 fold, P=4.5E-02. BCL2-interacting protein, however, which protects against cell death (108), was down-regulated -3.44 fold, P=2.2E-02. Genes important in cell adhesion, such as TRAF2 and NCK interacting kinase, involved in cytoskeleton regulation (109), and CD226 molecule, an intercellular adhesion molecule (110), were down-regulated after TI in GVHD, -7.8 fold, P=6.6E-03 and -5.35 fold, P=8.8E-04, respectively. In GVHD patients, several genes encoding NK cell receptors, which regulate the function of NK cells (92), were down-regulated. Killer cell lectin-like receptor subfamily C members 1 (-5.32 fold, P=2.6E-02), 2 (-2.37 fold, P=3.9E-02), and 3 (-2.40 fold, P=1.2E-02) were all down-regulated.

Accession no. (Genbank)	Description	Function	Fold change after TI			
			CTCL		GVHD	
			TI/Leuk	P-value	TI/Leuk	P-value
	DC-related genes					
BC005858	Fibronectin	Up-regulated in DC induced by GM-CSF + IL-4	No Change	-	92.08	NS
AF067286	TAP binding protein (tapasin)	Involved in MHC class I antigen presentation	No Change	-	3.86	3.2E-02
NM_001250	CD40 molecule	Involved in DC survival and cytokine secretion	No Change	-	3.81	1.5E-02
NM_006509	RELB (v-rel reticuloendotheliosis viral oncogene homolog B)	Up-regulated in activated DC, binds NFkB	No Change	-	3.27	1.5E-04
NM_004119	FLT3 (fms-related tyrosine kinase 3)	Induced on DC maturation, FLT3L is used to mature DC	No change	-	-9.09	4.0E-03
	Cell survival/apoptosis					
AB033060	Programmed cell death 6 receptor repressor	Part of apoptotic machinery controlled by T cell receptor	No Change	-	28.45	1.6E-02
AK001327	Tax1 (human T-cell leukemia virus type I) binding protein 3	Inhibits TNF-induced apoptosis	No Change	-	2.61	2.0E-02
AF116273	BCL2-associated athanogene	Enhances anti-apoptotic effects of BCL2	No Change	-	2.33	4.5E-02
U15172	BCL2/adenovirus E1B 19kDa interacting protein 1	Protects against cell death	No change	-	-3.44	2.2E-02
	Cell adhesion					
AF172268	TRAF2 and NCK interacting kinase	Involved in cytoskeleton regulation	No change	-	-7.88	6.6E-03
NM_006566	CD226 molecule	Intercellular adhesion molecule	No change	-	-5.35	8.8E-04
	NK cell related genes					
NM_002260	Killer cell lectin-like receptor subfamily C, member 1	Regulates function of NK cells	No change	-	-5.32	2.6E-02
AF022048	Killer cell lectin-like receptor subfamily C, member 3	Regulates function of NK cells	No change	-	-2.40	1.2E-02
L76666	Killer cell lectin-like receptor subfamily C, member 2	Regulates function of NK cells	No change	-	-2.37	3.9E-02

Table 13. Genes differentially expressed only in GVHD patients
Fold change in gene expression after TI, using the leukapheresis (Leuk) sample as a baseline. RNA was isolated from the CD4- cell fraction from 3 CTCL patients and 3 GVHD patients following leukapheresis and after ECP + overnight incubation (TI). All samples were processed identically and array data from 6 patients was analyzed using GeneSpring software to identify differentially expressed genes. P-values are listed. NS signifies "non-significant" and is listed in place of a P-value for genes where P is greater than 0.05.

qRT-PCR confirmation of microarray data

The expression of selected differentially expressed genes was confirmed by qRT-PCR using aliquots of the same RNA samples used for the microarrays (Figure 2). Despite the important differences in gene expression between patient groups, we chose to verify DC-

related genes that were similarly differentially expressed in CTCL and GVHD patients to validate the accuracy of our microarray data and to confirm the similarity of the changes in gene expression in these two patient groups. The qRT-PCR results correlated with the microarray results for all 4 selected genes (DC-LAMP, Indoleamine, CCR7, and CD14). Figure 2 shows the average transcriptional changes in these 4 selected genes across samples from 5 patients.

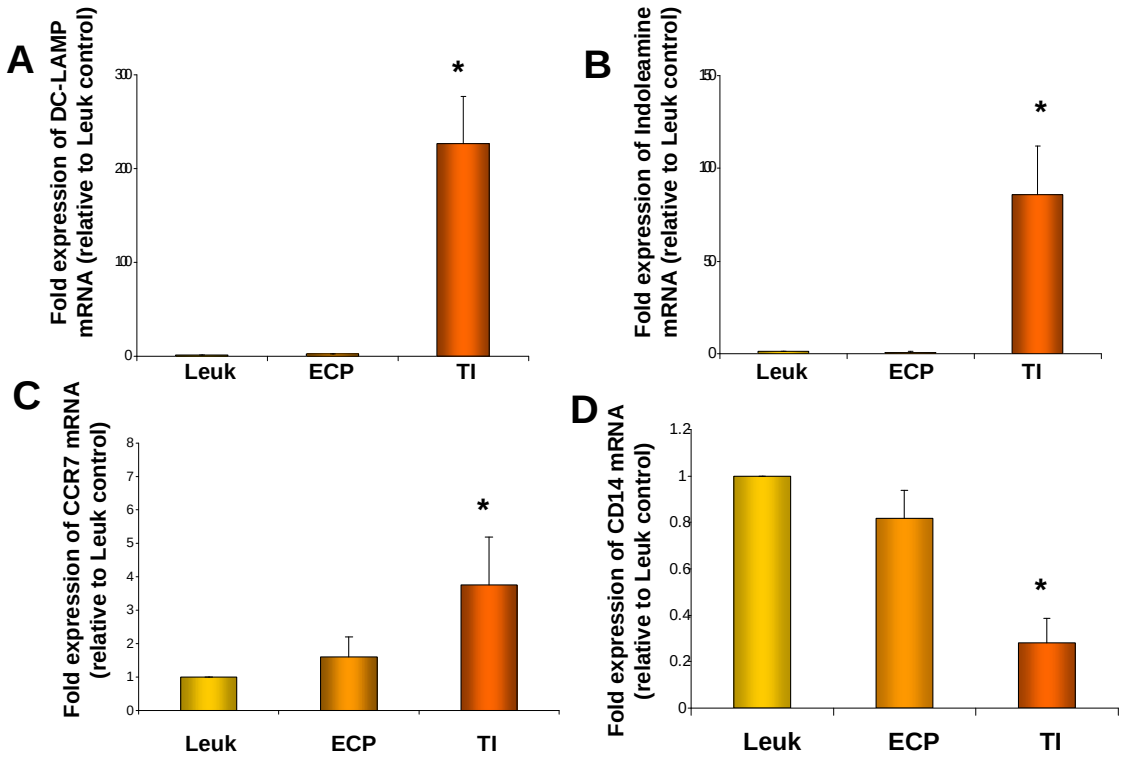

Figure 2: Quantitative RT-PCR confirmation of microarray data.
Fold expression of selected genes after ECP and TI relative to Leuk (control) expression levels. Data represents average fold change across 5 patients, error bars are standard error of the mean. **(A)** DC-LAMP was up-regulated 1.9± 0.3 fold in ECP vs Leuk samples and 226.5± 50.4 fold (P-value 0.008) in TI vs Leuk. **(B)** Indoleamine was unchanged after ECP and up-regulated 85.62± 26.6 fold after TI (P-Value 0.008). **(C)** CCR7 was up-regulated 1.60± 0.6 fold after ECP and 3.76± 1.4 fold after TI (P-value 0.01). **(D)** CD14 was down-regulated 1.3± 0.06 fold in ECP vs Leuk samples and was down-regulated 3.6± 0.04 fold in TI vs Leuk (P-value 0.006).

To verify that 1) the gene expression changes in these selected genes were taking place in the monocyte/DC population, and 2) that the leukapheresis sample was an appropriate baseline, we repeated this real-time PCR using patient samples that were depleted of CD4, CD8, CD19 positive cell fractions to enrich for the monocyte/DC population and had a pre-treatment peripheral blood draw as a baseline for gene expression levels. Samples (including new pre-treatment samples) from 4 patients undergoing ECP were treated with triple bead depletion of non-monocyte populations and RNA was isolated and qRT-PCR performed on 2 genes of particular interest (DC-LAMP, CD14). The observed gene expression trend in DC-LAMP and CD14 in these 4 patient samples confirms the microarray data and validates the use of the leukapheresis sample as a baseline (Figure 3).

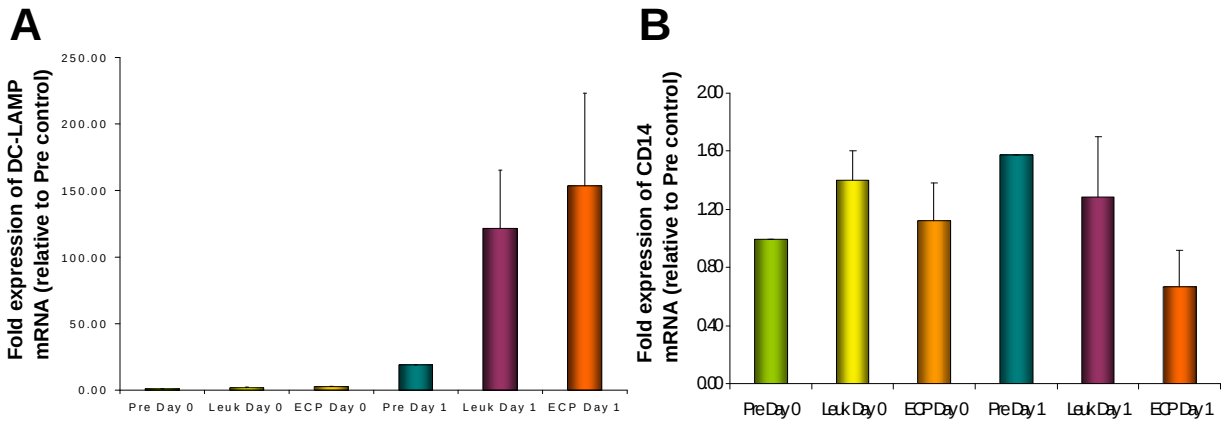

Figure 3 . Further qRT-PCR confirmation of microarray data using CD4, CD8, CD19 depleted samples from patients undergoing ECP.
Fold expression of selected genes after Leukapheresis, ECP, O/N incubation of pre-treatment sample, O/N incubation of leukapheresis sample, and O/N incubation of ECP (TI) relative to pre-treatment whole blood (control) expression levels. Data represents average fold change across 4 patients for graph A, and 3 patients for graph B. Error bars are standard error of the mean. **(A)** DC-LAMP was up-regulated 19.1 fold in Pre Day 1 samples, 121.6 ± 43.6 fold in Leuk Day 1 samples, and 153.58 ± 69.58 in TI treated samples. The trend towards upregulation did not reach significance. **(B)** CD14 was down-regulated after TI 1.5± 0.2 fold, demonstrating a downward trend that did not reach significance.

qRT-PCR validation of gene expression in normal controls

In order to verify that the induction of a mature DC gene expression profile after TI was not unique to CTCL and GVHD patients, we used qRT-PCR to study gene expression changes in TI-treated samples from healthy normal control leukapheresis donors. The transcriptional changes in 2 selected genes (DC-LAMP and CD86) were verified as shown in Figure 4, with similar TI-induced changes in gene expression as seen in patient samples.

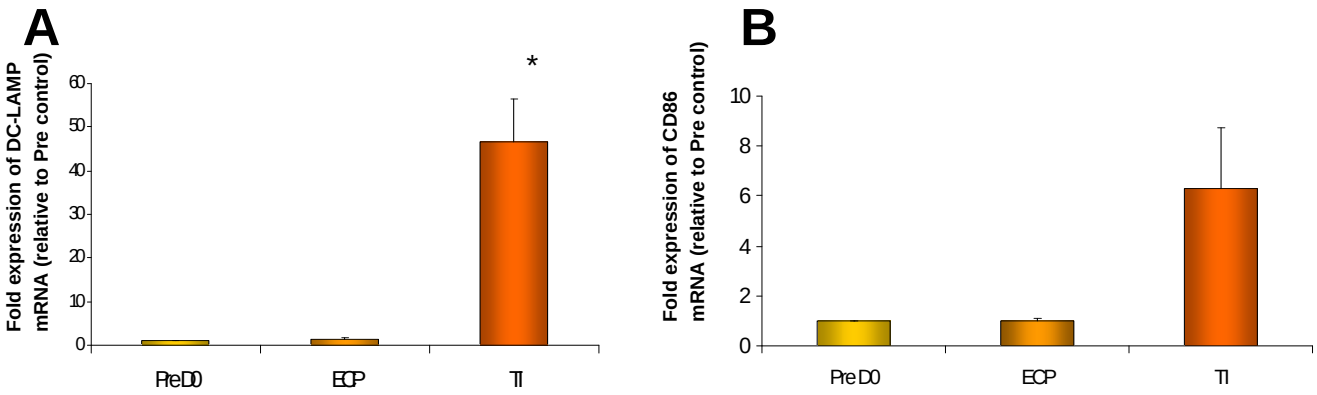

Figure 4: Gene expression changes in normal control samples after leukapheresis and bench-top ECP and TI procedures.
Fold expression of selected genes after a donated leukapheresis sample was treated with bench-top ECP apparatus (ECP sample underwent 3 hours of 8-MOP + UVA exposure) and subsequently incubated overnight (TI sample). The pre-treatment sample is used as baseline. Data represents average fold change across 3 healthy normal control individuals, error bars are standard error of the mean. **(A)** DC-LAMP was up-regulated 1.3± 0.4 fold in ECP vs Pre Day 0 samples and 46.67± 9.9 fold (P-value 0.01) in TI vs Pre Day 0. **(B)** CD86 was unchanged after ECP and up-regulated 6.28± 2.4 fold after TI, although the P-value did not reach significance (P = 0.09).

In addition, the time course of differential gene expression for selected genes (DC-LAMP, CD80, CD40, and GPNMB) was clarified through the analysis of transcript levels at several different time points during the ECP and TI procedure. This more detailed analysis was made possible by the use of a bench-top ECP apparatus, which is an

open access system allowing for acquisition of samples throughout the duration of the ECP procedure. The following samples were obtained from the normal control donors: 1) leukapheresis sample (baseline), 2) 1 hour ECP, 3) 2 hours ECP, and 4) 3 hours ECP. Half of each of these samples was incubated overnight (O/N), giving the following additional samples: 6) Leukapheresis O/N, 7) 1 hour ECP O/N, 8) 2 hour ECP O/N, 9) 3 hour ECP O/N, and 10) Transimmunization (TI), which was treated with the 3 hours of ECP and then cultured O/N in a 1 liter platelet storage bag. Not only did the gene expression changes in the monocyte population of normal controls correlate with the microarray findings, but also the transcript kinetics during this bench-top ECP procedure verified that the established TI procedure (ECP + overnight incubation of treated cells in a platelet storage bag) induces the largest and most significant gene expression changes consistent with a mature DC gene expression profile (Figure 5).

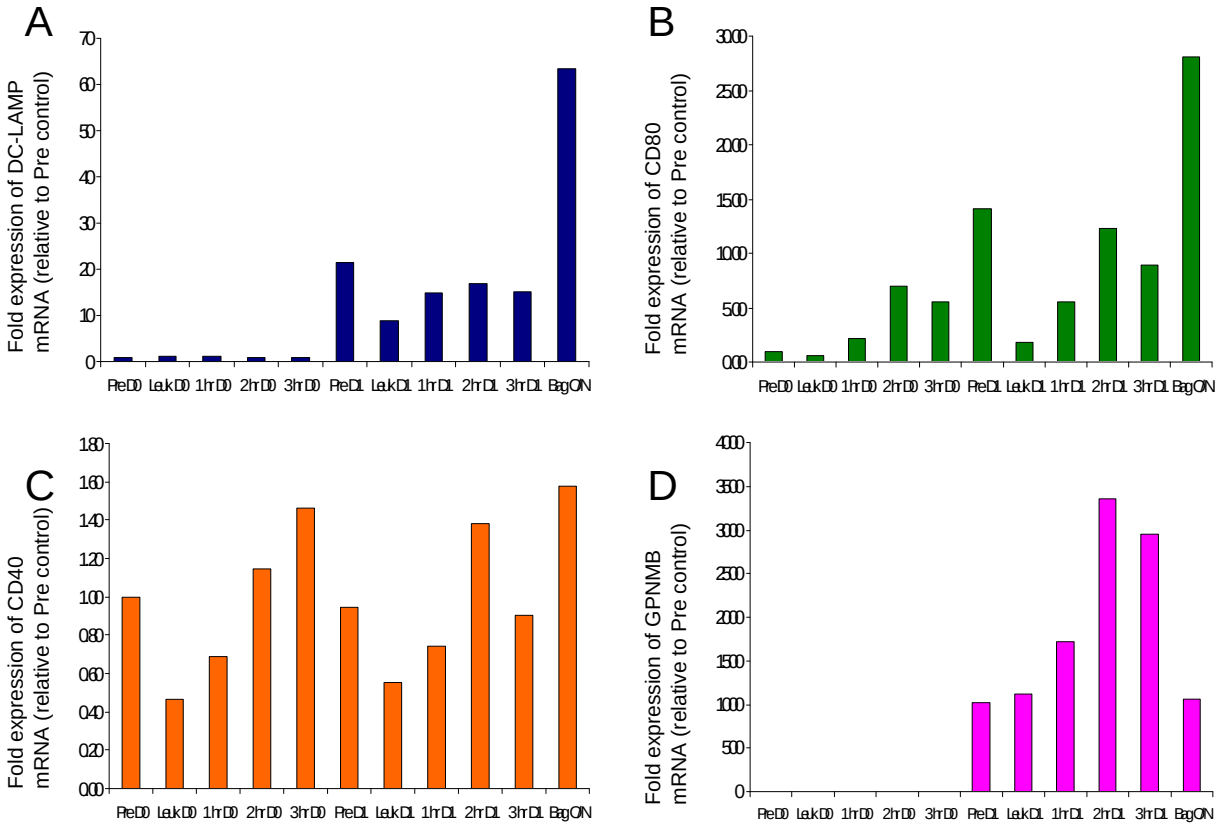

Figure 5 . Confirmation of gene expression changes in normal control leukapheresis donors
Fold expression change of selected genes in a representative normal control donor. This healthy individual underwent leukapheresis, which was treated with the TI procedure with samples taken at specific time-points. Fold change of selected genes (DC-LAMP, CD80, CD40, GPNMB) was measured using a pre-treatment whole blood sample as the baseline and is shown at the following time-points: immediately after leukapheresis aquisition (Leuk D0), after 1 hour of ECP treatment (1 hr D0), 2 hours ECP (2 hr D1), 3 hours ECP (3 hr D0), after overnight (O/N) incubation of the pre-treatment sample (Pre D1), O/N incubation of the leukapheresis (Leuk D1), 1 hour ECP sample incubated O/N (1 hr D1), 2 hours ECP sample O/N (2 hr D1), 3 hours ECP O/N (3 hr D3), and finally, after the full TI procedure including O/N culture in a platelet storage bag (Bag O/N). **(A)** DC-LAMP showed a trend of up-regulation during ECP + O/N incubation, with the most impressive increase in transcript (a 63.4 fold increase) occurring after TI. **(B)** CD80 also showed a trend of up-regulation. After TI we saw a 28.2 fold increase, which was the largest in any sample. **(C)** There was a slight increase in CD40 expression after 3 hours of ECP treatment and also in the 2 hr ECP O/N, 3 hr ECP O/N, and TI-treated samples. After TI the fold change was 1.6. **(D)** For GPNMB, a newly identified gene expressed in mature DC, we saw an increase in gene expression after overnight incubation, with the highest fold change occurring in the 2 hr D1 sample (up-regulated 3358.0 fold). After TI GPNMB was up-regulated 1069.0 fold, which correlates with our microarray data.

Pathway analysis using GeneGo

To identify pathways relevant to ECP and TI, we used MetaCore Software (GeneGo Inc, St Joseph, MI). This is a proprietary, manually curated database containing approximately 500 canonical signaling and metabolic pathways. After TI, there were 71 significantly involved pathways ($P \leq 0.05$). The 20 most significantly involved signaling pathways after TI are listed in Table 14. Several pathways related to regulation of apoptosis and cell cycle were significantly involved after TI. Given the degree of ex vivo manipulation of TI-treated cells, it seemed logical that genes related to apoptosis and cell survival would be affected by the TI procedure. There were 3 anti-apoptotic or pro-survival pathways significantly involved after TI: the anti-apoptotic TNFs/NFkB/Bcl-2 pathway, the AKT signaling pathway, and the anti-apoptotic TNFs/NFkB/IAP pathway. There are several other highly significantly involved pathways that are of particular interest. The chemokines and adhesion pathway was the only signaling pathway that was significantly involved after TI and importantly, also significantly involved after ECP (Table 15). The fact that this is the only signaling pathway that is significantly involved in both treatments emphasizes its potential importance. Activation of pathways related to modulation of the immune response, such as the alternative complement pathway and signaling mediated by IL-6 and IL-1 also occurred after TI (Table 14). Table 15 lists all the pathways that were significantly involved after ECP (12 significantly involved pathways in total). The RalA and RalB pathways, both highly significantly involved, play a role in chemotaxis and endocytosis, two important cell processes for newly activated DC. In addition to significant involvement of the chemokines and adhesion pathway, the more-specific integrin-mediated cell adhesion pathway is involved in the

mechanism of ECP. Taken together, the involvement of the chemokines and adhesion pathway in both TI and ECP as well as the involvement of integrin-mediated cell adhesion in ECP suggest that cell adhesion may initiate signaling cascades that could play an important role in the mechanism of both of these therapies. Because GeneGo software analyzes entire signaling networks, it is possible that the genes involved in the chemokines and adhesion pathway after ECP represent changes in gene expression that occur initially upon integrin binding, whereas the changes in gene expression that occur after TI represent much later events in the signaling and transcriptional cascade.

GeneGo Pathway Name	Total number of genes in pathway	Number of differentially expressed genes in pathway	P-Value
Apoptotic TNF-family pathways	40	20	1.3E-05
ATM/ATR regulation of G1/S checkpoint	42	20	3.1E-05
WNT signaling pathway	28	14	2.5E-04
Chemokines and adhesion	174	53	2.8E-04
Anti-apoptotic TNFs/NFkB/Bcl-2 pathway	42	18	4.0E-04
Putative SUMO-1 pathway	29	14	4.1E-04
TNFR1 signaling pathway	47	19	6.8E-04
Fas signaling cascades	44	18	7.9E-04
Transcription regulation of amino acid metabolism	41	17	9.2E-04
AKT signaling	57	21	1.5E-03
TPO signaling via Jak-Stat pathway	23	11	1.9E-03
Alternative complement pathway	23	11	1.9E-03
Brca1 as transcriptional regulator	30	13	2.3E-03
Activation of PKC via G-Protein coupled receptor	87	28	3.0E-03
JNK pathway	45	17	3.0E-03
N-Glycan biosynthesis p2	21	10	3.1E-03
Sialic-acid receptors (Siglecs) signaling	12	7	3.2E-03
Anti-apoptotic TNFs/NFkB/IAP pathway	31	13	3.2E-03
Signaling mediated by IL-6 and IL-1	38	15	3.2E-03

Table 14. List of 20 most significantly involved pathways after TI treatment.
GeneGo software was used to identify most significantly involved pathways in TI treatment. This pathway data was generated by uploading a gene list of all differentially expressed genes (combined data from 3 CTCL and 3 GVHD patients) with a fold change greater than or equal to 2 and P less than or equal to 0.05 after TI. The total number of genes in each GeneGo signaling pathway is listed, as is the number of pathway genes that were differentially expressed in our microarray data. The P-value represents the probability of having the observed number of differentially expressed genes on a given pathway by chance.

GeneGo Pathway Name	Total number of genes in pathway	Number of differentially expressed genes in pathway	P-Value
RalB regulation pathway	14	3	6.2E-04
Insulin regulation of glycogen metabolism	57	4	5.4E-03
IL9 signaling pathway	33	3	7.8E-03
RalA regulation pathway	35	3	9.1E-03
EPO-induced Jak-STAT pathway	37	3	1.1E-02
Glycolysis and gluconeogenesis	13	2	1.1E-02
cAMP signaling	115	5	1.4E-02
PDGF activation of prostacyclin synthesis	18	2	2.1E-02
Chemokines and adhesion	174	6	2.1E-02
Visual perception	19	2	2.3E-02
Integrin-mediated cell adhesion	92	4	2.7E-02
IL-10 signaling pathway	23	2	3.3E-02

Table 15. List of significantly involved pathways after ECP treatment.

GeneGo software was used to identify significantly involved pathways after ECP treatment. This pathway data was generated by uploading a gene list of all differentially expressed genes (combined data from 3 CTCL and 3 GVHD patients) with a fold change greater than or equal to 2 and P less than or equal to 0.05 after ECP. The total number of genes in each GeneGo signaling pathway is listed, as is the number of pathway genes that were differentially expressed in our microarray data. The P-value represents the probability of having the observed number of differentially expressed genes on a given pathway by chance.

Figure 6 shows the pathway map of the Chemokines and Adhesion Pathway. This diagram, generated by the GeneGo Software Program, shows all of the genes involved in this pathway and also demonstrates which of these genes are up or down regulated in our microarray data after TI.

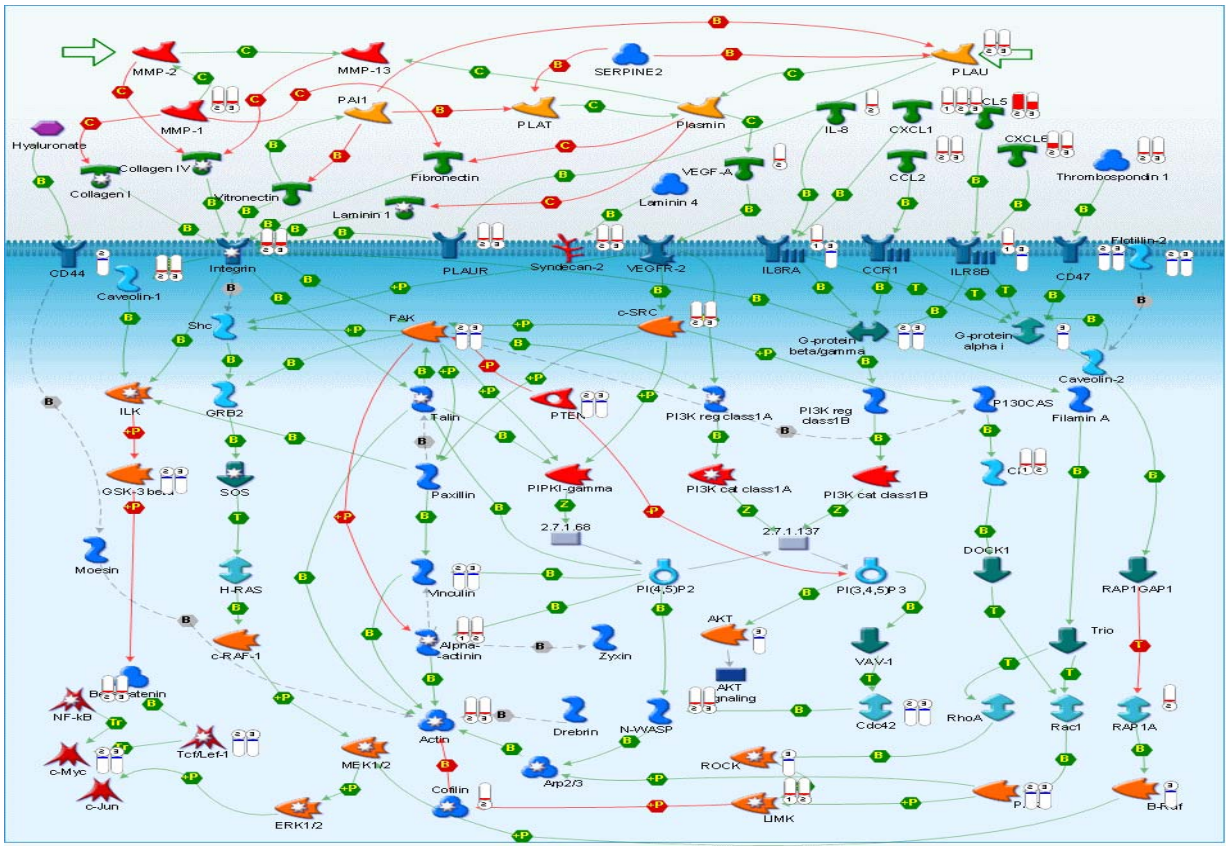

Figure 6: Chemokines and adhesion signaling pathway.
Pathway map shows all genes involved in chemokines and adhesion pathway as well as marking genes involved in pathway that were significantly up or down regulated in our data. The map was generated by uploading the microarray data (combined data set for all 6 patients) into GeneGo Software for pathway analysis. Thermometers indicate amount of differential gene expression for TI vs Leuk. Red thermometers indicate level of up-regulation, blue thermometers indicate level of down-regulation. Cell adhesion and activation of certain integrins and cell surface molecules play a role in initiating DC differentiation, so this pathway is of particular interest.

Figure 7 shows another pathway that is significantly involved in TI: the AKT Signaling Pathway. This pathway is thought to play a role in cell survival, particularly in the survival of DC (111). A recently published study used GeneGo Software to analyze the transcriptional networks involved in DC stimulated with TLR7 agonists (112). Researchers saw induction of anti-apoptotic pathways such as those involving NFkB and BCL2, as well as involvement of chemokine signaling networks (112). Unfortunately, it

is difficult to directly compare our pathway data to the data published in this other study (112), as the focus of that study was on transcription regulatory networks and protein-interaction networks, and not canonical signaling pathways (112).

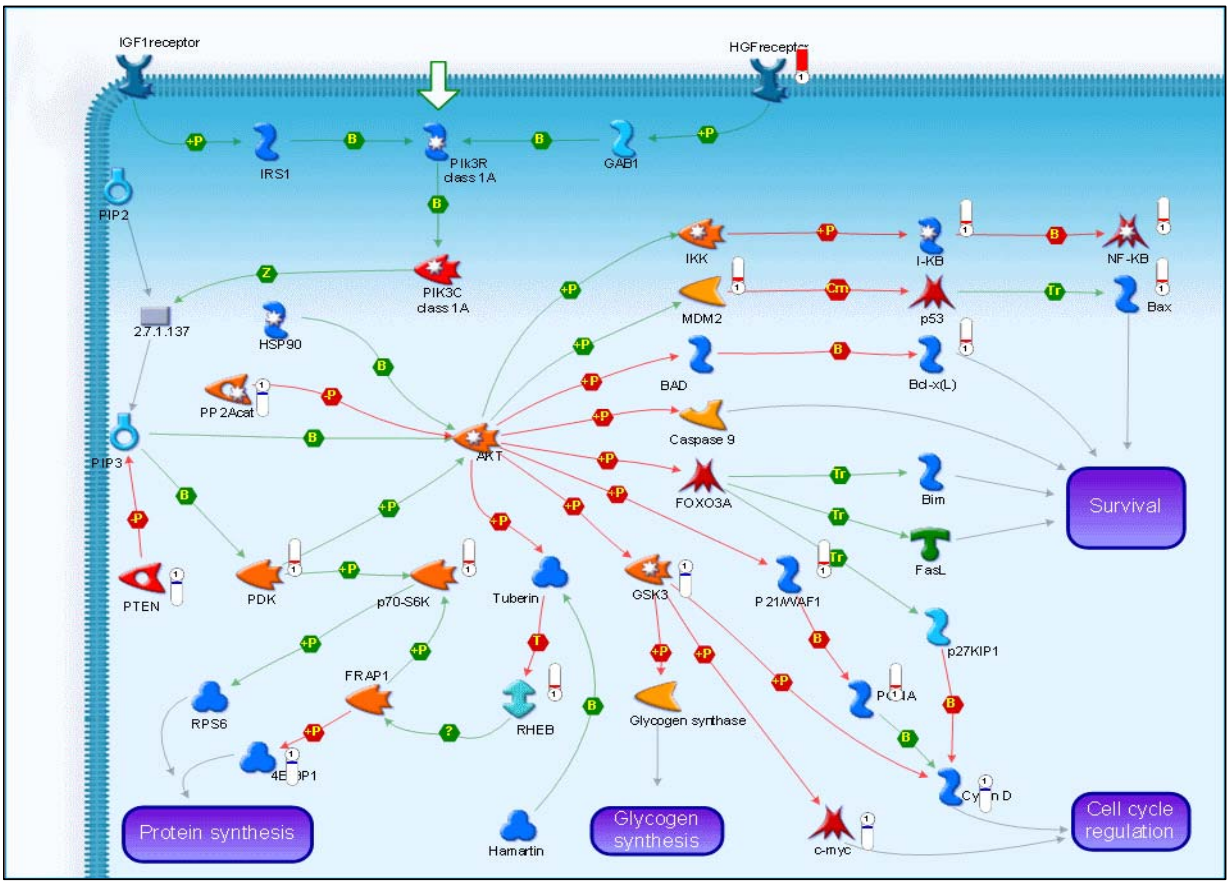

Figure 7: AKT signaling pathway.
Pathway map shows all genes involved in the AKT signaling pathway as well as marking genes involved in pathway that were significantly up or down regulated in our data. The map was generated by uploading the microarray data (combined data set for all 6 patients) into GeneGo Software for pathway analysis. Thermometers indicate amount of differential gene expression for TI vs Leuk. Red thermometers indicate level of up-regulation, blue thermometers indicate level of down-regulation. AKT is a critical regulator of DC lifespan, and over-expression of AKT has been shown to improve clinical efficacy of DC-based tumor vaccines (111).

Discussion

What distinguishes ECP from nearly all other selective cancer immunotherapies has been its clinical success in a significant subpopulation of patients. This success led to an expedited approval by the FDA for the treatment of CTCL, making ECP the first selective immunotherapy approved by the FDA for cancer. The high response rates in immunocompetent CTCL patients (113) have promoted widespread use of this therapy, not only in CTCL but also other serious T cell mediated diseases. ECP has been administered in more than 150 centers worldwide more than 250,000 times (1). It is effective and safe, with no limiting toxicity and remarkably few adverse reactions (2). This therapy is already currently available at a large percentage of leading tertiary care centers in the USA and Europe, making it easy to initiate treatment as well as large-scale clinical trials. Despite these significant successes, the fact remains that ECP has not achieved a universal acceptance in the world of cancer immunotherapy.

A likely reason for the hesitation by many cancer immunotherapists to embrace ECP is the fact that the clinical successes preceded a mechanistic understanding of this therapy. However in 2001, a potential mechanism for the mysterious efficacy of CTCL was elucidated (11) and subsequently used to rationally modify ECP, leading to the introduction of Transimmunization (TI) (12, 24). TI was conceived under the simple premise that the most efficient means for facilitating cell-to-cell contact between newly formed DC and newly apoptotic malignant cells was to incubate them together overnight (11). In conventional ECP, monocytes, which are activated by the treatment procedure, become DC after their return to the patient and malignant cells, rendered apoptotic by

exposure to 8-MOP + UVA, undergo apoptosis after their return to the patient. These two cell populations must encounter each other in the body post reinfusion. This is likely to occur in areas of sequestration of lymphoid cells, such as the spleen and lymph nodes, making these associations more likely. The efficacy of ECP, which is dependent on the interaction of DC and apoptotic cells, speaks to the incredible ability of our immune system to amplify a few key cellular events into a systemic immune response.

TI is an exciting and unique immunotherapy in that it has the same excellent safety profile as ECP, but is potentially more potent in terms of producing anti-tumor immune responses, since CTCL patients that were non-responsive to ECP improved clinically after TI therapy (24). The key cellular events that take place in TI are the same as those in ECP: apoptosis of the passaged lymphocytes, and activation of monocytes. Although seemingly simple, the intricacies of these cellular events contribute to the remarkable efficacy of these immunotherapies. For instance, the 8-MOP +UVA induced apoptosis of malignant lymphocytes is entirely unique in the field of cancer as a method of inducing apoptosis, since the activity of 8-MOP can be turned on and off with a light switch. When activated for just millionths of a second by long wave (low energy) ultraviolet light, 8-MOP forms cross-links in DNA (29). Through adjusting both the concentration of the drug and the intensity of UVA light, one can tightly focus the impact of the drug (29). Because it is biologically inert and safe in its inactivated form, 8-MOP is one of the most titratable potent drugs in clinical use. During the development of ECP, it was decided to use the lowest possible dose of 8-MOP, to avoid large-scale apoptosis and tumor lysis syndrome. In retrospect the lower dose of 8-MOP, which causes a more

gradual apoptosis, likely contributes to the ability of tumor antigens to be recognized by DC and successfully presented to cytotoxic CD8 T cells. The malignant cells are not blown to bits, but rather undergo a slower cell death, increasing the likelihood that surface proteins (tumor antigens) will remain intact.

The other key cellular event in both ECP and TI is the activation of the monocyte population. Monocytes are resistant to apoptosis induced by 8-MOP + UVA and maintain their ability to bind to plastic (10). Our group has previously published work demonstrating that large numbers of monocytes become activated after ECP, and following overnight incubation of ECP treated cells (TI), these monocytes progress further to develop within one day into actively phagocytic cells that express markers associated with the transition into the DC pathway (11). We showed that the differentiating DC were avidly phagocytic and engulfed apoptotic malignant T cells. The results of these prior studies suggested that TI induces tumor-loaded dendritic cells capable of modulating immune responses.

We set out to more fully characterize TI-induced DC and gain insight into their mechanism of induction. Our goal was to determine the gene expression pattern of TI-induced DC. We studied the effects of TI on the monocyte/DC population in CTCL and GVHD patients as well as in healthy normal individuals, in order to gain insight into whether the effects of treatment were generalizable across populations.

We sought to identify gene expression changes that occur during TI in order to more fully characterize TI-induced DC. Genes known to be involved in DC differentiation and maturation were differentially expressed after TI. Our results were in agreement with studies performed looking at gene expression changes in human monocyte-derived dendritic cells which were generated by culture for 5 days with GM-CSF + IL-4 followed by stimulation with LPS for 2 days, as well as DC generated by GM-CSF + IL-4 for 7 days followed by TNFalpha for an additional 7 days (30, 39). After our 24 hour TI procedure, we saw up-regulation of genes encoding DC-LAMP, CCR7, CD83, CD86, and MHC class I that were noted by others only after culturing monocytes for 7-14 days with cytokines. In addition to significant up-regulation of highly specific markers for mature DC, we also saw up-regulation of genes known to play an important role in DC function, such as the co-stimulatory molecule CD80 (B7-1, co-stimulatory signal for T cells) and the transcription factor NFkB, which controls the expression of numerous genes involved in inflammatory immune responses (115). The fact that TI induces gene expression changes associated with mature, activated dendritic cells in 24 hours is important since groups using more conventional methods of producing mature DC do not see these gene expression changes until the cells have been cultured for at least a week (30, 39). This data indicates that TI rapidly induces mature DC, and because the safety and efficacy of these DC have already been proven clinically, TI may represent a particularly valuable form of immunotherapy.

Considering the cellular events that occur during TI and their relevance to physiologic DC maturation, it may not be entirely surprising that TI is capable of

inducing such rapid DC maturation. In vivo, monocytes continuously emigrate from the blood into peripheral tissues. The process of migration across endothelial cell layers has been proposed to initiate monocyte to DC differentiation, with cell adhesion playing a crucial role (116). When migration and cell adhesion take place in the presence of phagocytic stimuli (such as apoptotic cells), a stronger maturation stimulus results, with DC reaching full maturity at an accelerated rate (116). The results of our pathway analysis, which showed that the chemokine and cell adhesion pathway is significantly involved in TI, indicate that cell adhesion may play a crucial role in the mechanism of Transimmunization. Because of the repetitive adhesion and disadhesion to the plastic treatment plate, it is likely that these physiologic signaling cascades initiated by adhesion are amplified, producing a strong maturation signal. This cell adhesion occurs in the presence of many potential "danger signals", which are also a crucial part of physiologic DC maturation. The presence of apoptotic lymphocytes may also provide an additional maturation stimulus (117).

Our data suggest that TI is capable of inducing a gene expression profile consistent with DC maturity regardless of disease state. Similar up-regulations of DC maturity genes were observed in CTCL, GVHD, and healthy normal controls. In addition to finding that TI leads to the up-regulation of genes specific for mature DC, we also found that the majority (60%) of genes that were differentially expressed after TI were the same in both CTCL and GVHD patients. This result is particularly interesting because ECP, the therapy upon which TI is based, causes much different immune responses in these two patient groups. ECP is thought to stimulate an anti-tumor

cytotoxic CD8 T cell response in CTCL patients, whereas, in GVHD, ECP is thought to selectively suppress the lymphocytes producing the graft-versus-host reaction (22). Our data showing up-regulation both of some genes associated with the ability to stimulate and others with the ability to suppress immune responses in both patient populations does not clarify the situation. For instance, the gene encoding indoleamine, a protein associated T cell suppression (53), is significantly up-regulated in both CTCL and GVHD patients after TI. However, the interleukin 1 receptor gene, which plays an important role in activating NFkB and mediating the inflammatory response (118), is also up-regulated in both patient populations after TI. So does TI induce immunostimulatory or immunosuppressive DC? The answer may in fact be that it is capable of inducing either kind of DC, depending on the environment in which the DC maturation process takes place. Researchers in the field of immunology have established that DC which encounter antigen in the presence of "maturation" signals (inflammation) are activated, up-regulate co-stimulatory molecules and are capable of inducing T cell proliferation and inflammation. DC which encounter antigen without inflammatory signals do not up-regulate co-stimulatory molecules and are tolerogenic (23). The advantage of TI is that the overnight culture system allows access to the newly differentiating DC and apoptotic cells, so adjuvant or cytokines could be added to the cell mixture to drive either immunostimulation or tolerance. It is possible that we are inducing one population of DC that may be "multi-potential", and because they express genes associated with both immunostimulation and immunosuppression, could be driven completely in one direction by the use of cytokines or adjuvant. Another possibility is that that we are inducing two separate populations of DC, one which is immunostimulatory and the other which is

immunosuppressive. Further studies are warranted to determine if there are multiple DC populations being induced. Alternatively, it is possible that from the start DC precursors from CTCL and GVHD patients have a different "genetic program" that has developed as a result of their environment. TI may act on these distinct sets of DC precursors in different ways, so that despite many genes being up-regulated in common, the cells still function differently in vivo. It is important to mention that in addition to the commonly differentially expressed genes there were many genes unique to CTCL and GVHD patients and perhaps these unique genes may provide clues about the pre-existing "genetic program" of these DC precursors. The most important message that can be derived from our gene expression data is that TI induces a mature gene expression profile and that these DC up-regulate genes associated with the ability to modulate immune responses. Characterization of the function of TI-induced DC is crucial and these experiments are currently underway in our laboratory.

Further characterization of TI's unique ability to induce DC maturation included an analysis of significantly involved signaling pathways. The results of this analysis suggest that two major groups of signaling pathways are involved: those associated with apoptosis and cell survival, and those associated with chemokines, cell adhesion, and the immune response. According to the GeneGo pathway description, the major apoptosis-related pathway, the apoptotic TNF-family pathway, is activated in response to DNA damage stimulus. There is also significant involvement of a pathway involved in regulation of the cell cycle (ATM/ATR regulation of G1/S checkpoint), described by GeneGo as a "DNA damage checkpoint and response to DNA damage stimulus".

Considering that cells undergoing TI are subjected to 8-MOP+UVA, which is known to cause DNA damage, it makes sense that these pathways are activated. However, previous studies have shown that the monocyte population is resistant to 8-MOP+UVA induced apoptosis (10). The involvement of several pro-survival pathways could provide an explanation for this. There are three pro-survival pathways that are significantly involved in ECP: the anti-apoptotic TNFs/NFkB/Bcl-2 pathway, the AKT signaling pathway, and the anti-apoptotic TNFs/NFkB/IAP pathway. The AKT signaling pathway is of particular interest because it is known to play a role in mediating DC survival (111). AKT is not only a critical regulator of DC lifespan; its activation is also thought to contribute to an enhanced ability of DC to stimulate T cell proliferation, activation, and long-term memory responses (111). Cancer immunologists have suggested that promoting activation of AKT can improve the clinical efficacy of DC-based tumor vaccines (111). The other major group of pathways involved in TI are the chemokines, cell adhesion, and the immune response pathways. The involvement of the chemokines and adhesion pathway in both ECP and TI supports the theory that adhesion to the plastic UVA exposure plate during ECP plays an important role in the mechanism of these therapies, likely activating monocytes and initiating DC differentiation. The involvement of the alternative complement pathway is interesting, as this pathway is associated with inflammatory immune responses. Also, the significant involvement of signaling mediated by IL-6 and IL-1 suggests TI-induced DC are up-regulating signaling cascades associated with inflammatory immune responses.

One of the benefits of microarray gene expression studies is that they produce large amounts of data and allow for analysis of overall patterns of gene expression. However, sometimes the sheer quantity of the data makes it difficult to navigate and therefore it can be helpful to have a focus while analyzing the data. We chose to focus on changes in gene expression that occurred in both CTCL and GVHD patients because this group represented the majority of differentially expressed genes after TI and contained many important DC and immune response related genes. However, upon initial analysis of the genes that are uniquely expressed in either CTCL or GVHD patients, it is clear that these genes may hold important clues to the mechanism of TI in these two patient groups. For instance, the marked up-regulation of heat shock proteins in CTCL patients could play an important role in the generation of the anti-tumor immune response (119). Heat shock protein 70, which is up-regulated in CTCL patients only after TI, plays a crucial role in cross-priming and the generation of antigen-specific cytotoxic T cell responses (119). In GVHD patients only, there was up-regulation of IL-19 and tryptophan 2,3 dioxygenase (TDO2). IL-19 is a transcriptional activator of IL-10, an immunosuppressive cytokine that has been implicated in the mechanism of ECP in GVHD patients (120). TDO2 is an enzyme closely related to indoleamine, and a recently published report suggests that indoleamine may play a protective role in GVHD, diminishing inflammation and disease severity (121). The up-regulation of a natural killer (NK) cell receptor gene in CTCL patients and the down-regulation of three NK receptor genes in GVHD patients likely represents changes in expression occurring in NK cells since these cells were in the population from which we isolated the RNA. The

generation of NK cells in CTCL would be desirable since they are capable of mediating potent anti-tumor cytotoxic responses (122).

TI was designed with the goal of improving the efficacy of ECP for the treatment of cancer. Because fully mature dendritic cells are more potent stimulators of CD8 T cell responses than immature DC (123), the fact that TI is capable of inducing mature DC in an efficient manner makes it ideal for this purpose. TI has been tested clinically in cancer patients with CTCL with impressive clinical results (24). As more is learned about TI and its underlying mechanism its uses may expand, but at the outset this treatment will be tested and optimized as a DC-based cancer immunotherapy. Improved immunotherapies are desperately needed for cancer patients, and with its ability to generate large numbers of mature DC Transimmunization holds great promise as a cancer immunotherapy.

The clinical successes of ECP and TI stand in stark contrast to the disappointing clinical findings of other forms of DC-based tumor vaccines. In melanoma, for which there has been the greatest number of DC-based tumor vaccine trials, the cumulative clinical response rate for Phase I/II studies of dendritic cell-based therapeutic vaccines is 14% (124). A greater response rate of 21% was achieved in a study using anti-CTLA4 in combination with a melanoma peptide-based vaccine, however 43% of patients showed grade III-IV autoimmune toxicities (125). This high level of autoimmune toxicity is concerning and contrary to the goal of immunotherapy, which is to provide targeted therapy with minimal toxicity. In prostate cancer, the clinical results of phase I and II studies of DC-based vaccines show a response rate ranging between 10-46% (126). A phase III clinical trial is currently underway to assess Dendreon's sipuleucel-T vaccine,

which uses autologous antigen-presenting cells loaded with a conjugate protein. This therapy holds some promise in terms of increasing survival although the clinical trials to date have not demonstrated an increase in time to disease progression (127). In head and neck cancer vaccine-based approaches have achieved limited success, with a peptide-based vaccine currently being evaluated in a phase I clinical trial (128). In colorectal cancer, a tumor-lysate-loaded DC vaccine was unable to induce long-lasting effective immune responses in patients (129). Researchers in ovarian cancer, frustrated with the failures of current strategies to induce significant anti-tumor immunity, have focused on overcoming tumor-mediated immune subversion through depleting T regulatory cells, although the clinical efficacy of this approach has not yet been tested (130). In general, the clinical efficacy of cancer vaccines has been surprisingly low, with an average objective response rate of 3.8% in patients with metastatic cancers (131). Many researchers feel these treatment strategies will not be widely adapted until their clinical efficacy is improved (132).

A targeted DC-based cancer immunotherapy with proven clinical efficacy may already exist in Transimmunization. This work demonstrates that Transimmunization induces a gene expression profile consistent with mature DC. Dr. Berger and colleagues have previously shown that these DC are loaded with apoptotic malignant cells, suggesting that during the process of TI-driven monocyte to DC differentiation the newly differentiating DC pass through an aggressively phagocytic stage, in which they can become loaded with tumor antigens (11). TI therefore represents an attractive strategy for the treatment of a multitude of different cancers, even solid tumors. If tumor cells

were harvested and rendered apoptotic, either by 8-MOP + UVA treatment or another method, they could be incubated overnight with a patient's TI treated cells. The newly differentiating DC could phagocytose the tumor cells and become loaded with tumor antigen before reinfusion into the patient the following day. This method circumvents the complex issues surrounding identification of specific tumor antigens and loading DC with these antigens. Whole tumor cells, expressing the full array of tumor antigens in that particular patient, could be used to load TI-induced DC with a diverse set of patient-specific tumor antigens. This is a particularly elegant design because the best antigens to target for anti-tumor vaccines for most cancers have not truly been defined (133) and may be unique to an individual patient's tumor (134).

The concept of using apoptotic whole tumor cells as a source of antigen for cancer immunotherapy is embraced by world-renowned DC biologists such as Jacque Banchereau (119). Dr. Banchereau's group and others have shown that ex-vivo-generated DCs loaded with killed tumor cells can generate clinical responses in cancer patients (119). Recently, this group reported that treating tumor cells with hyperthermia enhances cross priming of tumor-specific CTLs, likely via up-regulation of HSP70 (135). Hyperthermia is used because it stresses the tumor cells and up-regulates heat shock proteins, which play important roles in activating DC (135). In relating this to ECP, it is conceivable that ECP may be a type of stress treatment for CTCL cells. We saw up-regulation of heat shock proteins, signs of cellular stress, in CTCL patients. However, we do not know if heat shock proteins were specifically up-regulated in the malignant CD4+ cells because we depleted this cell population. Although we did not analyze the gene

expression in the CD4+ population in this current study, all of the CD4+ fractions isolated from the patient samples have been saved in archival storage and it would be interesting to go back and look at heat shock expression levels in these samples.

There are several characteristics of TI-induced DC that make TI a particularly appealing immunotherapy for cancer. First, TI generates mature DC in 24 hours, making it an extremely efficient method for producing mature DC. Because the induction of a mature DC phenotype occurs so rapidly, these DC are relatively synchronized in terms of their maturational state. Dr. Carole Berger has calculated that each TI treatment procedure induces approximately 350 million synchronized, mature DC (personal communication, Dr. Carole Berger, data not shown). This number was generated using the white blood count of normal control donors, and multiplying it by the percentage of monocytes and then the percentage of CD83+ monocytes induced by TI. Because mature DC are known to be superior generators of T cell responses (124), it is highly desirable to generate large numbers of these cells, all at the same maturation point. Second, TI-induces DC by a more physiologic mechanism than conventional culture methods, which involve at least a week in cell culture and flood monocytes with extremely high, non-physiologic concentrations of exogenous cytokines. In TI, cell adhesion pathways are activated in the presence of phagocytic stimuli and likely also danger signals released from apoptotic cells, mimicking physiologic circumstances under which monocytes undergo activation and differentiation into DC (116). Third, TI generates highly personalized and specific antigen-loaded DC for each patient. This therapy starts with a patient's own monocytes, and as these cells differentiate into DC they phagocytose the

patient's own tumor cells, allowing for the full array of tumor antigens in that patient to be presented. Finally, TI has proven clinical efficacy, making it unique in the field of cancer immunotherapy. TI is safe and readily available, as ECP treatment centers already exist around the world and minimal additional materials are necessary to perform the overnight incubation step.

TI has tremendous potential as a treatment for various immunogenic cancers. While TI has thus far only been performed on cancer patients, TI's precursor, ECP, is used not only for cancer treatment but also for the treatment of T-cell mediated immune diseases such as GVHD. TI may generate mature DC that could be modulated through the addition of exogenous agents to be inducers of tolerance, thus expanding the possible uses of TI. The ability to control the type of immune response induced may be provided by the access to all the constituents of the immune response present in the treatment culture bag.

The survival rate for many cancers remains frustratingly low, despite the dedication and long history of high-quality basic and clinical science in the field of cancer research. Immunotherapy for cancer is appealing because it has the potential to generate specific anti-tumor immune responses with little systemic toxicity. However, the current state of cancer immunotherapy, particularly in the area of DC-based tumor vaccines, is disappointing. Transimmunization is an ideal method for the generation of tumor-antigen loaded, mature DC with the ability to induce systemic anti-tumor immune

responses. Our hope is that the full potential of this powerful and unique immunotherapy will be realized and lead to improved outcomes for large numbers of patients.

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