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IMMUNOLOGICAL MECHANISMS OF EXTRACORPOREAL PHOTOPHERESIS IN CUTANEOUS T CELL LYMPHOMA AND GRAFT VERSUS HOST DISEASE

Lisa Shiue

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by

Lisa Harn-Ging Shiue, B.S.

APPROVED:

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Madeleine Duvic, M.D., Supervisory Professor

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Amin Alousi, M.D.

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Wei Cao, Ph.D.

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Dorothy Lewis, Ph.D.

_____

Greg Lizee, Ph.D.

_____

Xiao Ni, M.D., Ph.D.

_____

Stephen E. Ullrich, Ph.D.

APPROVED:

_____

Dean, The University of Texas Graduate School of Biomedical Sciences at Houston

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A

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by

Lisa Harn-Ging Shiue, B.S.

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DEDICATIONS

To my family.

To the patients suffering from L-CTCL and GVHD.

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Publication No._____

Lisa Harn-Ging Shiue, B.S.

Supervisory Professor: Madeleine Duvic, M.D.

Extracorporeal photopheresis (ECP) is an effective, low-risk immunomodulating therapy for leukemic cutaneous T cell lymphoma (L-CTCL) and graft versus host disease (GVHD), but whether the mechanism(s) of action in these two diseases is (are) identical or different is unclear. To determine the effects of ECP *in vivo*, we studied regulatory T cells (T-regs), cytotoxic T lymphocytes (CTLs), and dendritic cells (DCs) by immunofluorescence flow cytometry in 18 L-CTCL and 11 GVHD patients before and after ECP at Day 2, 1 month, 3 months, and 6 months. In this study, ECP was effective in 12/18 L-CTCL patients with a 66.7% overall response rate (ORR) and 6/11 GVHD patients with a 54.5% ORR. Prior to ECP, the percentages of $CD4^+Foxp3^+$ T cells in 9 L-CTCL patients were either lower (L-CTCL-Low, n=2) or higher (L-CTCL-High, n=7) than normal. Five of the 7 GVHD patients had high percentages of $CD4^+Foxp3^+$ T cells (GVHD-High). Six of 7 L-CTCL-High patients had >80% $CD4^+Foxp3^+$ T cells which were correlated with tumor cells, and were responders. Both L-CTCL-High and GVHD-High patients had decreased percentages of $CD4^+Foxp3^+$ and $CD4^+Foxp3^+CD25^-$ T cells after 3 months of treatment. $CD4^+Foxp3^+CD25^+$ T cells increased in GVHD-High patients but decreased in L-CTCL-High patients after 3 months of ECP. In addition, numbers of CTLs were abnormal. We confirmed that numbers of CTLs were low in L-CTCL patients, but high in GVHD patients prior to ECP. After ECP, CTLs increased after 1 month in 4/6 L-CTCL patients whereas CTLs decreased after 6

months in 3/3 GVHD patients. Myeloid (mDCs) and plasmacytoid DCs (pDCs) were also low at baseline in L-CTCL and GVHD patients confirming the DC defect. After 6 months of ECP, numbers and percentages of mDCs and pDCs increased in L-CTCL and GVHD. MDCs were favorably increased in 8/12 L-CTCL responders whereas pDCs were favorably increased in GVHD patients. These data suggest that ECP is favorably modulating the DC subsets. In L-CTCL patients, the mDCs may orchestrate Th1 cell responses to overcome immune suppression and facilitate disease regression. However, in GVHD patients, ECP is favorably down-regulating the immune system and may be facilitating immune tolerance to auto-or allo-antigens. In both L-CTCL and GVHD patients, DCs are modulated, but the T cell responses orchestrated by the DCs are different, suggesting that ECP modulates depending on the immune milieu.

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CD	Cluster of differentiation
CSSMs	Cell surface signaling molecules
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ECP	Extracorporeal photopheresis
Foxp3	Forkhead Transcription Factor
GVHD	Graft-versus-Host-Disease
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
L-CTCL	Leukemic Cutaneous T cell Lymphoma
mDC	Myeloid Dendritic cell
MHC	Major Histocompatability Complex
pDC	Plasmacytoid Dendritic cell
PRR	Pattern Recognition Receptors
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T-reg	$CD4^+$ Regulatory T cell

CHAPTER 1

GENERAL BACKGROUND

1.1 Elements of the Immune System

The immune system is comprised of differentiated hematopoeitic cells that arise from bone marrow hematopoietic stem cells (HSC) **(Figure 1.1)**. HSCs differentiate into various types of cells such as erythroid cells, platelets, myeloid cells such as neutrophils, basophils, eosinophils, monocytes, mast cells, natural killer cells, dendritic cells, natural killer T cells, and thymus derived cells (T cells), and B cells depending on the environment and stimuli [1]. For example, the HSCs that migrate to the thymus can develop into T cells where thymic epithelium –HSC interaction leads to deletion of naive T-cells recognizing self-antigens and promotes survival of other T-cells that will become part of the intrinsic host response to neo-antigens. After development, immune cells orchestrate host protection by responding against foreign antigens. For these reactions to occur normally, a proper maintenance of molecular and cellular balance is vital. Once awry, normal immunity is disrupted and disease with increased susceptibility to infection arises.

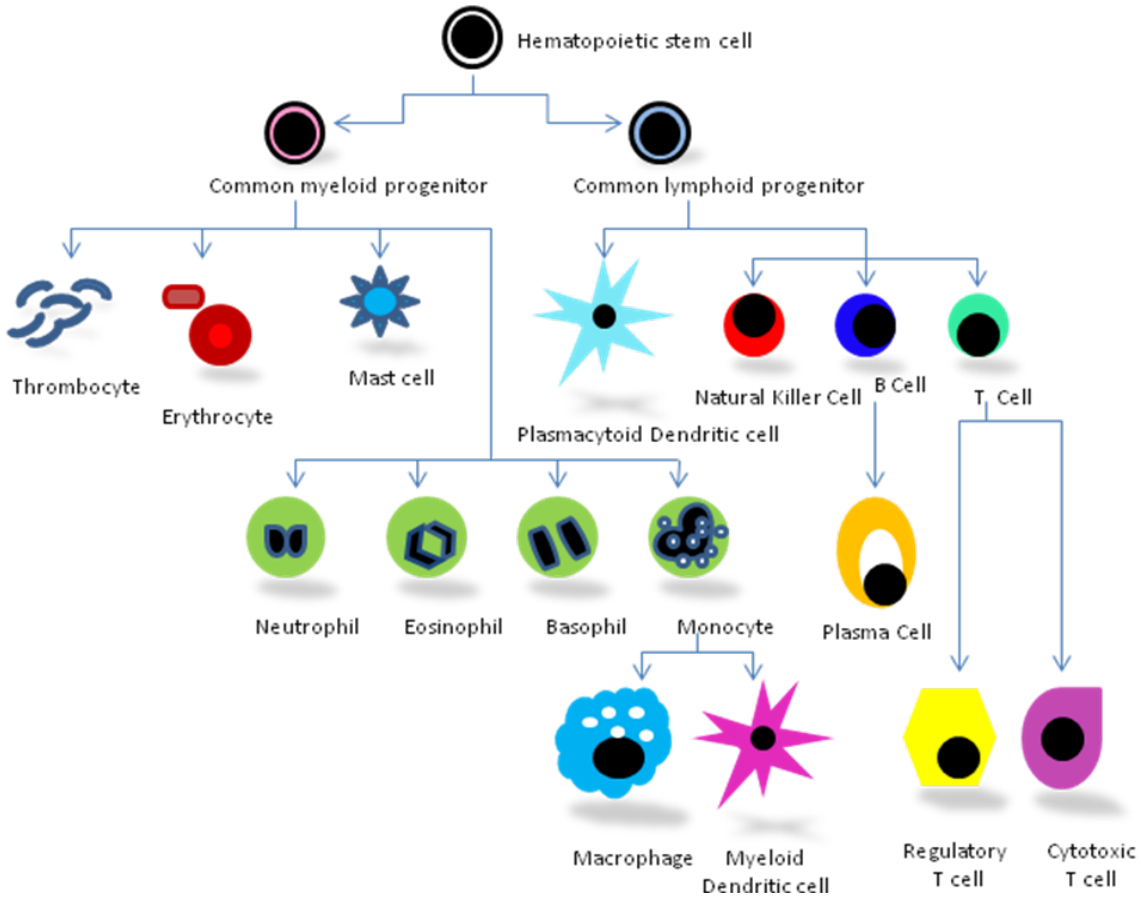


Figure 1.1. Simplified schematic of immune cell development.

1.2 Innate and Adaptive Immunity

Host defenses against pathogens are organized into two categories: 1) Innate and 2) Adaptive Immunity **(Figure 1.2)**. Innate immunity is the fast-acting, front-line defense against all pathogens and is non-specific. Adaptive immunity is activated by innate immunity and is a slow, antigen-specific response that can lead to formation of memory against foreign antigen.

Innate immunity comprises of surface tissues such as the skin and intestinal tract, molecules such as Toll-like receptors (TLRs), and cells including neutrophils, macrophages, eosinophils, and dendritic cells. The first line of defense in innate immunity includes the surface tissues which provide an inpenetrable barrier to the environment. The skin, intestinal tract, respiratory tract, and reproductive tract epithelia interact with the external environment and continuously encounter bacteria, fungi, viruses, and pathogenic parasites. As a result, these epithelia produce a diverse aramentium of antimicrobial proteins that can kill or inhibit microbial growth to manage the substantial microbial exposure. In addition, these surface tissues are challenged with defending a large surface area against pathogens while maintaining homeostasis of communities of commensal microorganisms. To cope with microbial challenges, epithelial antimicrobial proteins (AMP) are produced and protect mammalian body surfaces. AMPs are natural antibiotics that function to kill or inactivate microorganisms [2]. Defensins, cathelicidins, lysozyme, C-type lections (i.e. regenerating islet derived protein (REG) family, ribonucleases (RNAses, angiogenin 4 (ANG4), and S100 proteins (calprotectin, and psoriasin (S100A7)

are all distinct AMP protein families found in the skin and gut [2,3]. The physical surface tissues coupled with AMPs produced by epithelial cells provide a barrier to prevent pathogenic invasion. When pathogens evade anti-pathogenic defense, protection is activated at the molecular and cellular level. Phagocytic cells work quickly to contain the spread of invaders by sensing microbial components through Toll-like receptors (TLRs) as well as cellular pinocytosis and phagocytosis [4]. Neutrophils, macrophages, eosinophils, and dendritic cells (DCs) are types of phagocytic cells that can detect pathogens. In addition to their phagocytic capability, dendritic cells also express TLRs which can bind bacterial components such as Flaggellin or LPS found on gram-postive bacteria and gram-negative bacteria. This elicits an intracellular signaling casade that enhances cytokine secretion, upregulation of MHC and co-stimulatory molecules, and presentation of antigens.

Not only do DCs play a role in innate immunity, but they also have a role in adaptive immunity. They "bridge" innate and adaptive immunity by activating B and T cells through antigen presentation on their cell surfaces [5]. When B cells are triggered by DCs, a humoral response containing secretion of antigen specific antibodies occurs. At the same time, DCs activate $CD4^+$ helper and $CD8^+$ T cells that elicit T cell responses specific to the antigen [6]. Depending on the type of antigen, different $CD4^+$ T helper responses such as Th1, Th2, Th17, and T-reg can occur. Corresponding Th cells also secrete cytokines that promote cell interactions with other cells which are recruited to the infected site. For example, the Th1 response is mediated by $CD4^+$ helper T cells and $CD8^+$ T

cells that secrete IFN-γ, IL-2, and IL-12 to fight against viruses. The $CD4^+$ helper cells “help” to elicit a cytotoxic attack against the virus, mainly through $CD8^+$ cytotoxic T cells [7,8] .

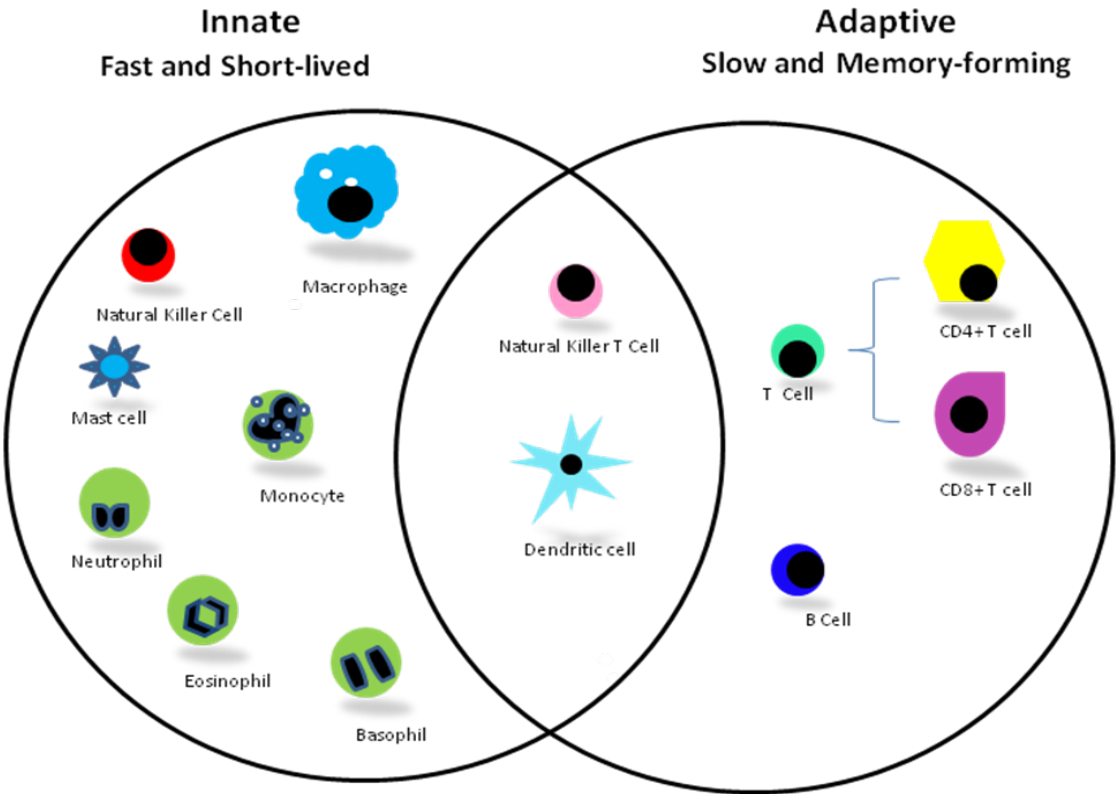


Figure 1.2. Diagram of cells involved in innate and adaptive immunity.

1.3 Dendritic Cells

The architecture of the immune system includes a wide variety of cells that help to maintain homeostasis. The balance between tolerance and immunity is tightly controlled by professional antigen presenting cells (APCs). Professional APCs have antigen-uptake machinery and MHC class II-peptide complexes on the cell surface and can elicit T or B cell responses. Dendritic cells (DC) are a type of professional APCs derived from hematopoietic precursors and have the capability to induce antigen-specific T or B cell responses. First discovered in the spleen about 40 years ago by Ralph Steinmen, DCs delineate from the common myeloid progenitors, develop through the lymphoid pathway, and differentiate from monocytes [9]. They exist in all lymphoid, non-lymphoid tissues, and circulate in blood. Their ubiquitious presence allows them to sample the environment through pattern recognition receptors (PPRs) such as TLRs or phagocytosis. Through phagocytosis, DCs process antigens for cell surface presentation to alert the other defense cells. Antigen presentation induces upregulation of co-stimulatory molecules such as B7 family molecules (e.g. CD80, CD86) and MHC molecules which allow interaction with multiple T cells and B cells in the periphery or in the lymph nodes [10] .

The interactions between DC MHC chains and co-stimulatory molecules with the T cell or B cell receptors initiates naïve T and B cell intracellular signaling that induces a primary immune response that result in extracellular cytokine secretion, extracellular and/or intracellular changes, and cellular differentiation **(Figure 1.3)**. Upregulation of stimulatory and MHC molecules on

DCs mark their transition from immature DCs to mature DCs status. Naïve T cells become differentiated and acquire specificity in their response against the foreign antigen. Naïve B cells also become differentiated into plasma cells that produce and secrete antibodies targeted against the foreign antigen [11].

Although the DC family is heterogenous, it consists of different subsets that each have specific functional characteristics. Two subsets that have been characterized in humans are myeloid DCs (mDC) and plasmacytoid DCs (pDC). The two subsets are distinguished by distinct expression of cell surface molecules and PPRs which determine their specialized functions. Myeloid DC (mDC) are $CD11c^+$ cells expressing myeloid markers (e.g. CD13, CD33, and CD11b). There are three subtypes of mDCs which can be identified by the expression of CD11c in combination with unique surface molecules CD1c (BDCA1), CD141 (BDCA3), and CD16 [12]. Immature mDCs reside in peripheral tissues and migrate to lymph nodes after their maturation during infection or inflammation to activate T cells and secrete IL-12 [13,14]. Plasmacytoid DCs (pDC) produce large amounts of type 1 IFNs in response to bacterial or viral stimuli. The high levels of type 1 IFNs enable communication between pDCs and mDCs, natural killer cells, and B cells. These IFNs stimulate mDCs to enhance T-cell activation and activate natural killer and B cells. During infection, single-stranded viral RNA or unmethylated CpG DNA motifs are recognized by TLR-7 and TLR-8, respectively, which are located in intracellular endosomes and lysosomes. In steady-state conditions, pDCs circulate through the body after entering the bloodstream and migrate to secondary lymphoid organs through

high endothelial venules. After inflammation, pDCs accumulate at the infectious site to take up antigens and then migrate to the lymph nodes to present the encountered antigen [15,16].

PDCs are $CD11c^-$ cells without myeloid markers but with plasmacytoid morphology, a well-developed Golgi apparatus, and rough endoplasmic reticulum. Although mDCs possess myeloid markers and pDCs do not, their developmental pathways remain unclear but cannot be categorized by the conventional myeloid or lymphoid pathway [17]. Therefore, it is challenging to identify mDCs and pDCs by lineage markers. The current method of identifying mDCs and pDCs by extraceullar immunofluorescence flow cytometry is by excluding lineage markers for other cells (CD3, CD14, CD16, CD19, CD20, CD56), while detecting expression of HLA-DR (MHC II), and CD11c or CD123 (IL-3Rα). Thus, mDCs are identified in this dissertation as $Lineage^-$, $HLA\text{-}DR^+CD11c^+$ and pDCs are identified as $Lineage^-HLA\text{-}DR^+CD123^+$.

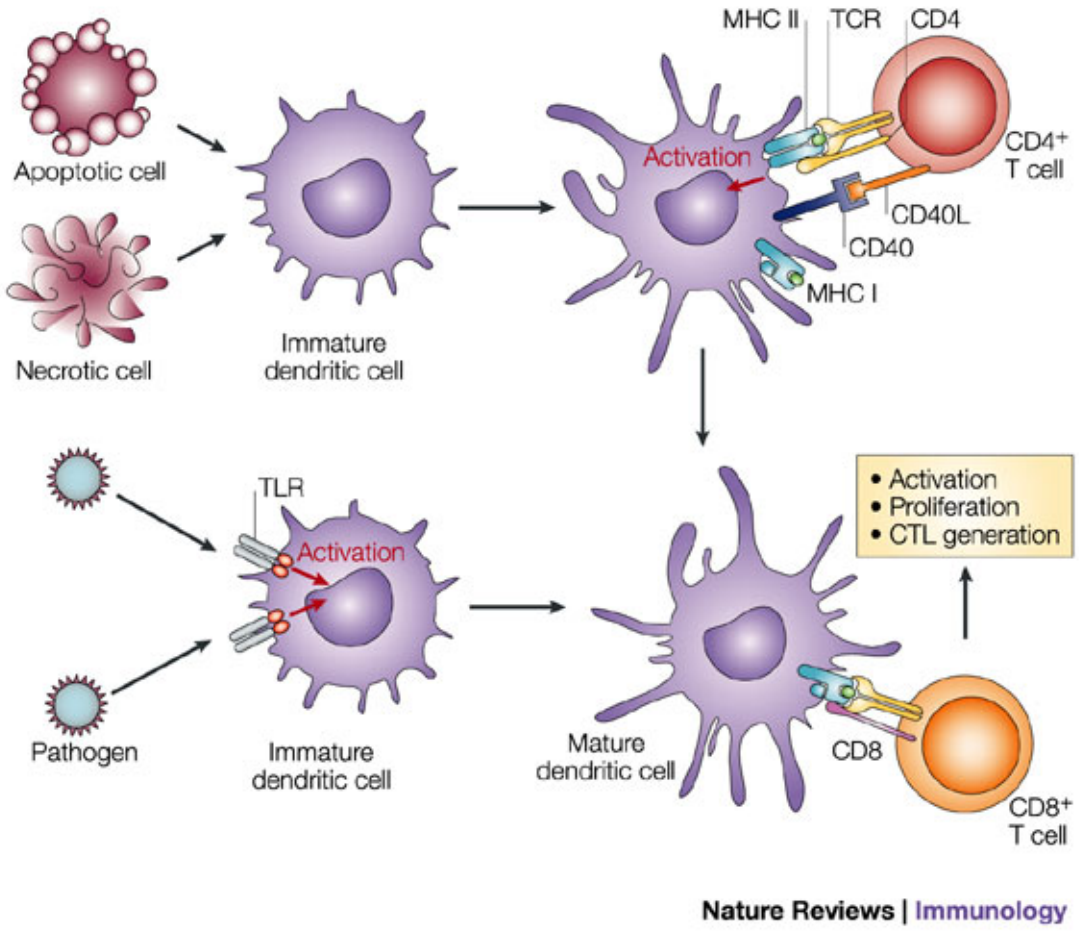


Figure 1.3. Adaptive Immunity: DC and T cell interactions. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, [18], copyright (2004).

1.4 T cells

T cells delineate from HSCs and mature in the thymus. Expression of self antigens in the thymic epithelium provides proper T cell education and development. T cells interact with DCs or thymic epithelial cells for instruction of proper function. Through a process termed negative selection, naïve T cells are tested against protein chains found on conjugate cells called major histocompatibility complexes (MHC). MHC I presents the self peptide and is found on all cells and distinguishes all cells as "self". It is vital that T cells do not strongly react with MHC I + self peptide, otherwise autoimmunity would occur. Thus, during developmental negative selection, T cells that do react to "self" antigens are deleted whereas T cells that do not react with "self" are retained and further differentiate into functionally distinct T-cell subgroups which react to and produce different cytokine profiles [19].

Although there are natural killer T cells and γδ T cells, two major types of αβ T cells include the $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T lymphocytes. CD4 and CD8 are surface molecules that are part of the T-cell receptor complexes which facilitate binding to MHC II or I on $CD4^+$ or $CD8^+$ cells, respectively [20,21] **(Figure 1.3)**.

Multiple types of $CD4^+$ T helper cells exist. Naïve T cells can differentiate into distinct $CD4^+$ lineages such as Th1, Th2, Th17, T-reg, and Tfh based on transcription factors and cytokines **(Figure 1.4A)**. The differentiation of $CD4^+$ Th cells was previously viewed as inconvertible linear developmental pathways; however more recently, current studies have challenged this classical view

suggesting that differentiated Th cells can change into other types of Th cells **(Figure 1.4B)** [22].

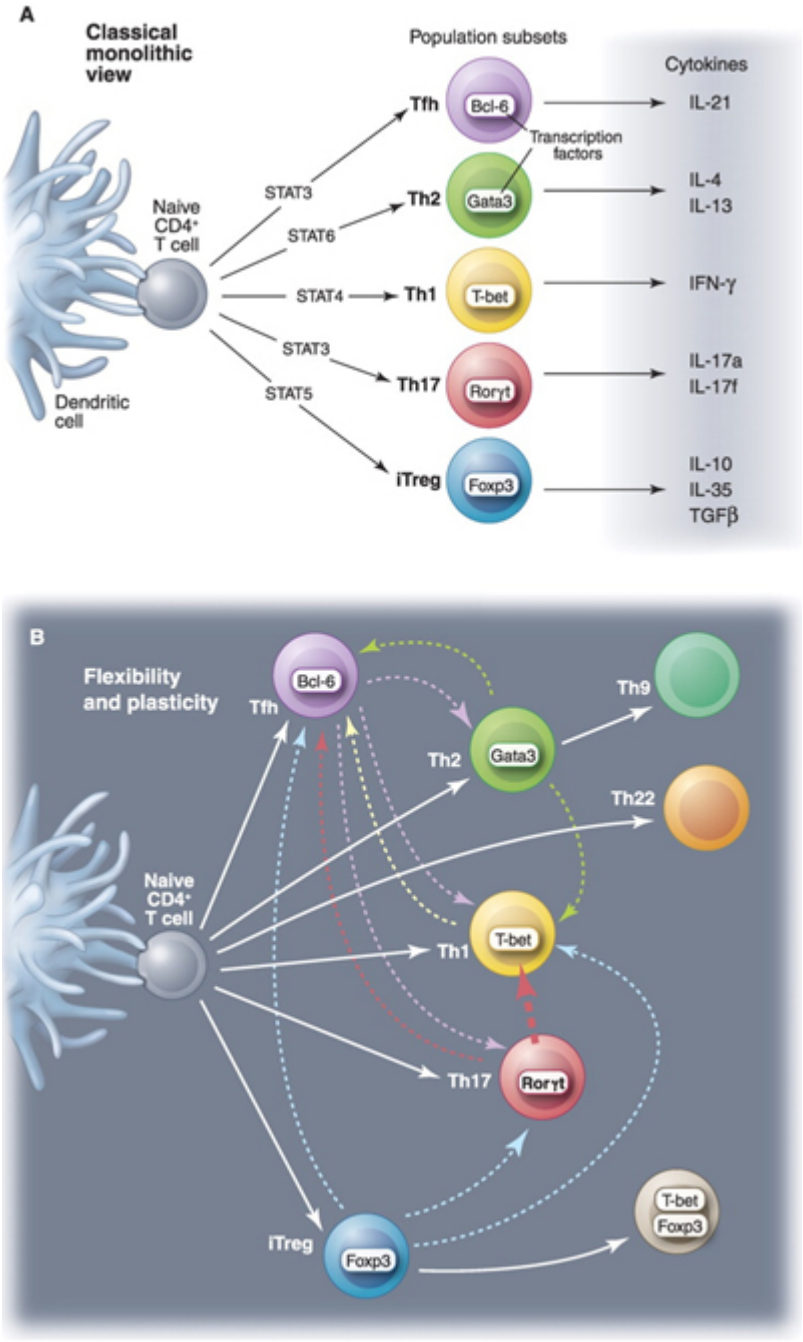


Figure 1.4. CD4$^+$ Th cell differentiation. A) Classical view of Th differentiation: Differentiation pathways are not convertible. B) Modern view of Th differentiation: Flexibility and plasticity of Th differentiation where differentiated Th cells can adopt new phenotypes. From [22]. Reprinted with permission from AAAS.

1.5 DC subsets and T cell immunity

Differences in mDC and pDC cellular markers underlie differences in cellular function. Although both mDCs and pDCs have functional plasticity to elicit an appropriate immune response through T cells, it is their reaction to specific stimuli and environment that promotes two major divergent responses T helper 1 (Th1) and T helper 2 (Th2) immune responses, which are associated with inflammation and allergy/antibody response, respectively. Th responses produce specific cytokines that aid in the orchestration of appropriate defenses. The Th1 response produces IL-2, IFN-γ, IL-12, and tumor necrosis factor (TNF) cytokines and the Th 2 response produces IL-4, IL-5, IL-10, and transforming growth factor (TGF-β) cytokines. At the cellular level, Th1 responses are mainly associated with cytotoxic T lymphocytes whereas Th2 responses are associated with induction of eosinophils and B cells. Since DCs conduct T cell responses, pDCs can elicit a Th2 response whereas mDCs favor a Th1 response [23]. When activated by CD40 ligand (CD40L), monocyte-derived mDCs induce Th1 development with IFN-γ and IL-12 production. However, when pDCs are activated by CD40L, they produce lower amounts of IL-12 and induce Th2 development. [24,25] **(Figure 1.5)**.

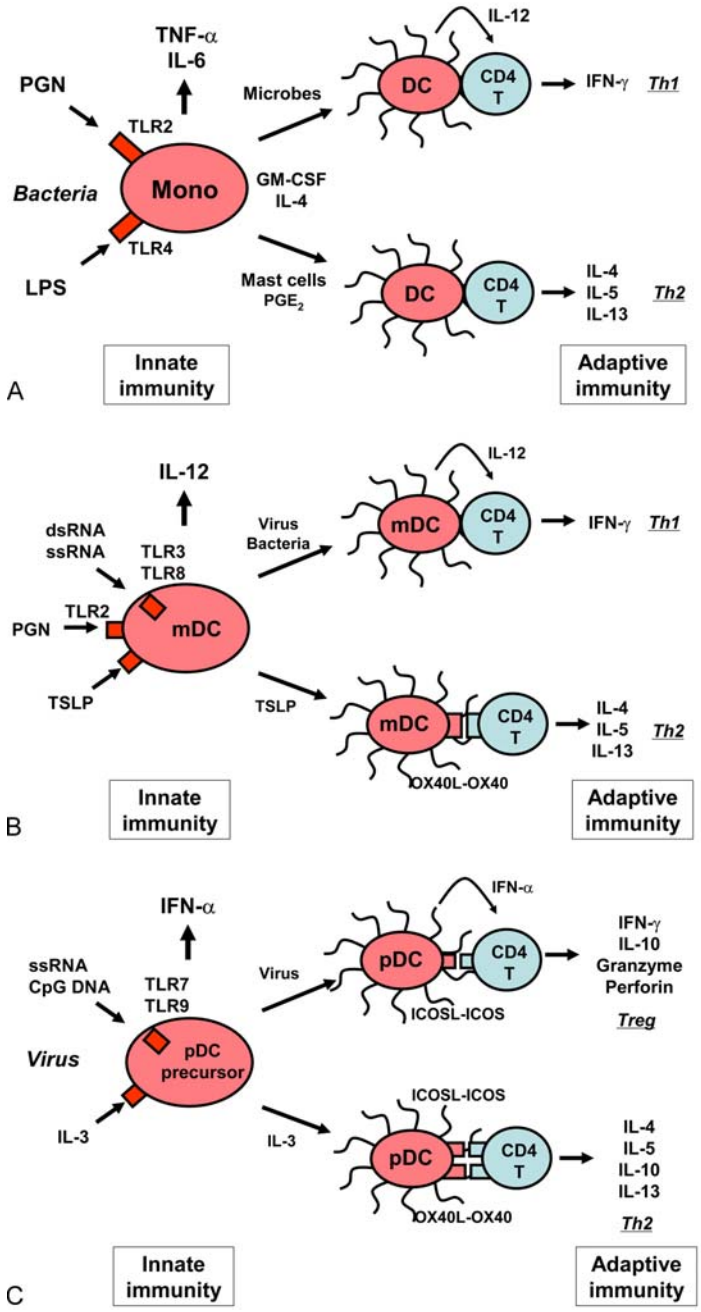


Figure 1.5. Human DC subsets induce different CD4$^+$ T cell responses based on environment and stimuli. A) Monocytes in innate immunity respond to bacterial components through Toll-like receptor (TLRs) and secrete pro-inflammatory cytokines, TNF-α and IL-6. Subsequently, monocyte-derived DCs induced by GM-CSF and IL-4 can induce a Th1 response against microbes. In contrast, moncyte-derived DCs stimulated by mast cells and PGE_2 can induce a Th2 response. B) In response to bacterial and viral components through TLRs, myeloid DCs (mDCs) secrete IL-12 and induce a Th1 response. However, when stimulated with Thymic stromal lymphoprotein (TSLP), mDCs can induce a Th2 response by OX40-OX40L interaction. C) Plasmacytoid DCs (pDCs) produce

IFN-α in response to viral components sensed through TLR7 and TLR9. pDCs stimulated with viruses induce cytotoxic regulatory T cells that secrete IL-10, whereas pDCs stimulated with IL-3 induce a Th2 response through OX40-OX40L ligation. ICOS ligand on pDCs induces IL-10 producing T cells, which are important in immunoregulation. From [8]. Reprinted with permission from Japanese Society of Allergology-Allergology International.

1.6 Regulatory T cells

Regulatory T cells (T-regs) are a subset of $CD4^+$ T helper cells that have immunosuppressive and immunoregulatory capabilities and are anergic or lack proliferative response upon T cell receptor activation. Discovered in 1969 in mice thymectomy experiments, the thymus-derived cell subset termed "suppressor cells" were found to be important in self-tolerance and preventing autoimmunity [26-28]. In 1995, suppressor cells were rediscovered and termed regulatory T cells. These suppressive T-regs were phenotypically characterized as $CD4^+$ T cells expressing CD25 (IL-2Rα) in mice [26,29]. However, there were caveats to the use of CD25 as a unique marker for T-regs. CD25 is also expressed on activated T cells and its expression is very dynamic. The $CD4^+CD25^+$ T cell population seems to be heterogeneous with a portion of cells exerting suppressive function, such as $CD4^+CD25^{high}$ T cells. However, in mice, $CD4^+CD25^-$ T cells also had suppressive function. Subsequently, in 2000 and 2001, suppressive Foxp3 and $CD25^{high}$ expressing $CD4^+$ T cells were found to be associated with suppressive T-regs in humans [30,31]. Although the spectrum of regulatory T cells includes multiple different T cell subsets, this dissertation will focus on $Foxp3^+$ T –regs.

Foxp3 is a forkhead transcription factor vital to the development and function of human regulatory T cells (T-regs). Foxp3 is vital because when mutated, there was a lack of suppressive T-regs and resulted in fatal multiorgan autoimmune disease in mice (Scurfy) and humans (Polyendocrinopathy, Enteropthy, X linked syndrome (IPEX)) [32-34]. When Foxp3 was ectopically

expressed in $CD4^+$ non-T-regs cells, suppressor function was conferred *in vitro* and *in vivo.* Thus, Foxp3 may be an exclusive marker for $CD4^+$ regulatory T cells.

T-regs comprise of 5-10% of total peripheral blood mononuclear cells. They functionally regulate a wide variety of immune cells such as $CD4^+$, $CD8^+$, natural killer, natural killer T cells, B cells, and antigen-presenting cells both *in vitro and in vivo* [35]. The unique capabilities of T-regs to regulate immune reactions allow them to prevent immune diseases such as autoimmunity and allergy. They also play a role in allograft tolerance as well as fetal-maternal tolerance during pregnancy. In addition to their normal regulatory function, T-regs can also impede anti-tumor responses to favor tumor progression. Many failures of cancer vaccines have been attributed to T-regs. However, T-regs also have potential use in clinical applications for dysregulated immunity. Cellular therapy involving *in vivo* expansion of T-reg in patients with autoimmune disease would be ideal for treatment. Likewise, strategies to remove T-regs may be helpful in augmenting a cytotoxic response to tumor antigens.

1.7 Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTL) confer immunity against viruses and cancer. Discovered in 1968, cytotoxic T lymphocytes' specific cell mediated destruction function allows for eradication of unwanted cells while leaving bystander cells intact [36]. Because of their ability to specifically target antigens on cells, they have been of immunologic interest for anti-tumor immunity and role in vaccination responses to prevent infectious diseases.

Their cytotoxic function to specifically kill target cells relies on the molecules present on the CTL cell surface and within the cytolytic synapse formed with target cells. CTLs express the $CD8^+$ molecule with the T cell receptor. In the cytolytic synapse, the molecules form a bull's eye with the TCR in the center. The TCR ligates with the MHC I on the target cells. At the CTL/target cell interface, cytolytic granules (perforin and granzymes) are released to lyse and kill the target cell [37]. Perforin, granules that perforate the cellular membrane, are first released and then granzymes enter the cell through the perforated membrane. To date, this is the well accepted mechanism of cytotoxic action [38]. However, much of the protein machinery used has yet to be identified and the cytotoxic process has yet to be completely elucidated.

Cytotoxic T lymphocytes can be idenfied by expression of cell surface CD8, CD69 marker and IFN-γ cytokine secretion. CD69, a transmembrane C-type lectin protein receptor involved in lymphocyte proliferation and transmission of external signals to the inside, is expressed when $CD8^+$ T lymphocytes are activated through docking of CD8 + TCR on target cell MHC class I + peptide [39,40]. CD69 activation along with cytotoxic function produces interferon-gamma (IFN-γ). IFN-γ is a type II IFN cytokine that signals through the JAK-STAT pathway and helps to promote an adaptive immune response against bacteria/viruses and tumors. It is secreted in $CD8^+$ T cells that have cytotoxic function [41,42], [43]. Thus, a current method of identifying CTL by flow cytometry is by intracellular expression of IFN-γ along with CD3, CD8, and CD69 markers [44-46].

1.8 Cutaneous T cell Lymphoma (CTCL): Immunopathogenesis

Cutaneous T-cell lymphomas (CTCL) are a heterogenous group of non-Hodgkin's peripheral T cell lymphomas that mainly impair skin. Mycosis Fungoides (MF) and Sézary Sydrome (SS) are the most common CTCLs. They are characterized by clonal skin-homing memory $CD4^+$ T cells and impaired immunity. Together they comprise of about 70% of CTCL cases and affect 6.4 per million persons. Approximately 1,500 new cases of MF/SS are reported every year and about 16,000-20,000 persons are found alive with MF/SS in the United States [47]. For the most part, CTCLs are incurable and thought to arise from uncontrolled proliferation and accumulation of atypical mature helper, memory clonal T lymphocytes [48-50]. Some patients with advanced MF and those with Sezary Syndrome defined as erythroderma and > 1000/μL atypical circulating malignant T cells in blood are called leukemic L-CTCL (L-CTCL)

1.9 Sézary Syndrome (SS): History and Clinical Presentation

First described by Albert Sézary in 1938, SS is a leukemia comprised of circulating cells with cerebriform nuclei, skin erythroderma, pruritus, and adenopathy. Patients with SS have the worst prognosis amongst all CTCL patients with a median overall survival (OS) of only 3 years [51]. Amongst the SS patients, we found that ≥10,000 Sézary cells/L is associated with the poorest prognosis with a median OS of 2.4 years whereas other patients have a median OS of >5 years [52]. SS patients typically expire from secondary and/or nosocomial infections, most often from Staphylococcus aureus (Staph) sepsis due to an impaired immune system, breaks in the integument and use of

catheters. SS patients have a high rate of Staph colonization. Staphylococci secrete erythro-toxins that cause erythroderma of skin when present in fentamolar concentrations. Exfoliative erythroderma is defined as pink or red skin over 80% of the body. It may be accompanied by pruritus, scaling, infiltration, keratoderma and ectropism. Due to the bright red color exhibited diffusely on the skin, SS has been nicknamed as the “red man syndrome” although erythroderma may also be seen with atopic dermatitis, psoriasis, PRP, and other skin diseases. The cause of SS is unknown but can be mimicked as exfoliative dermatitis, making diagnosis difficult.

SS was first thought to arise from MF and may do so in a subset of patients. Recent studies suggest that MF and SS can be separated based on the presence of distinct T cell subsets. MF cells are effector memory T cells whereas SS cells are central memory T cells [53]. Found both in the blood and skin, Sèzary cells are also currently recognized as $CD4^+$ T cells lacking CD26 or CD7 expression [54,55]. Clonal tumor T cell populations are sometimes identifiable by TCR Vβ clonality studies although some patients have a high percentages of cells that are not detected by the currently available antibodies [55].

1.10 CTCL skin and clinical pathology

Mycosis fungoides most often presents with patches or plaques while tumors appear later in the course. All three types of skin lesions contain skin homing epidermotropic $CD4^+$ helper memory T cells. SS, the leukemic variant, presents with exfoliative erythroderma (red scaly skin over > 80% of the body)

and >1000 circulating atypical $CD4^{+}CD26^{-}$ T cells, with common bone marrow and lymph node involvement [49,50]. SS can arise de novo or evolve from pre-existing MF [56]. $CD8^{+}$ and CD4, CD8 double negative variants have also been described. Although SS cells are slow growing malignant cells and are characteristically anergic, accumulation of these tumor cells in the skin disrupts normal immune barrier function. In addition to skin involvement, the presence of tumor cells in blood, bone marrow, and lymph nodes is associated with worse outcomes [57]

MF typically begins as patches or plaques similar in clinical appearance to eczema or psoriasis. Because early MF is a chronic eczematous dermatitis, a definitive biopsy is often delayed. Breaks in the skin barrier found as a feature of eczema which may be due to a negative mutation in Filaggrin can induce release of cytokines, epidermal proliferation and attraction of inflammatory T cells. In early MF, an infiltrate of atypical lymphocytes with hyperchromatic, convoluted, cerebrifom nuclei are found around vessels in the dermis. Epidermatropism is a key feature required for the diagnosis of MF—either as single cells along the dermis-epidermal junction or as collections of cells known as Pautrier's microabscesses composed as clusters of T cells surrounding Langerhan cells. However, in contrast, de novo SS patients' biopsies contain perivascular rather than epidermotorpic atypical lymphocytic infiltrates and the diagnosis is often missed or delayed [58].

1.11 CTCL tumor T cell abnormalities (Immunophenotype)

Abnormal T lymphocytes found in CTCL lesions are aberrant in development, cell migration, cell survival, cell proliferation, and apoptosis. Although malignant cells are memory $CD4^+CD45RO^+$ T helper cells in both MF and SS, recent studies classified MF as an outgrowth of effector memory T cells whereas SS are central memory T cells [53,59]. In addition to this distinction, cellular markers also differentiate MF and SS. Compared to SS cells, MF cells are more localized to the skin. In MF cell lines, the level of skin-homing chemokine receptor, CCR4, expression is much higher than in SS cell lines suggesting that MF cells are more localized to the epidermis [60]. Additionally, SS cells have a different phenotype from MF. SS cells are currently identified as $CD4^+CD26^-$ and sometimes $CD4^+CD7^-$ and expansion of a specific TCR Vβ clone. CXCL13, a chemokine found to be high in SS cells, induces not only skin but lymph node homing properties when combined with CCR7 agonists expressed in the lymph nodes, CCL19 and CCL21 [61].

1.12 Impaired immunity in CTCL

Normal immunity with T cell diversity allows protection from various antigens. The variations of T cells are created by the T cell receptor (TCR) rearrangements at the genomic level and expression on the surface of T cells. Unique TCR are created by deletions in genomic DNA capable of recognizing foreign antigens presented on MHC. In L-CTCL, single clones predominate and the normal T cell repertoire is lost. These clones can be detected by flow cytometry and PCR amplification of TCRVβ or Vγ genes. Decrease of T-cell receptor excision circles during early T cell development in association with loss

of T cell repertoire complexity through CDR3 spectratyping contribute to development of clones [59,62]. The diversity of T cell repertoire was recovered when patients were successful treated [63].

Due to the loss of T cell variety in L-CTCL patients, $CD8^+$ T cells are abnormally low and consequently hypothesized that the numbers of cytotoxic T lymphocytes are also low due to depressed cell-mediated cytotoxicity marked by Th2 dominance with eosinophilia and elevated serum IgE and IgA [64-66]. This imbalance also reduces the activity of natural killer cells.

Since $CD4^+$ T cells are directed by DC or antigen presenting cells through TCR:MHC II antigen presentation, it has been reported that DC are 'helping hands' in L-CTCL progression [67]. The two main DC subsets in humans, Lineage 1^- HLA-DR^+CD11c^+myeloid DCs and Lineage 1^-HLA-DR^+CD123^+plasmacytoid DCs, were found to be defective in numbers and function in SS patients [68]. The failure of DCs to elicit normal responses and prevent tumor cell outgrowth suggest an immunopathogenic role of DC. Stimulaton of malignant L-CTCL cells with immature dendritic cells loaded with apoptotic-T-cells *in vitro* exhibited a T-reg like phenotype [69]. Not only did this study provide evidence of DC eliciting L-CTCL outgrowth, but it also suggests that L-CTCL is an outgrowth of T-regs. However, conflicting studies show a lack of Foxp3 expression and suppressive function in skin and blood of L-CTCL patients, suggesting that L-CTCL is not a malignancy of T-regs, but normal T-regs may be a favorable prognostic factor [70-72]. Of interest, a recent study by Heid et al reported findings of a subgroup of L-CTCL patients with malignant cells

that were functionally suppressive and were $CD4^{+}Foxp3^{+}CD25^{-}$T cells [73]. Taken together, these studies reveal the controversy over whether L-CTCL malignant cells are T-regs or not. Thus, the role of T-regs in L-CTCL remains unclear.

1.13 SS and current treatments

Since SS affects the skin as well as the blood, palliative topical treatment agents are used but are ineffective for anything except symptom control. Immunomodulatory and biological response modifiers that increase Th1 immunity such as IFN-alpha or oral retinoids, or fusion proteins are first line therapy combined with extracorporeal photopheresis (ECP). Second line therapies include histone deacetylases (HDAC) inhibitors, and experimental monoclonal antibodies directed to tumor cells **(Table 1)**. Our recent studies have shown that Syndecan-4, a heparin sulfate proteoglycan, expressed on activated T cells can be depleted with dendritic cell-associated heparan sulfate proteoglycan-dependent integrin ligand (DC-HIL) conjugated with saporin (a toxin) suggesting a novel opportunity to treat T cell mediated diseases [74].

Effective treatment options to reduce tumor burden and regain normal T-cell function are limited to ECP and biological response modifiers (BRM) while other available therapies are immunosuppressive [75]. Treatment induced immunosuppression coupled with disease-related immunosuppression means that these patients frequently succumb to opportunistic infections especially Staph aureus. Although a possible curative therapy for SS is non-ablative allogeneic human stem cell transplantation (HSCT), it is feasible for only a subset

of younger healthy patients and may cause mortality from infection, disease reoccurrence, and chronic graft-versus-host disease (cGVHD) [76]. Treatment of GVHD requires major immunosuppressive therapy which can lead to opportunistic infections. Therapy with extracorporeal photopheresis (ECP) is effective with minimal toxicity and favorably modulates immunity. Due to its beneficial immunomodulatory effects, the mechanism of ECP action *in vivo* is under investigation.

1.14 Graft versus Host disease: Immunopathogenesis

Graft versus host disease (GVHD) is an immunologic disorder that limits wide use of allogeneic stem cell/bone marrow transplant therapy. The donor immune cells in the transplant recognize host cells as being foreign based on HLA mismatch. The donor cells mount an immunological attack resulting in damage to organs including the skin, gut, liver, eyes, or lung.

In 1966, Billingham established 3 criteria necessary for GVHD: 1) Transplanted graft has immunologically competent cells. 2) The host/recipient is histoincompatible or has antigens that are lacking from the transplant so that the host appears foreign. 3) The host/recipient is immunologically innocuous or immunocompromised [77]. Two types of GVHD are characterized by occurrence based on time after transplant. Acute GVHD is defined as occurring within 100 days post-transplant and chronic GVHD is defined as disease that occurs after 100 days post-transplant. In acute GVHD, a retrospective analysis revealed that 81% patients had skin involvement, 54% had gastrointestinal (GI) involvement, and 50% had liver involvement [78].

According to Ferrara et al, GVHD is considered as a three-step process: 1) tissue damage to the recipient by pre-existing infections and/or radiation/chemotherapy pre-transplant conditioning regimen and antigen presenting cell activation; 2) T cell activation and clonal expansion of donor T cells; and 3) cellular and inflammatory factors [79]. Prior to the infusion of donor cells, the patient's tissues have already been damaged by a host of factors such as underlying disease and its treatment, infection, and the condition regimen involving drugs and radiation. This damage activates host antigen presenting cells (APCs) that are vital in activating donor T cells. High intensity chemoradiotherapy also activates APCs. Inflammatory cytokines such as TNF-alpha and IL-1 are secreted from host tissues as a result of total body irradiation. Subsequently, they induce endothelial apoptosis leading to epithelial cell damage in the GI tract. GI damage may amplify severity of GVHD if microbial products invade the systemic circulation. These inflammatory and microbial products such as lipopolysaccharide (LPS) are mediators of an immune response or tolerance. They are immune 'danger signals' [80]. APCs activated by these danger signals can steer the immune response towards activation or tolerance [81].

At the molecular and cellular level, major histocompatibililty complex (MHC), $CD4^+$ and $CD8^+$ T cells, and cytokines are the main disease mediators. The MHC is vital in the recognition of foreign antigens that invade the immune system as it is unique to each individual. MHC encodes heterogenous protein chains that present antigen to T cells through the T cell receptor and communicates in an autologous or matched setting. The TCR: MHC interaction

directs the T cells activity by activating or inactivating it. In the case of GVHD, graft T cells that fail to recognize the MHC proteins on host cells mount an immunologic attack against the host cells. Since the host is seen as foreign due to MHC mismatch, the graft T cells attack the host cells. However, if there is a match at the MHC loci, GVHD can also occur due to incompatibility of minor histocompatible antigens. Since $CD4^+$ and $CD8^+$ T cells are matched by MHC II and MHC I, respectively, they elicit a T cell attack due to the lack of self recognition **(Table 1.1)**. T cells are previously educated in the thymus to recognize self and delete autoreactive cells in a process termed “negative selection” [82]. Thus, the MHC and minor histocompatible antigen mismatch attributes to the disrupted normal immunity leading to GVHD.

Table 1.1. Comparison of L-CTCL and GVHD disease characteristics and treatment options.

	L-CTCL-Sézary	**GVHD**
Clinical Manifestations	Skin-Erythroderma, pruritus	Skin-Scleroderma
Disease mediated cell type	$CD4^+$ T cells: $CD4^+CD26^-$ Low $CD8^+$ T cells	$CD4^+$ T cells $CD8^+$ T cells
Cytokines	Th2 dominant: IL-4, IL-5, Decreased IL-2 and IFN-γ	Th1 dominant: IL-2, IL-12, TNF-α, IFN-γ
Treatment strategy and options	IMMUNOMODULATORY: Extracorporeal photopheresis plus interferon-γ,α, bexarotene, Denileukin Difitox, retinoids, steroids, Stem cell transplant - allo IMMUNOSUPRESSIVE: campath, chemotherapy, targeted antibodies, HDAC inhibitors.	IMMUNOSUPPRESSIVE: Extracorporeal photopheresis, Glucocorticoids , cyclosporine, tacrolimus, rituximab

1.15. Extracorporeal Photopheresis (ECP): History and Procedure

Initially developed in 1987 for the treatment of patients with high numbers of circulating SS cells and with erythrodermic L-CTCL, ECP is extremely well-tolerated with minimal side effects, improves patients' quality of life, and increases overall survival [83]. ECP was first approved by the FDA in 1987 for the treatment of erythrodermic L-CTCL based on a multicenter clinical trial by Edelson et al. Response rates between 54% and 66% have been reported in L-CTCL patients with about 10% complete responders [84,85]. In the ECP procedure, $5x10^9$ white blood cells (5-10% of PBMCs) are removed by leukopheresis, sensitized ex vivo with a plant-derived psoralen drug, 8-methoxypsoralen (8-MOP), and irradiated with 1.5 J/cm^2 ultraviolet (UVA) light. The cells are re-infused back into the hosts' circulation **(Figure 1.6)**. Not only is ECP used to treat SS, but it is also effectively used to treat diseases of an inflammatory/autoimmune etiology such as scleroderma, scleromyxedema, fibrosing nephrogenic dermatopathy, organ transplant rejection, and inflammatory bowel disease, etc [86-90]. How ECP can favorably treat a disease of malignant T-cells with a possible over-abundance of T-regs and a Th2 phenotype as well as GVHD with a lack of T-regs and a Th1 phenotype is not yet understood.

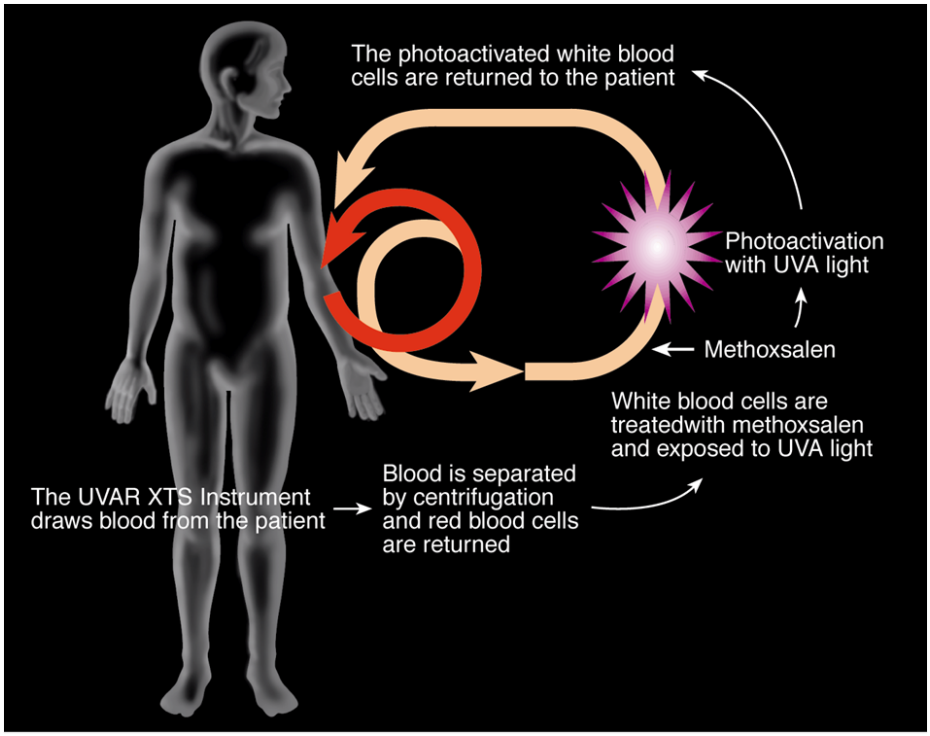


Figure 1.6. Schematic view of ECP procedure. Reprinted with permission from Therakos.

1.16. ECP: Apoptosis

SS tumor cells have aberrant apoptotic pathways including decreased Fas/Fas L and c-myc expression [91]. These defects in cell death or apoptosis are hypothesized to contribute to the accumulation and imbalance of tumor T cells within the blood and skin.

Following ECP, the radiated tumor cells undergo apoptosis at 24 hours after treatment [92]. How apoptosis and cell cycle arrest occurs is in part attributed to the DNA damage caused by covalently crosslinking thymidine bases in lymphoma cells elicited by the 8-MOP and UVA light [93-96].

Apoptosis is a programmed cell death process and apoptotic cells induced by ECP have effects on the patients' immune system. Apoptotic cells following ECP are sensed by the immune system by phagocytic dendritic cells. These "professional antigen presenting" cells control immune responses by sampling the environment. Following the uptake of apoptotic cells, DCs can promote an immunologically innocuous and/or tolerizing effect. However, in some instances, apoptotic cells provide an attractive immunogenic antigen source to DCs for cross-priming cytotoxic T lymphocytes. Since only a small portion of the total tumor lymphocytes are treated by ECP and prolonged clinical responses can be observed, ECP is hypothesized to induce beneficial immunomodulating effects *in vivo.* What occurs after the apoptotic cells are re-infused into SS and GVHD patients and how it affects the patients' immune system *in vivo* are largely unknown and is the subject of this dissertation.

1.17 Immunomodulation by ECP: Vaccination Theory vs. Tolerance

The classic hypothesis of how ECP works upon re-infusion of photo-damaged PBMCs into the host's circulation is the 'vaccination' theory [97]. Part of the ECP procedure circulates psoralen-treated white blood cells within a plastic chamber during UVA irradiation leading to photoactivation of 8-MOP. Upon photoactivation, 8-MOP intercalates into the DNA of tumor lymphocytes and induces apoptosis. Monocytes, however, are suggested to differentiate into dendritic cells and become more receptive to the uptake and processing of apoptotic cells within the plastic chamber. Studies prolonging interactions with DCs and apoptotic tumor cells by overnight incubation called "transimmunization" have shown to be therapeutically effective when reinfused into the patient the next day [98,99]. Transimmunization suggests that a therapeutically significant immunobiologic modifying response orchestrated by DCs is capable by ECP to specifically target tumors cells supports the vaccination theory of ECP mechanism of action after re-infusion. As these apoptotic tumor lymphocytes are a source of tumor antigen, re-infusion of tumor-antigen loaded DCs is hypothesized to induce a vaccination response [67,98,100]. Mature dendritic cells facilitate in vivo $CD8^+$ T-cell maturation into cytotoxic T cells that elicit a tumor specific attack on SS tumor cells while preserving non-pathogenic cells [67,98,101]. Thus, DC subsets may provide evidence of ECP response. The two DC subsets myeloid (mDC) and plasmacytoid DCs (pDC) in normal humans have been found to induce Th1 and Th2 responses, respectively [23]. In SS patients, DC subsets have been found to be depressed compared to healthy

controls [68]. Efforts to increase DCs would be logical for therapy and increase of a specific DC subset may be indicative of the *in vivo* T cell response.

Since $CD8^+$ T-cells are dramatically reduced in many patients with SS and low CD8 numbers are associated with poor prognosis and poor response to ECP, strategies to increase their numbers may be helpful in treating SS. ECP treated patients with close to normal levels of $CD8^+$ T cells are observed to respond better [102,103]. Modulation of the immune system by ECP appears to restore the production of Th1 cytokines and may allow monocytes to become effective tumor-antigen presenting cells to induce cytotoxic responses against tumor T cells [104-107] . Additionally, these Th1 cytokines may promote "normal" immunity in SS patients required for cellular immunity, and defense against opportunistic infections [108].

However, in GVHD patients, the vaccination theory is illogical. Increase of $CD8^+$ T cells and Th1 cytokines would exacerbate GVHD. A previous report showed that in GVHD, treatment with ECP is associated with a shift from predominantly a Th1 (IL-2, INF-γ) to a Th2 (IL-4, IL-10) immune response [109], suggesting that ECP is down-regulating Th1 immunity. Thus, it is proposed that ECP works by immunosuppression. When apoptotic cells are endocytosed by DCs, DCs may elicit $CD4^+$ regulatory T cells (T-regs). A recent study reported $CD4^+25^{high}$ T cells, or T-regs increased in GVHD patients but decreased in L-CTCL patients after six months of ECP [110]. These findings suggest that immunosuppression through T-regs occurs in GVHD patients but not in L-CTCL patients and favors the immunostimulatory or vaccination hypothesis in L-CTCL

patients. In addition to GVHD, other Th1-mediated conditions such as transplantation and autoimmune disorders benefit with immunosuppression of inflammatory responses. In these disorders, immunotolerant DCs are generated through the uptake of apoptotic cells and induce T-regs, cells that suppress immunity [111]. One previous study on the effect of ECP in the GVHD murine model reported that increased T-regs improved disease [112]. Other studies in transplantation show that T-regs are induced after ECP and prevent GVHD and suppress autoimmunity [113-115]. Thus, it is currently well accepted that T-regs suppress the pathogenic cells in Th1 mediated diseases, but what specific types of DCs or where the T-regs originate are unclear. Further validation of the mechanism of ECP in L-CTCL and GVHD patients is needed and could provide information for better treatment. It is hypothesized that the mechanism of ECP action in GVHD is through immunotolerance and induction of regulatory T cells while immunostimulation and induction of cytotoxic T lymphocytes occur in L-CTCL.

1.18 Dissertation Objective and Hypothesis

The *overall objective of this investigation* is to elucidate the immunological mechanisms of ECP and determine whether they are similar or different in L-CTCL and GVHD patients. Regulatory T lymphocytes, Cytotoxic $CD8^+$ T lymphocytes, and Dendritic cell subsets from peripheral blood in L-CTCL versus GVHD patients will be examined. Studies will be associated with clinical response to ECP and the mechanism will be compared between the two patient

populations. To execute this objective, I utilized peripheral blood mononuclear cells (PBMC) isolated from peripheral blood samples taken from L-CTCL and GVHD patients pre- and post-ECP at Day 2, 1 month, 3 months, and 6 months, whenever possible. **The *central hypothesis* is that ECP works differently in L-CTCL and GVHD and that cyotoxic $CD8^+$ T lymphocytes will be induced in L-CTCL whereas in GVHD T-regs will be induced.** My specific aims were: **1)** To determine the prevalence of functional $CD4^+CD25^{high/+}Foxp3^+$ regulatory T-cells (T-regs) in clinically responsive L-CTCL patients after ECP treatment. **2)** To determine the prevalence of functional $CD8^+$ cytotoxic 'killer' T-cells in clinically responsive L-CTCL and GVHD patients after ECP treatment. **3)** To characterize the dendritic cell (DC) subsets in L-CTCL and GVHD patients on ECP and determine a correlation in ECP-responsive patients. The *general rationale* of this study is to identify the immune cell subsets that are efficacious in treating L-CTCL or GVHD or that are associated with the immunological mechanism of ECP in L-CTCL versus GVHD patients ex vivo. The ***significance*** of this study is to better understand how ECP works immunologically to improve treatment of patients.

I tested this hypothesis and accomplished the proposed objective by addressing the aforementioned specific aims.

Specific Aim 1: I determined the prevalence of regulatory T cells in L-CTCL patients and GVHD patients before and after treatment. Percentages of

$CD4^+Foxp3^+$ were high in 7/9 L-CTCL (L-CTCL-High) patients and in 5/7 GVHD (GVHD-High) patients studied. In 6/7 L-CTCL-High patients, percentages of $CD4^+Foxp3^+$ T cells correlated with percentages of malignant $CD4^+CD26^-$ T cells suggesting that these malignant T cells have the T-reg phenotype. Thus, it was determined there were 6 L-CTCL patients had the malignant T-reg phenotype and 3 L-CTCL patients did not have a malignant T-reg phenotype. After 3 months of ECP, the L-CTCL patients with a malignant T-reg phenotype had decreased $CD4^+CD26^-$ tumor T cells and $CD4^+Foxp3^+$, $CD4^+Foxp3^+CD25^-$, and $CD4^+Foxp3^+CD25^+$ T cells whereas the non-malignant T-reg phenotype patients had no change. In the GVHD patients, there was a decrease of $CD4^+Foxp3^+$ and $CD4^+Foxp3^+CD25^-$ T cells, and increase of $CD4^+Foxp3^+CD25^+$ T cells after 3 months of treatment.

Specific Aim 2: I confirmed the low levels of $CD8^+$ T cells in 6 L-CTCL patients but high levels of $CD8^+$ T cells in 3 GVHD patients before ECP. After ECP, the $CD3^+CD8^+$, $CD8^+CD69^+$, $CD8^+IFN\text{-}y^+$ T cells increased after 1 month in L-CTCL patients, but decreased in GVHD patients.

Specific Aim 3: I confirmed that DCs were low in L-CTCL and GVHD patients before treatment. Numbers and percentages of mDCs and pDCs increased in L-CTCL and GVHD patients after ECP treatment. CSSMs increased on mDCs and pDCs. In L-CTCL patients, numbers and percentages of activated mDCs were

increased within some responders. However, in GVHD patients, there were increased numbers and percentages of pDCs.

CHAPTER 2

EFFICACIES OF EXTRACORPOREAL PHOTOPHERESIS IN PATIENTS WITH L-CTCL AND GVHD IN THIS STUDY

2.1 Background and Rationale

ECP is extremely well-tolerated with minimal side effects, improves patients' quality of life, and increases overall survival [83]. In CTCL patients, the overall response rate is between 54% and 66% with a 14%-33.3% complete response rate [85,116]. In GVHD patients, 76% of patients had improved skin manifestations with 38% complete responses [117].

To achieve more complete responses, biological response modifiers (BRM) are administered with ECP in L-CTCL patients. Since ECP+BRM affect the immune system, its immunologic mechanism(s) of action *in vivo* is of interest and is not fully understood in L-CTCL patients.

L-CTCL patients have both blood and skin symptoms. Malignant cells, $CD4^+CD26^-$ T cells, are circulating in blood impairing immunity and the skin is erythrodermic. Laboratory studies to enumerate these clinical presentations are used. By flow cytometry, absolute numbers and percentages of $CD4^+CD26^-$ T cells can be measured in blood samples at baseline and following ECP therapy. Skin involvement is calculated using a modified severity weighted assessment tool (mSWAT) score that measures the extent and severity of skin involved in patch, plaque, and tumor. In this study, these two parameters, numbers of $CD4^+CD26^-$ T cells and mSWAT score, were used to clinically assess disease progression or regression. Therefore, during ECP therapy both blood and skin

improvements were assessed by one physician (M. Duvic) in L-CTCL patients. Skin response was defined as a > 50% decrease in the mSWAT score at baseline. Response in the blood was subsets or the TCR Vβ clone, where available.

2.2 Patients and Methods

Please see Chapter 7.

2.3 Results

Eighteen L-CTCL patients completed the 6-month treatment course. Their individual characteristics and responses are shown in **Table 2.1.** Demographics and response rate are summarized in **Table 2.2**. The cohort consisted of more Caucasians (16/18, 88.9%) and males (13/18, 72.2%) with a median age of 67.0 (54-79) years. All patients had ≥ 39.0 mSWAT except pt. # 17 who had normal skin (mSWAT 5.0). Confluent erythema (≥ 87.0 mSWAT) was observed in 7 patients who met the criteria for SS. According to the revised criteria for MF/SS staging by ISCL/EORTC, 16/ 18 patients were diagnosed with MF/SS stage IV (8-SS IVA and 8-SS IVB) and 2 were staged as MF/SS IIIB. Patient #8 had MF/SS stage IIIB in addition to chronic GVHD. With the exception of patient #4 (20.6%), all patients had ≥ 50% circulating $CD4^+CD26^-$ tumor T cells. Fifteen of 18 patients (83.3%) had B2 (High tumor burden, >1000/μL $CD4^+$ $CD26^-$ T cells) and 3/18 (16.7%) had B1 (Low tumor burden, <1000/μL $CD4^+CD26^-$ T cells). In 16/18 patients, dominant TCRvβ clones were identified.

All L-CTCL patients were previously treated with skin directed therapies, but none were treated with radiation, chemotherapy, or immunosuppressive agents.

Table 2.1. Clinical summary of L-CTCL patients on extracorporeal photopheresis

Pt.#	Age/Gender/Race	Stage	ECP Cycles	Additional Therapy	TCR vβ%	Skin mSWAT	Blood $CD4^+CD26^-$ T cells		Response		
							%	μL	Skin	Blood	Overall
1	58/F/C	SS IVA	7	Bexarotene IFN-α	Vβ (70.0)	63.0	64.2	1751.0	MR	SD	MR
2	66/M/C	SS/MF IIIB	10	Bexarotene IFN- α	Vβ22 (91.0)	100.0	82.2	413.0	PR	CR	PR
3	71/M/AA	SS IVB (HTLV+)	7	Bexarotene IFN- α	None	100.0	55.5	525.0	SD	PD	PD
4	54/M/C	MF IVA	7	None	Vβ17 (54.0)	39.0	20.6	69.0	SD	SD	SD
5	74/F/C	SS IVB	11	Bexarotene	Vβ (85.0)	93.0	89.9	3928.0	PR	SD	PR
6	74/M/C	MF/SS IVB (BM+)	7	None	Vβ8 (73.7)	74.0	73.7	2218.0	MR	PR	PR
7	67/M/C	SS IVA	12	Bexarotene	Vβ (83.0)	87.0	79.1	1671.0	PR	SD	PR
8**	61/M/C	GVHD, MF/SS IIIB	25	IFN- α	Vβ (76.0)	77.0	64.4	227.0	SD	MR	MR
9	66/F/C	SS IVB	13	Bexarotene IFN- α	Vβ2 (97.0)	100.0	93.8	17981.0	SD	SD	SD
10	74/M/C	SS/MF IVA	11	None	Vβ7.2 (93.0)	48.1	85.9	2752.0	PR	SD	PR
11	79/M/C	SS IVB (BM+)	9	Bexarotene	Vβ 13.6 (97.1)	54.0	87.5	11581.0	PR	SD	PR
12*	71/M/C	SS/MF IVA	10	Bexarotene IFN- α	Vβ 17 (94.0)	100.0	56.0	1284.6	SD	PD	PD
13	78/F/AA	SS IVB	10	None	Vβ 13.6 (95.0)	47.0	94.5	9977.0	PD	SD	PD
14	63/M/C	SS IVB (BM+)	10	IFN- α	Vβ (94.0)	49.0	94.7	22751.0	PR	SD	PR
15	73/M/C	SS IVA	10	Bexarotene	Vβ 18.0 (95.0)	47.0	77.1	2600.0	SD	SD	SD
16	67/F/C	SS IVA	6	Bexarotene	n/d	67.0	91.9	4300.0	PR	SD	PR
17***	63/M/C	SS IVA	6	None	Vβ 5.1 (95.0)	5.0	91.7	5139.0	MR	SD	MR
18	61/M/C	SS IVB	8	None	Vβ 7.1 (97.0)	87.0	91.7	5500.0	MR	SD	MR

yrs = years, M=male, F=female, C=Caucasian, AA-African American, MR=minimal response, SD=stable disease, PR=partial response, PD=progressive disease,TCR=T-cell receptor, pts=patients, BM=bone marrow, GVHD=Graft-versus-host disease, SS=Sézary Syndrome, MF=Mycosis Fungoides, HTLV=Human T Lymphotrophic Virus, n/d = not done, mSWAT=modified severity weight assessment tool, ECP=Extracorporeal photopheresis

*$CD4^+CD7^-$ T cell phenotype present instead of $CD4^+CD26^-$.

** Patient with both GVHD and MF/SS who underwent the greatest number of ECP cycles (25).

***Patient with normal skin and malignant T-reg phenotype $CD4^+Foxp3^+$ (Shiue, LH et al 2012)

Table 2.2. L-CTCL Patient demographics and response rate.

Characteristic	L-CTCL (n=18)	
Gender	Male (n=13, 72.2%) Female (n =5, 27.8%)	
Median Age (range)	67 (54-79) years	
Race	Caucasian (n=16, 88.9%) African American (n=2, 11.1%)	
Stage-no. of patients (%)	SS/MF IIIB-11.1% SS/MF IVA-44.4% SS IVB-44.4%	
Tumor burden (TB)	High TB (>1000/µl) 83.3% Low TB (<1000 /µl) 16.7%	
TCRVβ+ pts (%)	88.9%	
Median ECP cycles after 6 mos (Range)	9.9 (6-25)	
Response - no. of pts (%)		
Complete response (CR)	none	
Partial response (PR)	n=8 (44.4%)	66.7% Response Rate
Minor response (MR)	n=4 (22.2)	
Stable disease (SD)	n=3 (16.7%)	
Progressive disease (PD)	n=3 (16.7%)	
Deceased	none	
Combination therapies	ECP alone (n=6) ECP + Bexarotene (n=5) ECP + IFN-α (n=2) ECP+ Bexarotene+IFN-α (n=5)	

At recruitment, all patients were beginning ECP for the first time. Six of 18 L-CTCL patients were treated with ECP only. For lack of response, biological response modifiers were added at 3 or more months after initial ECP. Five received only Bexarotene at 150 or 225 mg per day, two received only interferon alpha at a dose of 3 milllion daily or three times per week (+IFN-α), and five received both Bexarotene and IFN-α. The average number of ECP cycles administrated to L-CTCL patients was 9.9 over a six-month treatment period.

At the end of study or 6 months, the overall response rate to ECP was 66.7%,(12/18) with 8 patients achieving partial responses (PR, 44.4%) and 4 with minor responses (MR, 22.2%). There were no complete responses. The non-response rate was 33.3% (6/18) with 3 patients having stable disease (SD, 16.7%) and 3 with progressive disease (PD, 16.7%). Responses were determined by changes in mSWAT scores **(Figure 2.1A)** and/or circulating $CD4^+CD26^-$ tumor T cells **(Figure 2.1B)** from baseline shown for all patients.

At baseline, there were no significant differences in mSWAT between responders and non-responders. Similarly, at baseline, there were no significant differences in $CD4^+CD26^-$ T cells between responders and non-responders. After 6 months, there was a decrease in $CD4^+CD26^-$ T cells in both responders **(Figure 2.1C)** and non-responders **(Figure 2.1D)**. The mSWAT significantly decreased in responders (67.0→23.7, p=0.0004) but not in non-responders (72.2→73.4, p=0.63).

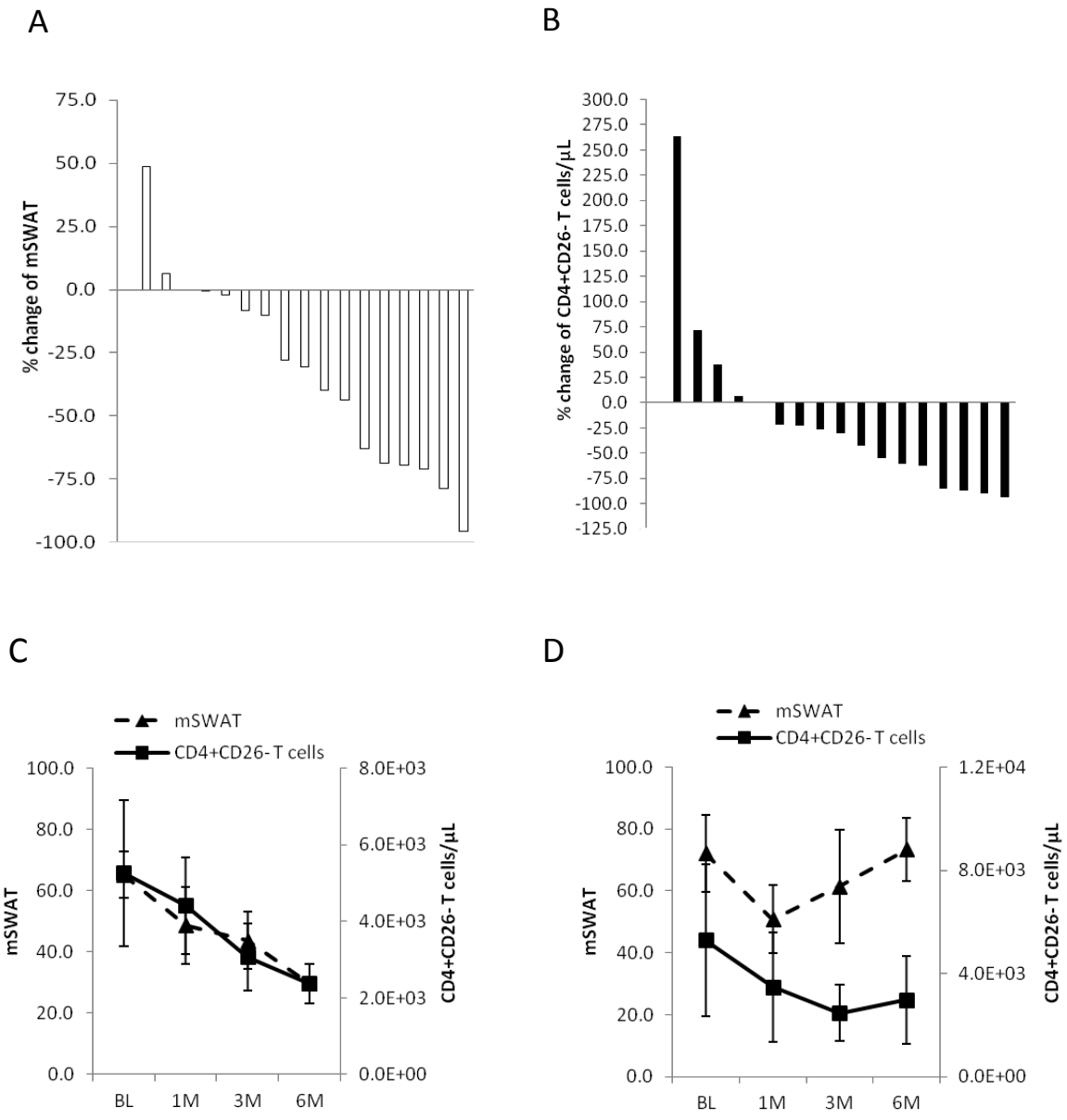


Figure 2.1

Figure 2.1. Clinical skin (mSWAT) and blood response ($CD4^+CD26^-$ T cells) from baseline (BL) to 6 months (6M) of ECP-BRM therapy

A) Percentage change in mSWAT from baseline (BL) to 6 months (6M) in all 18 patients.
B) Percentage change in CD4+CD26- T cells/μL from baseline (BL) to 6 months (6M) in all 18 patients.
C) Skin (mSWAT, ▲) and blood (CD4+CD26- T cells/μL, ■) response at baseline (BL), 1 month (1M), 3 months (3M), and 6 months (6M) in 12 responding patients.
D) Skin (mSWAT, ▲) and blood (CD4+CD26- T cells /μL, ■) response at BL, 1M, 3M, and 6M in 6 non-responding patients.

Eleven GVHD patients summarized in **Table 2.3** who enrolled in this study had either a non-L-CTCL primary lymphoma or leukemia. They developed acute and chronic GVHD after matched non-ablative stem cell transplantation. All patients had skin involvement, and all received standard immunosuppressive therapy for GVHD in addition to ECP. GVHD patients received an average of 14.6 to 29.9 cycles of ECP treatment in a 3 to 6-month period. After 3 to 6 months treatment, 6 of 11 (54.5%) had achieved skin and/or other organ improvements **(Table 2.4).**

Table 2.3. Clinical summary of GVHD patients on extracorporeal photopheresis

GVHD Patient #	Age/Sex/ Race	Primary Disease	Type of Donor	Type of GVHD	Organs affected	Concurrent therapies	ECP cycles at 3/6 months	Clinical Response at 3 months	Clinical response at 6 months
1	60/F/C	NHL	Match unrelated	De novo chronic	Skin (sclerodermoid), Mouth	Tacrolimus Methylprednisolone	7/12	No Response	GVHD progression
2	50/M/C	NHL	Match related sibling	Acute, Grade 4 of skin	Skin	Steroids, Mycophenolate, Methylprednisolone	5/n/a stopped ECP	Skin-Partial response	n/a
3	29/M/C	CLL	Cord blood	Late acute	Skin (non-sclerodermoid), Gastrointestinal	Mycophenolate, Prednisone, Tacrolimus	14/25	No response	Partial response
4	52/M/C	CML	Match related transplant	Classic chronic	Skin (sclerodermoid), eyes, liver, lung	Steroids, Erythromycin, Prednisone	9/25	Stable disease	Eye-no response Skin-no response Lungs-partial response Liver-complete response Overall-partial response
5	62/M/C	CLL	Match unrelated	Classic Chronic	Skin(sclerodermoid), liver, ,oral, eyes	Tacrolimus	19/43	Skin-Partial response Liver-Complete response Eye-no response Mouth-partial response	Skin - Partial response Liver-Complete response Eye -no response Mouth-partial response
6	55/F/C	SLL	Match Related Donor-from male donor	Classic chronic	Skin(sclerodermoid), joints- fascia, mouth, liver, eye	Tacrolimus, Methylprednisolone	23/37	Skin-Progression Eye-Progression Liver-normal	Skin-minor response fascia -Improved Mouth-partial responses
7	50/M/C	CLL	Match related donor	Overlap syndrome (both chronic & acute)	Skin (scleroderma), fascia, liver, mouth	Mycophenolate , Methylprednisolone, Imatinib, Tacrolimus	n/a	Skin-Partial response Fascia-Partial response Liver-Complete response Mouth- Complete response	Skin-Partial response Fascia-Partial response Liver-Complete response Mouth- Complete response
8	23/M/C	Aplastic anemia	Match related	Late acute	Liver, skin-involved but not active	Methylprednisolone, Tacrolimus	14/n/a stopped ECP	No Response	No response-progression
9	55/F/C	AML	Match related	Classic chronic	Skin, fascia, liver, Gastrointestinal tract (acute)	Prednisone, Tacrolimus,	11/35	No response	No response
10	52/M/C	HL→ B cell lymphoma	HLA matched sibling	De novo chronic	skin (sclerodermoid), Mouth, eye, lung	Steroids, Azithromycin	21/32	Stable disease (Skin, Eyes, Lung, Mouth)	Stable disease (Eyes, Lung) Partial response (Skin, Mouth)
11	62/F/C	Myelodysplastic syndrome / follicular lymphoma	Match-sibling	Overlap syndrome-both acute & chronic	Skin (sclerodermoid, macular popular)	Tacrolimus, Methyl prednisone	23/30	Stable Disease	Stable Disease

GVHD: Graft versus Host Disease, F: Female, M: Male, C: Caucasian, ECP: Extracorporeal Photopheresis, n/a: not available, HL: Hodgkin's Lymphoma, NHL=Non-Hodgkin's Lymphoma, AML: Acute myelogenous leukemia, CLL: Chronic lymphocytic leukemia, CML: Chronic myelogenous leukemia, SLL=Small Lymphocytic Lymphoma

Table 2.4. GVHD patients' demographics and response rate.

Characteristic	GVHD (n=11)	
Gender	Male (n=7, 63.6%) Female (n =4, 36.4%)	
Median Age (range)	52 (23-62) years	
Race	Caucasian (n=11, 100%)	
Type of GVHD-no. of patients (%)	Acute-9.0% Late acute-18.2% Denovo, chronic-18.2% Classic Chronic-36.4% Overlap (Acute & Chronic)-18.2%	
Median ECP cycles (Range)	After 3 mos: 14 (5-23) After 6 mos: 31 (12-43)	
Response - no. of pts (%)		
Complete response (CR)	none	
Partial response (PR)	n=4 (36.4%)	54.5% response rate
Minor response (MR)	n=1 (9.0%)	
Stable disease (SD)	n=1 (9.0%)	
Progressive disease (PD)	n=2 (18.2%)	
No Response (NR)	n=2 (18.2%)	
Response not available	n=1	
Combination therapies	ECP alone (n=0) ECP + Tacrolimus (n=8) ECP + Steroids (n=3)	

2.4 Discussion & Conclusions

Since 12 of 18 L-CTCL patients (66.7%) and 6 of 11 GVHD patients (54.5%) achieved clinical responses with ECP, this study further confirms the efficacy of ECP in treatment for L-CTCL and GVHD [85,116,117]. A confounding factor in this study is that 12 of 18 L-CTCL and all GVHD patients received additional immunomodulatory therapies initiated at 1 or 3 months after starting ECP. These include Bexarotene and IFN-α in L-CTCL patients and steroids and Tacrolimus in GVHD patients. We therefore cannot rule out effects of these agents on response and the modulation on DCs, T-regs, and CTLs in these patients. However, combined immunotherapy is widely used in both diseases, and a study devoid of these agents is not currently possible. Of interest, 4 of 6 (66.7%) L-CTCL patients who received ECP alone and 8 of 12 (66.7%) who received ECP with immunomodulatory therapy achieved the same overall clinical response rates, suggesting that most of the beneficial response may be attributed to ECP.

CHAPTER 3

EFFECT OF EXTRACORPOREAL PHOTOPHERESIS ON REGULATORY T CELLS IN PATIENTS WITH L-CTCL AND GVHD

3.1. Background and Rationale

Regulatory T cells (T-reg) are immunosuppressive T cells that regulate immune reactions. The $CD4^+$ $Foxp3^+CD25^+$ T-reg phenotype is the most understood type, but other phenotypes exist. $CD4^+Foxp3^+$ T cells were termed "naturally occurring" T-regs to distinguish them from *in vitro* generated "suppressor cells" [118]. A recent study reported that $CD4^+$ $Foxp3^+CD25^-$ T cells found in the periphery are reservoirs of inactive T-regs ready to activate when necessary [119].

Because of the existence of multiple T-reg phenotypes, data on T-regs in L-CTCL are limited and controversial. Both T-regs and L-CTCL cells share similar characteristics. They are both $CD4^+$ and anergic T cells. Therefore, it is thought that the L-CTCL malignant clones may be T-regs. Whether L-CTCL tumor cells are T-regs or not or if there is heterogeneity among L-CTCL patients is unclear. As an expansion of anergic $CD4^+$ T cells, MF/SS tumor cells suppress anti-tumor immunity and secrete Th2 cytokines [69,72,73]. Heid et al found a subset of SS patients with malignant suppressive $CD4^+$ $Foxp3^+CD25^-$ T cells and reported that tumors may be T-regs [50]. Conversely, in other studies, the T-reg phenotype was absent in L-CTCL [70,71,120].

ECP has been hypothesized to induce T-regs in both autoimmune/inflammatory diseases and in Graft-versus-Host Disease (GVHD);

however, it is unknown if T-regs are also induced by ECP in L-CTCL patients. To address this question, we examined three $Foxp3^+$ T cell subsets in L-CTCL and GVHD patients before and after ECP treatment at Day 2, 1 month, 3 months, and 6 months-2 years. We investigated $CD4^+Foxp3^+$, $CD4^+$ $Foxp3^+$ $CD25^-$, and $CD4^+$ $Foxp3^+CD25^+$ T cells within peripheral blood mononuclear cells (PBMCs) by immunofluorescence flow cytometry and Foxp3 mRNA within PBMCs by quantitative real time PCR.

3.2 Patients and Methods

Please refer to Chapter 7

3.3 Results

Patients and clinical response

Please refer to Chapter 2

$CD4^+$ $Foxp3^+$ $CD25^-$ and $CD4^+Foxp3^+CD25^+$ T cell subsets differ in patients with L-CTCL and GVHD at baseline.

Percentages of $CD4^+Foxp3^+$ T cells were significantly higher than normal (8.3±2.6%) in both L-CTCL and GVHD. Between the L-CTCL (n=9, 66.8±41.2%, p=0.03) and GVHD (n=7, 66.2±42.0, p=0.05) patients, percentages of $CD4^+Foxp3^+$ T cells were similar **(Figure 3.1A)**. Although percentages of $CD4^+Foxp3^+$ T cells were similar in L-CTCL and GVHD, percentages of $CD4^+$ $Foxp3^+CD25^-$ and $CD4^+$ $Foxp3^+CD25^+$ T cell subsets differed between the disease groups. Percentages of $CD4^+$ $Foxp3^+CD25^-$ T cells were higher in L-CTCL (48.5±32.3%) versus GVHD (22.9±25.3%) patients **(Figure 3.1B)** whereas

$CD4^+$ $Foxp3^+CD25^+$ T cells were significantly lower in L-CTCL (17.2±18.1%, p=0.04) versus GVHD (47.1±36.1%) patients **(Figure 3.1C)**.

The percentages of $CD4^+Foxp3^+$ T cells within L-CTCL patients were either higher (L-CTCL-High, 85.5±20.8%, n=7) or lower (L-CTCL-Low, 1.4±1.1%, n=2) than normal donors (ND) **(Figure 3.1D)**. No L-CTCL patients were within normal range (5.7-10.9%). Within GVHD patients, percentages of $CD4^+Foxp3^+$ T cells were significantly higher than normal in 5/7 patients (GVHD-High, 90.6±5.7%, $p=4.5*10^7$), lower than normal in 1/7 patients (GVHD-Low), and normal in 1/7 patients (GVHD-Normal) **(Figure 3.1E)**. Interestingly, 77.8% (7/9) of L-CTCL patients (L-CTCL-High) and 71.4% (5/7) of GVHD patients (GVHD-High) had significantly higher than normal percentages of $CD4^+Foxp3^+$ T cells **(Figure 3.2C)**.

$CD4^+$ $Foxp3^+CD25^-$ and $CD4^+$ $Foxp3^+CD25^+$ T cell subsets differ between the L-CTCL-High and GVHD-High patients

Since the majority of L-CTCL and GVHD patients had high percentages of $CD4^+Foxp3^+$ T cells, we next compared the $CD4^+Foxp3^+CD25^-$ and $CD4^+Foxp3^+CD25^+$ T cell subsets between the L-CTCL-High and GVHD-High patients. L-CTCL-High patients (62.1±20.5%, p= 0.03) had significantly higher percentages of $CD4^+$ $Foxp3^+CD25^-$ T cells than GVHD-High patients (27.4±25.4%) **(Figure 3.2B,D)**. In contrast, percentages of $CD4^+$ $Foxp3^+CD25^+$ T cells were significantly lower in L-CTCL-High patients (22.1±17.7%, p=0.009) compared to GVHD-High patients (63.6±27.5%) **(Figure 3.2B, E)**. These data

suggest that L-CTCL and GVHD patients may be harboring pathogenic $Foxp3^+$ phenotype cells prior to treatment.

Malignant T cells with a $Foxp3^+$ phenotype in 6/7 L-CTCL-High patients at baseline

Whether the tumor cells are $Foxp3^+$ regulatory T cells (T-regs) in at least some L-CTCL patients is controversial. Berger et al reported that L-CTCL is a malignancy of T-regs [69], but other studies show a lack of malignant T cells with a $Foxp3^+$ phenotype in majority of L-CTCL patients [70,71,73]. In our study, we investigated 9/18 patients' T-reg phenotypes and their correlation to malignant T-cell populations as defined by their expression of $CD4^+CD26^-$ [121].

We found that patients with greater than 50% of $CD4^+CD26^-$ T cells also had greater than 50% of $CD4^+Foxp3^+$ T cells. There is a correlation between $CD4^+$ $Foxp3^+$ and $CD4^+$ $Foxp3^+$ $CD25^-$ T cells and malignant $CD4^+CD26^-$ T cells in 6/7 L-CTCL-High patients **(Figure 3.3A)**, but no correlation in 1 L-CTCL-High and the 2 L-CTCL-Low patients **(Figure 3.3B)**. As expected, the percentages of malignant cells and $CD4^+Foxp3^+$ T cells positively correlated within L-CTCL High patients (R=0.54) **(Figure 3.3C)**. There was also a positive correlation between percentages of malignant cells and $CD4^+Foxp3^+CD25^-$ T cells (R=0.58) suggesting that these malignant cells have a $Foxp3^+$ phenotype but CD25 negative **(Figure 3.3D).** There was a negative correlation between malignant and $CD4^+$ $Foxp3^+CD25^+$ T cells (R=-0.34) **(Figure 3.3E).** All T cell percentages

and numbers in these L-CTCL-High and L-CTCL-Low patients are summarized in **Table 3.2**.

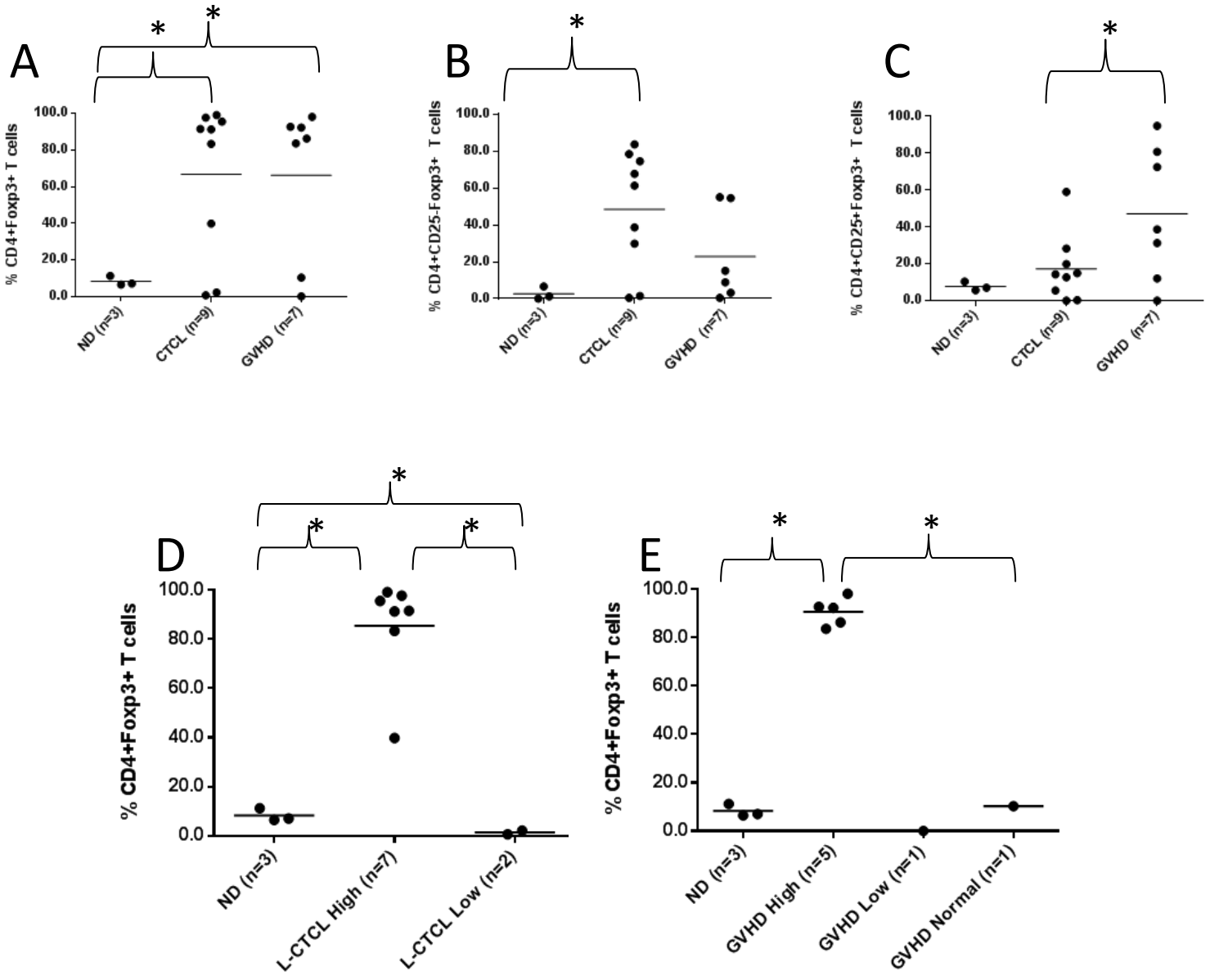


Figure 3.1

Figure 3.1. Foxp3^{+} T cells in L-CTCL and GVHD patients.

A) Dot graph displaying the percentages of CD4^{+}Foxp3^{+} T cells in L-CTCL and GVHD patients at baseline.
B) Dot graph displaying the percentages of CD4^{+} Foxp3^{+}CD25^{-} T cells in L-CTCL and GVHD patients at baseline.
C) Dot graph displaying the percentages of CD4^{+} Foxp3^{+}CD25^{+} T cells in L-CTCL and GVHD patients at baseline.
D) Dot graph displaying the percentages of CD4^{+}Foxp3^{+} T cells in L-CTCL patients with High (L-CTCL-High) and Low Foxp3 (L-CTCL-Low) at baseline.
E) Dot graph displaying the percentages of CD4^{+}Foxp3^{+} T cells in GVHD patients with High (L-CTCL-High), Low (L-CTCL-Low), and Normal (L-CTCL-Normal) Foxp3 at baseline.

* denotes statistical significance, p-value<0.05

Table 3.1. T lymphocyte parameters in normal donors (ND) versus L-CTCL versus GVHD patients before ECP.

	ND (n=3)	L-CTCL (n=9)	GVHD (n=7)	p-value
CD4+Foxp3+ (µl)	9.9±7.9	3640.7±7226.2	76.0±158.9	0.22
CD4+Foxp3+ (%)	8.3±2.6	66.8±41.2*	66.2±42.0*	0.98
CD4+ Foxp3+ CD25-(µl)	3.7±4.2	3067.7±6126.9	40.3±95.9	0.22
CD4+ Foxp3+ CD25-(%)	2.6±3.5	48.5±32.3*	22.9±25.3	0.13
CD4+ Foxp3+CD25+(µl)	10.6±11.9	555.9±1079.5	36.4±65.6	0.2
CD4+ Foxp3+ CD25+(%)	7.7±2.4	17.2±18.1	47.1±36.1	0.05
CD4+CD25high (µl)	n/a	285.8±569.3	n/a	n/a
CD4+CD25high (%)	0.6±0.5	3.2±5.4	9.6±6.0	0.07
Foxp3 mRNA	0.9±0.5	27.0±48.9*	0.1±0.1	0.30

p-value between L-CTCL and GVHD
*= p<0.05 compared to normal

Table 3.2. T lymphocyte parameters in normal donors (ND) versus L-CTCL-High versus L-CTCL-Low patients before ECP.

	ND (n=3)	L-CTCL-High (n=7)	L-CTCL-Low (n=2)	p-value
CD4+Foxp3+ (µl)	9.9±7.9	4674.2±8001.0	23.3±29.1	0.0009
CD4+Foxp3+ (%)	8.3±2.6	85.5±20.8*	1.4±1.1	0.5
CD4+ Foxp3+ CD25-(µl)	3.7±4.2	3933.7±6790.7	36.8±49.1	0.5
CD4+ Foxp3+ CD25-(%)	2.6±3.5	62.1±20.5*	0.98±0.7	0.005
CD4+ Foxp3+CD25+(µl)	10.6±11.9	714.1±1192.5	1.86±2.5	0.4
CD4+ Foxp3+CD25+(%)	7.7±2.4	22.1±17.7	0.2±0.2	0.1
CD4+CD25high (µl)	n/a	107.8±80.7	908.8±1247.3	0.07
CD4+CD25high (%)	0.6±0.5	1.7±2.2	8.5±11.6	0.1
Foxp3 mRNA	0.9±0.5	30.9±51.4	0.2	n/a
$CD4^{+}CD26^{-}$ (µl)	n/a	5208.4±7933.0	6288.5±5216.3	0.8
$CD4^{+}CD26^{-}$ (%)	n/a	78.4±14.8	85.8±12.3	0.5

p-value between L-CTCL High and L-CTCL Low
*= p<0.05 compared to normal

Table 3.3. T lymphocyte parameters in normal donors (ND) versus GVHD-High versus GVHD-Low versus GVHD-Normal patients before ECP.

	ND (n=3)	GVHD-High (n=5)	GVHD-Low (n=1)	GVHD-Normal (n=1)	p-value
CD4+Foxp3+ (µl)	9.9±7.9	106.2±184.1	0.2	0.8	0.4
CD4+Foxp3+ (%)	8.3±2.6	90.6±5.7	92.2	10.3	$4.5*10^7$
CD4+ Foxp3+ CD25-(µl)	3.7±4.2	56.4±112.5	0	0.03	0.5
CD4+ Foxp3+ CD25-(%)	2.6±3.5	27.4±0.2	54.6	0.4	0.2
CD4+ Foxp3+CD25+(µl)	10.6±11.9	50.9±74.4	0	0.9	0.4
CD4+ Foxp3+CD25+(%)	7.7±2.4	63.6±27.5	38.7	12.0	0.01
CD4+CD25high (µl)	n/a	n/a	n/a	n/a	n/a
CD4+CD25high (%)	0.6±0.5	10.4±6.6	n/a	6.3	0.001
Foxp3 mRNA	0.9±0.5	0.1±0.03	n/a	0.3	0.02

p-value between GVHD High and ND

Table 3.4. T lymphocyte parameters in normal donors (ND) versus L-CTCL-High versus GVHD-High patients before ECP.

	ND (n=3)	L-CTCL-High (n=7)	GVHD-High (n=5)	p-value
CD4+Foxp3+ (µl)	9.9±7.9	4674.2±8001.0	106.2±184.1	0.2
CD4+Foxp3+ (%)	8.3±2.6	85.5±20.8*	90.6±5.7*	0.6
CD4+ Foxp3+ CD25-(µl)	3.7±4.2	3933.7±6790.7	56.4±112.5	0.2
CD4+ Foxp3+ CD25-(%)	2.6±3.5	62.1±20.5*	27.4±25.4	0.03
CD4+ Foxp3+CD25+(µl)	10.6±11.9	714.1±1192.5	50.8±74.4	0.2
CD4+ Foxp3+CD25+(%)	7.7±2.4	22.1±17.7	63.6±27.5*	0.009
CD4+CD25high (µl)	n/a	107.80.7	n/a	n/a
CD4+CD25high (%)	0.6±0.5	1.7±2.2	10.4±6.6*	0.009
Foxp3 mRNA	0.9±0.5	30.9±51.4	0.07±0.03*	0.5

p-value between L-CTCL-High and GVHD-High
*= p<0.05 compared to normal

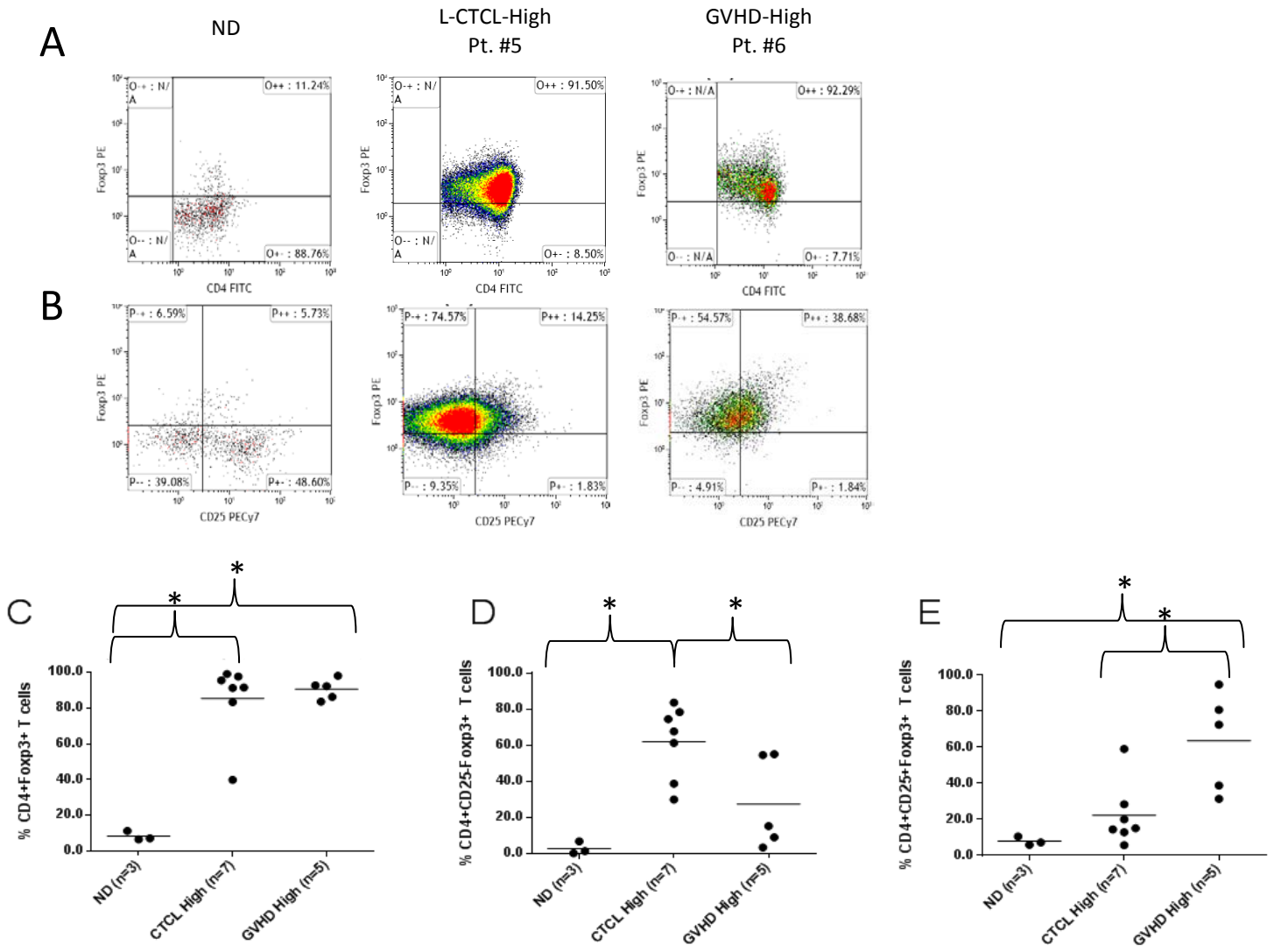


Figure 3.2

Figure 3.2. Division of patients based on baseline percentages of $CD4^+Foxp3^+$ T cells by flow cytometry.

A) Representative flow cytometry dot plots of $CD4^+Foxp3^+$ T cells (upper right quadrant) from a normal donor (ND, left), patient 5 (Pt. 5-CTCL-High $CD4^+Foxp3^+$ T cells, middle), and patient 6 (Pt. 6-GVHD-High $CD4^+Foxp3^+$ T cells, right).

B) Representative flow cytometry dot plots of $CD4^+$ $Foxp3^+CD25^-$T cells (upper left quadrant) and $CD4^+$ $Foxp3^+CD25^+$T cells (upper right quadrant) from normal donor (ND, left), patient 5 (Pt. 5-CTCL-High $CD4^+Foxp3^+$ T cells, middle), and patient 6 (Pt. 6-GVHD-High $CD4^+Foxp3^+$ T cells, right).

C) Patients were divided according to the percentages of $CD4^+Foxp3^+$ T cells in peripheral blood. Dot plot displaying percentages of $CD4^+Foxp3^+$ T cells in normal donors (ND, n=3), CTCL-High (n=7), and GVHD-High (n=5).

D) Dot graph displaying the percentages of $CD4^+$ $Foxp3^+CD25^-$T cells in normal donors (ND, n=3), CTCL-High (n=7), and GVHD-High (n=5).

E) Dot graph displaying the percentages of $CD4^+$ $Foxp3^+CD25^+$T cells in normal donors (ND, n=3), CTCL-High (n=7), and GVHD-High (n=5).

* denotes statistical significance, $p<0.05$

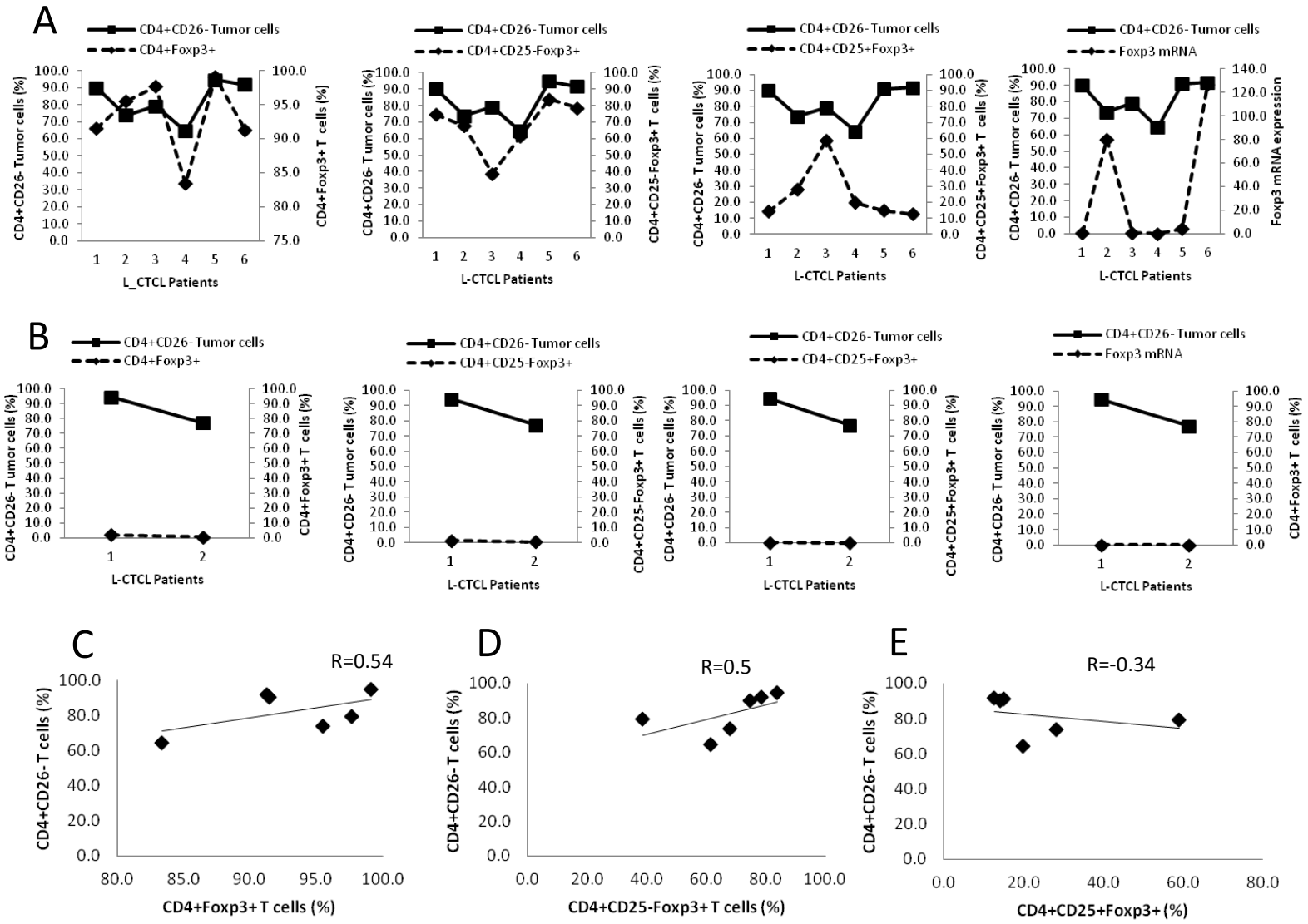


Figure 3.3

Figure 3.3. Correlation analysis of $Foxp3^+$ T cell subsets and malignant $CD4^+CD26^-$ T cells in L-CTCL-High and L-CTCL-Low patients

A) Comparison of percentages of malignant $CD4^+CD26^-$ T cells and percentages of $CD4^+Foxp3^+$ T cells, $CD4^+$ $Foxp3^+CD25^-$ T cells, $CD4^+$ $Foxp3^+CD25^+$T cells, and Foxp3 mRNA(from left to right) in the 6 L-CTCL-High patients.

B) Comparison of percentages of $CD4^+CD26^-$ tumor T cells and percentages of $CD4^+Foxp3^+$ T cells, $CD4^+$ $Foxp3^+CD25^-$T cells, $CD4^+$ $Foxp3^+CD25^+$T cells, and Foxp3 mRNA(from left to right) in the 2 L-CTCL-Low patients.

C) Scatter plot revealing a positive correlation between $CD4^+CD26^-$ T cells and $CD4^+Foxp3^+$ T cells in the 6 L-CTCL-High patients.

D) Scatter plot revealing a positive correlation between $CD4^+CD26^-$ T cells and $CD4^+$ $Foxp3^+CD25^-$T cells in the 6 L-CTCL-High patients.

E) Scatter plot revealing a negative correlation between $CD4^+CD26^-$ T cells and $CD4^+$ $Foxp3^+CD25^+$T cells in the 6 L-CTCL-High patients.

Decrease of malignant cells with Foxp3$^+$ T-reg phenotype after treatment with ECP in L-CTCL patients

We next studied the effects of ECP on CD4$^+$Foxp3$^+$, CD4$^+$ Foxp3$^+$CD25$^-$, and CD4$^+$Foxp3$^+$CD25$^+$ T cells in L-CTCL High **(Table 3.5).** We found a decrease in percentages and numbers of CD4$^+$Foxp3$^+$ **(Figure 3.3A)**, CD4$^+$ Foxp3$^+$CD25$^-$ **(Figure 3.3B)**, and CD4$^+$ Foxp3$^+$CD25$^+$ T cells **(Figure 3.3C)** in the L-CTCL-High through the treatment timepoints. Six of the 7 L-CTCL-High patients have the malignant Foxp3$^+$ phenotype and were responders to ECP treatment. Therefore, this data show that these malignant Foxp3$^+$ phenotype cells decrease with treatment suggesting that there is an association to response.

Decrease of Foxp3$^+$ T cells after treatment with ECP in GVHD patients

In the GVHD-High Foxp3 patients, the effects of ECP on percentages and number of CD4$^+$Foxp3$^+$, CD4$^+$ Foxp3$^+$CD25$^-$, and CD4$^+$Foxp3$^+$CD25$^+$ T cells in GVHD-High patients are summarized in **Table 3.6**. Percentages of CD4$^+$Foxp3$^+$ T cells remained the same after 3-4 months of treatment. However, percentages of CD4$^+$Foxp3$^+$CD25$^-$ decreased while CD4$^+$Foxp3$^+$CD25$^+$ T cells trended to increase after 3-4 months of treatment **(Figure 3.3D-F)**. There were 4/5 responders within these patients.

Table 3.5. Percentages (mean±SD) and numbers of T lymphocytes and Foxp3 mRNA in L-CTCL-High patients before and after ECP.

	BL	D2	1M	3M-4M	6M-10M	1YR-2YR
CD4+Foxp3+ (µl)	4674.2±8001.0	3300.9±3656.9	990.3±896.1	3015.6	314.9±521.2	5.05
CD4+Foxp3+ (%)	85.5±20.8*	94.4±3.9	68.9±51.5	52.5±56.5	64.6±44.9	98.1
CD4+CD25-Foxp3+ (µl)	3933.7±6790.7	2644.4±2866.5	1177.8±499.7	4991.8	357.5±493.1	2.3
CD4+CD25-Foxp3+ (%)	62.1±20.5*	70.2±8.9	54.9±40.1	44.5±45.3	42.9±26.6	44.3
CD4+CD25+Foxp3+ (µl)	714.1±1192.5	649.1±88.3	187.3±158.7	494.5±699.2	58.1±92.9	2.8
CD4+CD25+Foxp3+ (%)	22.1±17.7	24.0±12.8	13.8±12.4	7.9±10.3	20.7±22.8	53.8
CD4+CD25high (µl)	107.8±80.7	n/a	97.1±87.8	75.0±66.3	243.6±368.5	n/a
CD4+CD25high (%)	1.7±2.2	2.1±1.9	1.1±0.6	1.2±0.7	3.4±3.9	n/a
Foxp3 mRNA (n=7)	30.9±51.4	36.0±67.0	20.8±40.8	11.3±19.5	19.2±39.9	n/a
CD4+CD26- (µl)	5208.4±7933.0	n/a	3878.3±5109.7	2318.6±2446.4	2456.3±1907.0	n/a
CD4+CD26- (%)	78.4±14.8	n/a	72.8±20.3	69.2±26.2	72.5±25.0	n/a

n/a: not available

Table 3.6. Percentages (mean±SD) and numbers of T lymphocytes and Foxp3 mRNA in GVHD-High patients before and after ECP.

	BL	D2	1M	3M-4M	6M-10M	1YR-2YR
CD4+Foxp3+ (µl)	106.2±184.1	13.0±19.0	18	10.1±6.4	22.2	n/a
CD4+Foxp3+ (%)	90.6±5.7*	57.7±51.3	85.9	96.0±2.1	86.2	n/a
CD4+CD25-Foxp3+ (µl)	56.4±112.5	1.3±1.1	11.2	0.4±0.1	2.3	n/a
CD4+CD25-Foxp3+ (%)	27.4±25.4	3.1±2.9	53.5	4.4±2.7	8.8	n/a
CD4+CD25+Foxp3+ (µl)	50.8±74.4	12.3 ±17.9	7.4	9.7±6.3	19.9	n/a
CD4+CD25+Foxp3+ (%)	63.6±27.5*	55.1±48.8	35.2	91.9±2.4	77.4	n/a
CD4+CD25high (µl)	n/a	n/a	n/a	n/a	n/a	n/a
CD4+CD25high (%)	10.4±6.6*	15.7±14.1	8.9±8.3	10.4±4.5	n/a	n/a
Foxp3 mRNA (n=3)	0.07±0.03*	0.05±0.02	0.05±0.02	0.05	n/a	n/a

n/a: not available

*

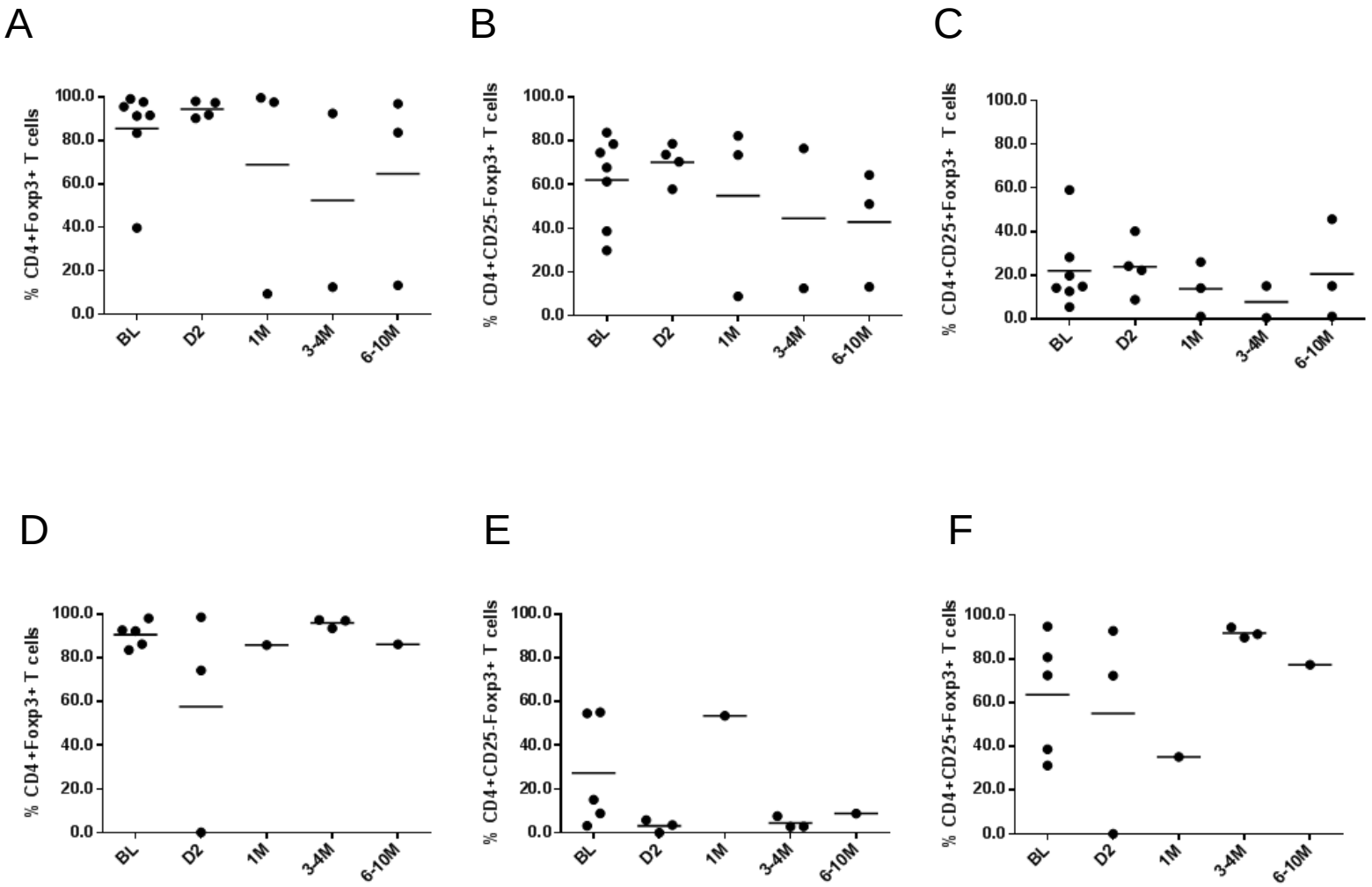


Figure 3.4

Figure 3.4. Changes in numbers of Foxp3$^+$ T cell subsets after ECP-BRM in L-CTCL-High and GVHD-High patients by flow cytometry.

A) Dot graph displaying the percentages of CD4$^+$Foxp3$^+$ T cells in L-CTCL-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).
B) Dot graph displaying the percentages of CD4$^+$ Foxp3$^+$CD25$^-$ T cells in L-CTCL-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).
C) Dot graph displaying the percentages of CD4$^+$ Foxp3$^+$CD25$^+$ T cells in L-CTCL-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).
D) Dot graph displaying the percentages of CD4$^+$Foxp3$^+$ T cells in GVHD-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).
E) Dot graph displaying the percentages of CD4$^+$ Foxp3$^+$CD25$^-$ T cells in GVHD-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).
F) Dot graph displaying the percentages of CD4$^+$Foxp3$^+$CD25$^+$ T cells in GVHD-High patients at baseline (BL), day 2 (D2), 1 month (1M), 3-4 months (3-4M), and 6-10 months (6-10M).

Table 3.7. Summary of GVHD patients' characteristics studied in this chapter.

Parameters	GVHD Patients (n=7)
Disease	NHL (n=2) CLL (n=3) CML (n=1) SLL (n=1)
Type of GVHD	Chronic GVHD (n=4) Acute GVHD (n=2) Overlap (n=1)
Donor	Match Related (n=4) Match Unrelated (n=2) Cord (n=1)
Organ affected	Skin (n=7)
Prophylaxis for GVHD	Tacrolimus +Methotrexate (n=5)
Concurrent Therapies	ECP + Tacrolimus (n=5)
Response	
At 3 months	PR (n=3); NR (n=2); SD (n=1); PD (n=1)
At 6 months	PR (n=5); PD (n=1); n/a (n=1)
Longer Outcomes	Deceased (n=5); Alive (n=2)

3.4. Discussion & Conclusions

In this small study of 9 L-CTCL patients with leukemic blood involvement (L-CTCL) 6/9 or 66.7% were found to have a malignant T-reg phenotype cells that correlated with the $CD4^{+}CD26^{-}$ Sézary cell phenotype. Both populations of cells decreased after ECP+BRM treatments suggesting that the treatments favorably modulate peripheral blood levels of $CD4^{+}Foxp3^{+}$ T cells. All 6 of the L CTCL patients whose blood had >80% $CD4^{+}Foxp3^{+}$ cells were also responders to ECP-BRM. The other 3 patients did not have a malignant T-reg phenotype and did not respond. We conclude that patients with malignant T-reg circulating cells should be treated differently than patients whose blood has non-malignant T-reg populations. ECP+BRM is an effective therapy for malignant T-reg L-CTCL patients but may not be as effective in non-malignant T-reg L-CTCL patients.

Surprisingly, 5/7 GVHD patients had high $CD4^{+}Foxp3^{+}$ T cells at baseline and 4/5 were responders. These data suggest that GVHD patients may have had prior $Foxp3^{+}$ pathogenic cells. We hypothesized that the initial disease treated with transplantation may have been a malignancy of $Foxp3^{+}$ T-regs. Two of the 5 GVHD patients with high Foxp3 had Non-Hodgkin's Lymphoma (NHL), 1 had Chronic Lymphocytic Leukemia (CLL), 1 had Chronic Myelogenous Leukemia (CML), and 1 had Small Lymphocytic Lymphoma (SLL). All the GVHD patients' characteristics studied in this chapter are summarized in **Table 3.7**. Although the literature is scarce, it has been reported that there is a high frequency of T-regs in CLL patients and correlates with disease progression [122]. Our data suggest that the high Foxp3 T-regs found in these GVHD High

patients may be remanent of their initial disease prior to transplantation. However, they may play a role in GVHD pathogenesis and may inhibit the graft versus tumor effect. This data challenges the current thought that T-regs may be beneficial for GVHD patients. Further studies are needed.

The immunosuppression in L-CTCL patients is attributed to many factors but can be mainly explained by the loss of T- cell repertoire [59]. Having both malignant clonal T cells that produce Th2 cytokines and T-reg type cells would severely inhibit normal immune processes such as cell mediated cytotoxicity. Although previous studies have found a lack of Foxp3 expression in L-CTCL patients [70,71,120], Heid et al first reported that clonal tumor cells with regulatory function express Foxp3 but lack CD25 typically found on $CD4^+$ T-regs in 40% of Sezary patients [73]. Our findings agree with Heid that L-CTCL or SS tumor cells are $Foxp3^+CD4^+$ T cells lacking CD25, the alpha chain of the IL-2 receptor. CD25 is a widely accepted marker for T-regs, however, more recent studies have reported that Foxp3 is a more specific marker for T-regs especially for development of regulatory function. Foxp3-transduced into $CD4^+CD25^-$ T cells elicited suppression of T cell proliferation and autoimmune disease and inflammatory bowel disease *in vivo* [123]. Another study characterized T-regs in $CD25^-CD45RB^{low}$ $CD4^+$ T cell population by Foxp3 expression and found their suppressive activity *in vitro* was similar to $CD4^+CD25^+$ T –regs [124]. Taken together, these studies indicate that expression of CD25 on $CD4^+$ T cell is insufficient to identify a cell as a T-reg and Foxp3 may be the more dominant marker. Thus, heterogeneity amongst cells with suppressive function may exist.

After ECP+BRM, 6 of 9 (66.7%) L-CTCL patients achieved clinical responses confirming the efficacy of ECP reported by others [85,116]. L-CTCL-High (n=7) had an 85.7% (6 of 7) response rate whereas L-CTCL-Low patients had a 0% response rate. All the patients with >50% of $CD4^+Foxp3^+$ T cells that were comparable to $CD4^+CD26^-$ T cells responded to ECP+BRM. Responses to ECP were associated with reduction in the numbers and percentages of malignant $CD4^+$ $Foxp3^+$ T cells. Although a lower response rate was observed in patients with a non-malignant $Foxp3^+$ phenotype, their responses may be attributed to other mechanisms. Increased cytotoxic activity may be responsible for early responses seen in the patients with non-malignant $Foxp3^+$ phenotype as we observed an increase in numbers of $CD8^+IFN\text{-}\gamma^+$ T cells after 1 month of treatment.

From these results, we found that patients can be divided into two groups: one with high $CD4^+Foxp3^+$ T cells and a second with low $CD4^+Foxp3^+$ T cells. Biological differences in these 2 cohorts suggest differences in treatment may be warranted. Because Foxp3 may be a biomarker for malignancy in CTCL, therapies targeting Foxp3 may be a novel treatment approach. Diagnostic tests to detect Foxp3 expression should be incorporated into standard care to identify patients with a T-reg phenotype. In addition, patients should be monitored for treatment response by flow cytometry of peripheral blood instead of solely using response observed in the skin. This study for the first time examines the immunobiological effects of ECP+BRM in L-CTCL patients with a malignant T-reg phenotype. It provides information to improve diagnosis and treatment of

heterogeneous L-CTCL patients and suggest that L-CTCL patients with malignant T-regs are better responders to ECP+BRM than patients without malignant T-regs. ECP+BRM may be a good treatment option in patients with malignant T-regs.

CHAPTER 4

EFFECT OF EXTRACORPOREAL PHOTOPHERESIS ON CYTOTOXIC T LYMPHOCYTES IN PATIENTS WITH L-CTCL AND GVHD PATIENTS

4.1 Background and Rationale

Cytotoxic T lymphocytes (CTL) are a subset of T lymphocytes that arise from common lymphoid progenitors differentiated from hematopoietic stem cells. CTL by definition express the $CD8^+$ molecule with the T cell receptor and kills other immune cells. They have been of immunologic interest for potential rejection of tumors and role in vaccination responses. CTL cytotoxic function was first identified in 1968 using alloantigen-activated thymus derived cells [36]. After their initial discovery, further research revealed specificity of CTL [125]. This led to further exploration in vaccines where they may be induced to prevent infectious diseases and limit tumor formation and progression.

At the molecular level, the cytotoxic function is mediated through exocytosis of cytotoxic granules and cytokine secretion in response to MHC class I antigenic peptides. By way of T cell receptor and CD8 ligand docking on the target cell MHC class I+peptide, cytotoxic granules, perforin and granzymes, participate in lysing and killing the target cell [42,125]. Perforin granules perforate the cellular membrane first and then release granzymes into the target cells. This also causes the activation of the $CD8^+$ T lymphocyte which induces the expression of CD69. This transmembrane C-type lectin protein is involved in lymphocyte proliferation and is a receptor that transmits signals from outside to

inside the lymphocyte [39,40]. The cytokine, interferon-gamma (IFN-γ), is secreted in $CD8^+$ T cells that have cytotoxic function [41,42]. IFN-γ is a type II IFN that signals through the JAK-STAT pathway and helps to promote an adaptive immune response against bacteria/viruses and tumors [43]. Thus, a current method of identifying CTL by flow cytometry is by intracellular expression of IFN-γ along with CD3, CD8, and CD69 markers [44-46].

Extracorporeal photopheresis, an immunomodulating therapy that induces lymphocyte apoptosis by UVA-psoralen, is used to treat cutaneous T cell lymphoma and graft-versus-host disease patients. ECP is hypothesized to have an effect on $CD8^+$ T cells in L-CTCL patients [108]. Prior to therapy, $CD8^+$ T cells are abnormally low and L-CTCL patients with close to normal levels of $CD8^+$ T cells do better on ECP [64]. Thus, it was hypothesized that ECP's mechanisms of action is by the engulfment of apoptotic cells by antigen-presenting cells to induce or increase CTL and their activity [98]. However, in GVHD, CTL numbers are elevated and are thought to be primary mediators of disease yet ECP is clinically beneficial [112]. Thus, we hypothesize that ECP would decrease CTL numbers and their activity in GVHD. Since it is currently unclear how ECP modulates CTL, we investigated its effects on $CD8^+$ T lymphocyte numbers and their cytotoxicity in L-CTCL and GVHD patients.

4.2 Patients and Methods

Please refer to Chapter 7.

4.3 Results

Patients and clinical responses

All L-CTCL **(Table 2.1)** and GVHD patients' **(Table 2.3)** demographics and characteristics are reported. The clinical characteristics and response rates of all L-CTCL and GVHD are summarized in **Table 2.2 and 2.4**.

Based on sample availability, 6/18 L-CTCL patients and 3/11 GVHD patients were analyzed for $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells. The 6 L-CTCL patients' demographics and characteristics are summarized in **Table 4.1**. The 3 GVHD patients' demographics and characteristics are summarized in **Table 4.2**. The L-CTCL patients were diagnosed with Sézary Syndrome with a T cell receptor clone present in 5/6 patients and one patient with late stage MF. There was 1 patient (pt.12) who had a $CD4^+CD26^+$ T cell rather than $CD4^+CD26^-$ population present. Patients' ages ranged from 58-79 years with a mean age of 70 years. There were more males (n=4, 66.7%) than females (n=2, 33.3%) in this study. Five out of six patients were concurrently on treatments with ECP. They were either on Bexarotene or IFN-α or combination (Bexarotene+IFN-α). Two patients (pt. #1 and #3) were treated with Bexarotene at the beginning of ECP treatment (study baseline). The other patients had Bexarotene and/or IFN-α addition after 1 month of ECP treatment except pt #4. Average number of ECP cycles at 6 months was 9.2 (range 7-12). All the GVHD patients had skin involvement. Two patients were diagnosed with classic chronic GVHD and the other had late acute GVHD. Ages ranged from 29-55 and the mean age was 45.3 years. There were more males

(n=2; 66.7%) than females (n=1, 33.3%). GVHD patients were on combination therapies including Tacrolimus. ECP cycles ranged from 25-37 at 6 months after initial ECP treatment.

In SS patients, the blood responses measured by decreased $CD4^{+}CD26^{-}$ T cell numbers are as follows: CR (n=0), PR (n=0), MR (n=0), SD (n=5), and PD (n=1). The skin responses measured by decreased mSWAT are as follows: CR (n=0), PR (n=1), MR (n=2), SD (n=2), PD (n=1). The inclusion of skin and blood responses resulted in overall responses as follows: CR (n=0), PR (n=1), MR (n=2), SD (n=1), PD (n=2). Responders have CR, PR, MR, or SD. Response rates were 83.3% for blood, 83.3% for skin, and 66.7% overall in SS patients.

The responses were graded in the disease affected skin as a regression of erythroderma typical of cGVHD. The overall response rate in this study was 100% partial responders in GVHD patients. Clinical characteristics for L-CTCL and GVHD patients are summarized in **Table 4.3**.

Table 4.1. L-CTCL patients' demographics and characteristics.

Pt. #	Age/Sex/Race	Diagnosis	SWAT	$CD4^{+}CD26^{-}$ (%)	TCR vβ (%)	ECP cycles	Concurrent Therapies
1	58/F/C	SS IVA	63.0	64.2	n/a	7	Bexarotene, IFN-α
2	79/M/C	SS IVB (BM+)	54.0	87.5	Vβ 13.6 (97.1%)	10	Bexarotene
3	71/M/C	SS/MF IVA	100.0	56.0	Vβ 17 (94.0%)	12	Bexarotene, IFN-α
4	78/F/AA	SS IVB (BM+)	47.0	94.5	Vβ 13.6 (95.0%)	10	none
5	73/M/C	SS IVA	47.0	77.1	Vβ 18.0 (95.0%)	9	Bexarotene
6	61/M/C	SS IVB	87.0	91.7	Vβ 7.1 (97.0%)	7	Bexarotene

Pt.= patient, F=Female, M=Male, C=Caucasian, AA=African American, ECP= Extracorporeal Photopheresis, n/a=not available, BM=bone marrow,TCR=T cell receptor, SS=Sezary Syndrome, MF=Mycosis Fungoides, IFN=interferon

Table 4.2. GVHD patients' demographics and characteristics.

Pt. #	Age/ Sex/ Race	Disease Transplant done	Type of Donor	Type of GVHD	Organs affected	ECP cycles	Concurrent Therapies
1	29/M/ C	CLL	Cord	Late aGVHD	Skin (non-sclerodermoid), GI (late aGVHD)	25	Cellcept, prednisone (80mg, 2x daily), tacrolimus
2	52/M/ C	CML	Match Related transplant	Classic cGVHD (Progres-sive cGVHD)	Skin (scleroder-moid, eyes, liver, lung	25	Steroids, erythromycin, prednisone (60mg)
3	55/F/C	SLL	Match Related Donor-from male donor	Classic cGVHD-prior hx of aGVHD	Skin (scleroderm-oid), joints-fasicitis, mouth, liver, eye	37	Tacrolimus (6mg), Medrol (16 mg)

GVHD=Graft versus Host Disease, aGVHD= acute GVHD, cGVHD=chronic GVHD, F=Female, M=Male, C=Caucasian, ECP= Extracorporeal Photopheresis, n/a=not available, CLL=Chronic lymphocytic leukemia, CML=Chronic myelogenous leukemia, SLL=Small Lymphocytic Lymphoma

Table 4.3. Clinical summary of L-CTCL and GVHD patients.

Characteristic	L-CTCL (n=6)	GVHD (n=3)
Sex	Male (n=4, 66.7%) Female (n =2, 33.3%)	Male (n=2, 66.7%) Female (n=1, 33.3%)
Age (Mean)	70 (58-79) years	45.3 (29-55) years
Race	Caucasian (n=5) African Amer. (n=1)	Caucasian (n=3)
Diagnosis	Sezary Syndrome IVA (n=3) Sezary Syndrome IVB (n=3)	Late Acute GVHD (n=1) Classic Chronic GVHD (n=2)
Tumor burden (TB)	High TB (>1000/μl) 83.3% Low TB (<1000 /μl) 16.7%	n/a
TCR vβ (≥95%)	Yes (n=5, 83.3%)	n/a
ECP cycles after 6 mos (Mean)	9.2 (7-12)	29 (25-37)
Clinical Response	SD (n=5, 83.3%) PD (n=1, 16.7%)	PR (100%)
Combination therapies	Bexarotene (n=5, 83.3%) IFN-α (n=2, 33.3%) Bexarotene+IFN-α (n=2, 33.3%)	Tacrolimus (100%)

Low levels of $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells at baseline in L-CTCL patients but high in GVHD patients

Absolute values of $CD8^+$ T cells subsets were compared between normal donors, L-CTCL, and GVHD patients at baseline prior to ECP treatment. The absolute numbers of $CD3^+CD8^+$ T cells were statistically lowest ($p<0.01$) in L-CTCL patients (n=6, 12.9±12.4 /µl) compared to normal donors (n=3, 596.6 ± 412.8 /µl). The highest absolute numbers were found in GVHD patients' blood (878.9 ± 1513.6 /µl) **(Figure 4.1A)**. Similarly, $CD3^+CD8^+CD69^+$ T cells were lowest in L-CTCL patients (6.3±6.5 /µl) compared to normal donors (288.5 ± 185.6 /µl, $p<0.01$) and GVHD (758.8 ± 1314.0 /µl) patients **(Figure 4.1B)**. Interestingly, the $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells were also lowest in L-CTCL patients (4.0 ±6.3) compared to normal donors (160.1±111.3, $p<0.01$) and GVHD patients (456.9±791.2) **(Figure 4.1C), (Table 4.4)**.

Table 4.4. Baseline numbers of $CD8^+$ T cell subsets in normal donors and patients with L-CTCL and GVHD.

	$CD3^+CD8^+$ (/µl)	$CD3^+CD8^+CD69^+$ (/µl)	$CD3^+CD8^+$ IFN- γ^+ (/µl)
Normal donor (n=3)	596.6 ± 412.8	288.5 ± 185.6	160.1±111.3
L-CTCL (n=6)	12.9±12.4	6.3±6.5	4.0 ±6.3
GVHD (n=3)	878.9 ± 1513.6	758.8 ± 1314.0	456.9±791.2

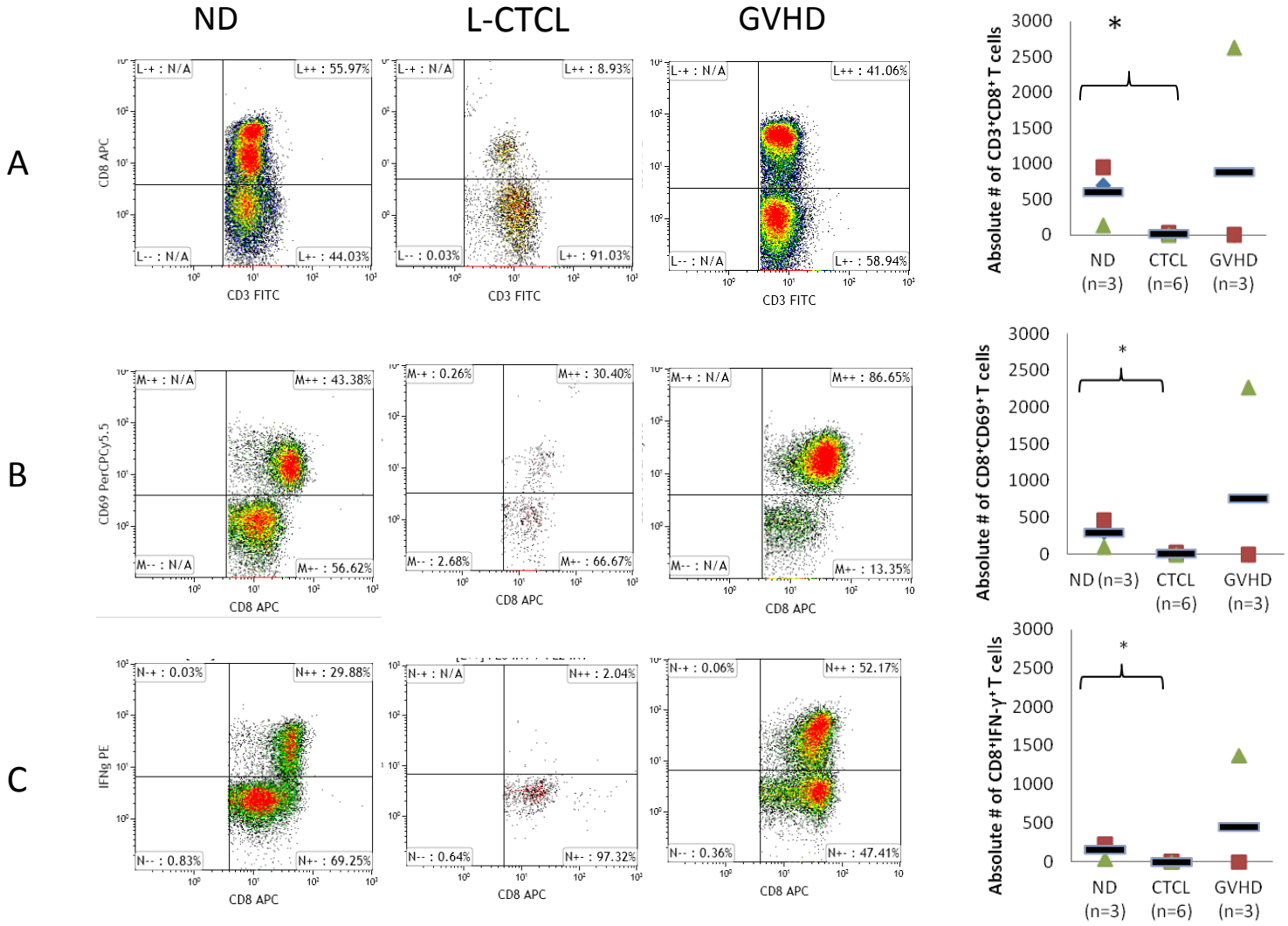


Figure 4.1

* p<0.01

Figure 4.1. Flow cytometry dot plots and graphs of numbers of $CD8^+$ T cell subsets before treatment.

A) $CD3^+CD8^+$ double positive T cells (top right quadrant) in a normal donor (ND), L-CTCL #1, and GVHD patient #3. Graph (top right) shows absolute numbers of $CD3^+CD8^+$ T cells in normal and disease groups where black bars represent averages.

B) $CD3^+CD8^+CD69^+$ triple positive T cells (top right quadrant) in a normal donor (ND), L-CTCL , and GVHD patient. Graph (middle right) shows distribution of absolute numbers of $CD3^+CD8^+CD69^+$ T cells in normal and disease groups where the black bars represent averages.

C) $CD3^+CD8^+IFN\text{-}\gamma^+$ triple positive T cells (top right quadrant) in a normal donor (ND), L-CTCL, and GVHD patient. Graph (lower right) shows distribution of absolute numbers of $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells in normal and disease groups where the black bars represent averages.

Increase in $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, $CD3^+CD8^+INF\text{-}\gamma^+$ T cells after ECP treatment in L-CTCL patients

Five out of six L-CTCL patients had increased numbers of $CD3^+CD8^+$ and $CD8^+CD69^+$ T cells after one month and 3 months to 1 year. On average, $CD3^+CD8^+$ and $CD3^+CD8^+CD69^+$ numbers spiked at Day 2 (117.3 ±187.8, 89.5 ±146.0) and decreased after one month (101.7±196.6, 73.4±145.9) and 3 month-1 year (60.3±123.3, 45.7±98.9)**.** Numbers were still higher than baseline (12.9±12.4, 6.3±6.5) after 3 months-1 year post-ECP **(Figure 4.2A,B), (Table 4.5)**.

Four of the five patients with follow-up at one month had increased $CD3^+CD8^+$ IFN-γ^+ T cells from baseline. Three of the five patients with follow-up at 3 months-1 year also had increased $CD3^+CD8^+$IFN-γ^+ T cells from baseline. On average, the numbers increased at Day 2 (7.8±7.4) and again at 1 month (9.6±11.5) but were lower at 3 months-1 year (3.7±7.0) and almost back to baseline levels (4.0±6.3) **(Figure 4.2C), (Table 4.5).**

Table 4.5. Mean numbers of $CD8^+$ T cell subsets pre- and post-ECP in L-CTCL patients

			Post-ECP		p-value
	Pre-ECP	Day 2-1 week	1 M	3M-1YR	
$CD3^+CD8^+$ (/µl)	12.9±12.4	117.3 ±187.8	101.7±196.6	60.3±123.3	0.02
$CD3^+CD8^+CD69^+$ (/µl)	6.3±6.5	89.5 ±146.0	73.4±145.9	45.7±98.9	0.02
$CD3^+CD8^+$ IFN- γ^+ (/µl)	4.0±6.3	7.8±7.4	9.6±11.5	3.7±7.0	0.02

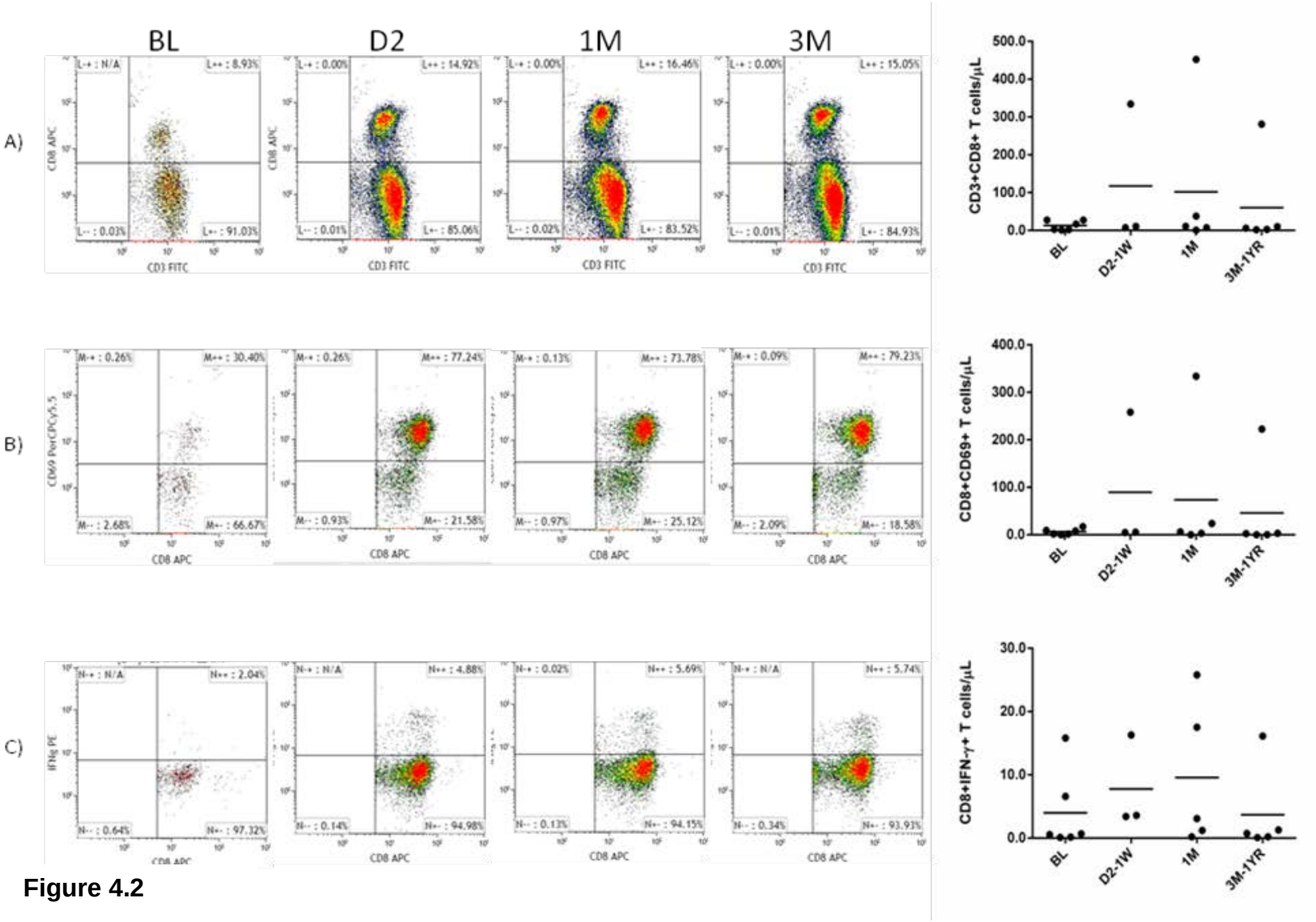


Figure 4.2

Figure 4.2. Flow cytometry analysis of $CD8^+$, $CD8^+CD69^+$, and $CD8^+$IFN-γ^+ T cells in L-CTCL patients (n=6) at baseline and after ECP+BRM treatment. Representative flow cytometry dot plots (left to right) from a responding L-CTCL patient #1 and dot graphs of absolute numbers of (far right) A) $CD3^+CD8^+$, B) $CD8^+CD69^+$, and C) $CD8^+$IFN-γ^+ T cells at baseline (BL), Day 2 (D2)-1week (1W), 1 month (1M), and 3 months (3M)-1 year (1YR).

$CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells decreased in GVHD patients treated with ECP

Three GVHD patients had follow-up time points at 3 months of ECP therapy. All of them had decreased numbers of $CD3^+CD8^+$ and $CD8^+CD69^+$ T cells from baseline (878.9 ±1513.6, 758.8±1314.0) to post –ECP 3 months (1.8±2.4, 0.3±0.5). On average, the $CD3^+CD8^+$ T cell levels decreased from baseline (878.9 ±1513.6) to day 2 (0.9±0.2) dramatically and remained low at 1 month (10.02) , 3 months (1.8±2.4), and 6 months (60.9) post-ECP. Similarly, on average, the $CD3^+CD8^+CD69^+$ T cell levels dramatically decreased from baseline (758.8±1314.0) to day 2 (0.1±0.02) and remained low at 1 month (0.2), 3 months (0.3±0.5), and 6 months (32.4) post-ECP.

$CD3^+CD8^+IFN\text{-}\gamma^+$ T cells also decreased from baseline (456.9±791.1) to 3 months (0.2±0.2) post-ECP in all the GVHD patients. On average, the levels dramatically decreased from baseline (456.9±791.1) to Day 2 (0.1±0.0) and remained low at 1 month (0.08), 3 months (0.2±0.2), and 6 months (9.1) post-ECP **(Figure 4.3, Table 4.6)**.

Table 4.6. Mean numbers of $CD8^+$ T cell subsets pre- and post-ECP in GVHD patients.

		Post-ECP			
	Pre-ECP	**Day 2**	**1 mo**	**3 mos**	**6mos**
$CD3^+CD8^+$ (/µl)	878.9 ±1513.6	0.9±0.2	10.02	1.8±2.4	60.9
$CD3^+CD8^+CD69^+$ (/µl)	758.8±1314.0	0.1±0.02	0.2	0.3±0.5	32.4
$CD3^+CD8^+$ IFN -γ^+ (/µl)	456.9±791.1	0.1±0.0	0.08	0.2±0.2	9.1

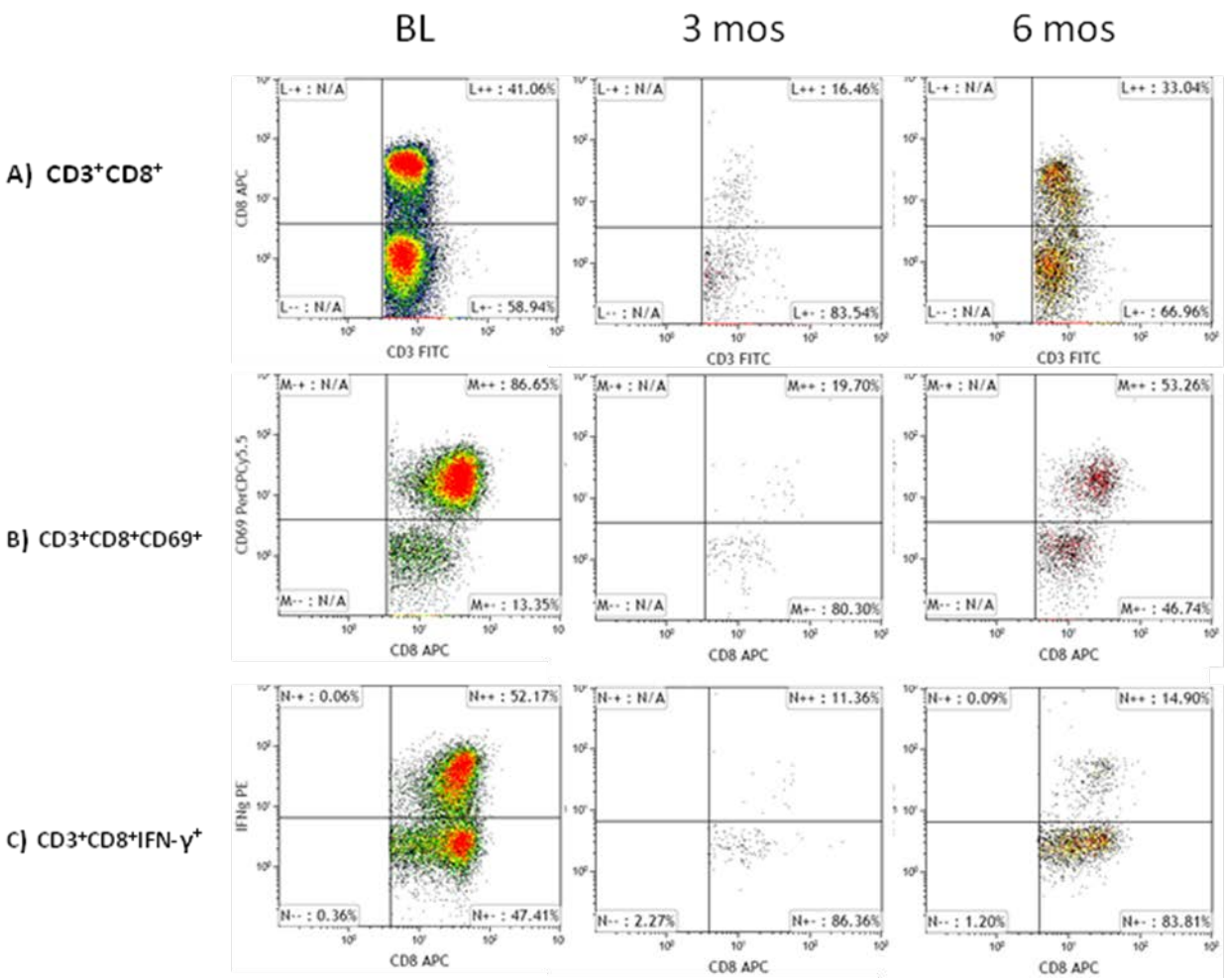


Figure 4.3

Figure 4.3. Flow cytometry dot plots of numbers of $CD8^+$ T cell subsets after treatment in GVHD patient #3 who responded.

A) $CD3^+CD8^+$ double positive T cells (top right quadrant in each panel) at baseline (BL), 3 months (mos), and 6 mos post-ECP (top from left to right).

B) $CD3^+CD8^+CD69^+$ triple positive T cells (top right quadrant in each panel) at BL, 3 mos, and 6 mos post-ECP (middle from left to right).

C) $CD3^+CD8^+$IFN-γ^+ triple positive T cells (top right quadrant in each panel) at BL, 3 mos, and 6 mos post-ECP (bottom from left to right).

The correlation of $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells to clinical response in L-CTCL patients

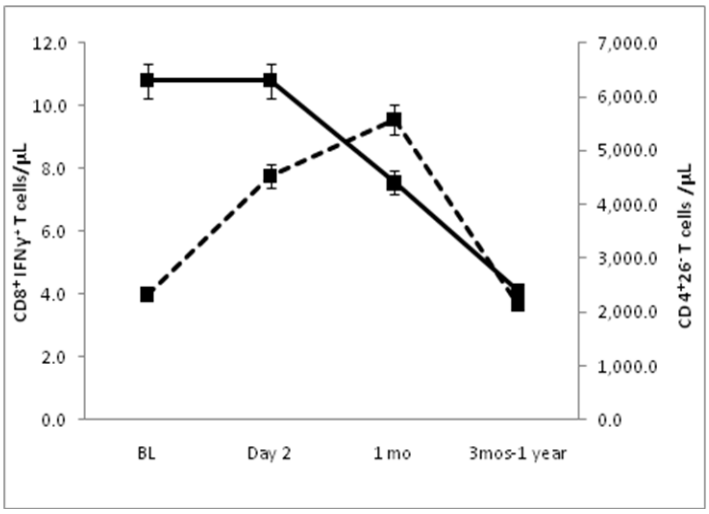
On average, the numbers of $CD4^+CD26^-$ SS tumor cells decreased from baseline (6281.8 /µl), to 1 month (4440.5 /µl), and to 6 months (2354.4 /µl) in selected SS patients who had cells available for *in vitro* analysis (n=6). However, numbers of $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells increased from baseline (3.99 /µl) to Day 2 (7.77/ µl) to 1 month (9.56 /µl), but then decreased at 3 months-1 year (3.69 /µl) post-ECP **(Figure 4.4A)**.

This change in $CD8^+$ cells were associated with improvement and worsening of skin involvement as determined by mSWAT measurements of skin involvement. Mean mSWAT scores decreased from baseline (68.0), to 1 month (45.7) but increased again at 3 months -1 year (54.3) in these SS patients (n=6). There was an inverse relation between mSWAT improvement and increase in numbers of $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells suggesting that induction of CD8+ cytotoxicity favorably effects clinical L-CTCL expression. **(Figure 4.4B)**.

There were 3/6 responders within the SS patients studied for activated and functional IFN-γ producing $CD8^+$ T cells. Responding 3 patients had higher numbers of $CD3^+CD8^+$ (23.7±3.5), $CD3^+CD8^+CD69^+$ (11.5±2.8) and $CD3^+CD8^+IFN\text{-}\gamma^+$ (7.7±4.4) T cells prior to treatment compared to the three non-responders (2.1±0.6; 1.2±0.5; 0.3±0.2) **(Table 4.7).** Although, in non-responders there was an increase in numbers of $CD8^+$ T cell subsets after treatment at 1 month, the numbers of $CD3^+CD8^+$ (**Table 4.8**, 9.0±1.2), $CD3^+CD8^+CD69^+$ (**Table 4.9**, 4.5±1.2) and $CD3^+CD8^+IFN\text{-}\gamma^+$ (**Table 4.10**, 2.2±0.8) remained low. In

responders, the numbers of $CD3^+CD8^+$ (163.5±144.8, 6.9-fold, **Table 4.8**), $CD3^+CD8^+CD69^+$ (119.3±107.5, 10.3-fold, **Table 4.9**), and $CD3^+CD8^+IFN\text{-}\gamma^+$ (14.5±7.5, 1.9-fold, **Table 4.10**) were higher than non-responders and increased from baseline after only 1 month of ECP. These results suggest that although these $CD8^+$ subset numbers remained low, their activation status and cytotoxic function improved with ECP treatment and may be related to overall clinical response.

A)



B)

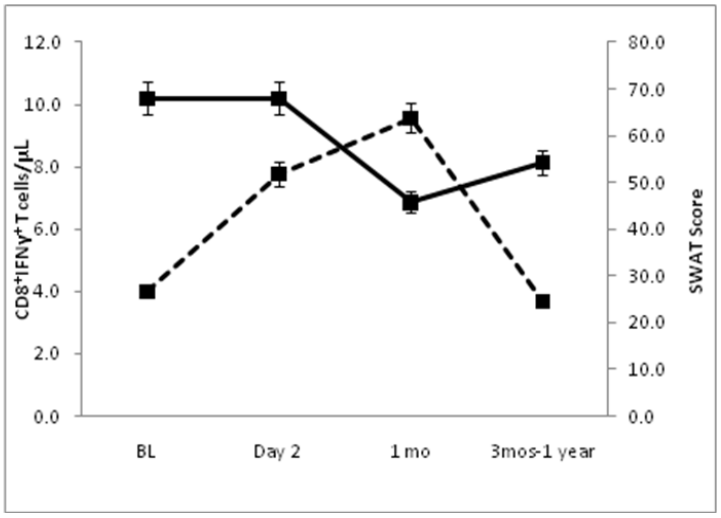


Figure 4.4

Figure 4.4. Clinical response and $CD3^+CD8^+IFN\text{-}\gamma^+$ cytotoxic T cells in L-CTCL patients.

A) Comparison of $CD8^+$ $IFN\text{-}\gamma^+$ T cells (dashed line) numbers to SS tumor cells, $CD4^+CD26^-$, (solid line) T cell numbers before and after treatment.

B) Comparison of $CD8^+$ $IFN\text{-}\gamma^+$ T cells (dashed line) numbers mSWAT score (solid line) before and after treatment.

Table 4.7. Comparison of $CD8^+$ T cells in L-CTCL responders and non-responders at baseline.

	Responders (n=3)	Non-responders (n=3)
$CD3^+CD8^+$	23.7±6.1	2.1±1.1
$CD8^+CD69^+$	11.5±4.8	1.2±0.9
$CD8^+IFN\text{-}\gamma^+$	7.7±7.7	0.3±0.3

Table 4.8. Numbers of $CD3^+CD8^+$ T cells in L-CTCL responders and non-responders before and after treatment.

	BL	D2-1W	1M	3M-1YR
Responders (n=3)				
1	27.2	334.2	452.4	280.9
11	27.2	n/a	0.5	2.6
18	16.7	7.3	37.7	2.0
Mean±SD	23.7±6.1	170.8±231.2	163.5±250.9	95.2±160.9
Non-Responders (n=3)				
12	1.0	n/a	n/a	10.3
13	3.1	10.5	7.4	n/a
15	2.3	n/a	10.5	5.9
Mean±SD	2.1±1.1	10.5	9.0±2.2	8.1±3.1

Table 4.9. Numbers of $CD8^+CD69^+$ T cells in L-CTCL responders and non-responders before and after treatment.

	BL	D2-1W	1M	3M-1YR
Responders (n=3)				
1	8.3	258.1	333.8	222.6
11	17.1	n/a	0.3	0.2
18	9.2	4.7	23.8	0.1
Mean±SD	11.5±4.8	131.4±179.2	119.3±186.1	74.3±128.4
Non-Responders (n=3)				
12	0.2	n/a	n/a	2.5
13	1.8	5.6	3.0	n/a
15	1.5	n/a	6.0	3.1
Mean±SD	1.2±0.9	5.6	4.5±2.1	2.8±0.4

Table 4.10. Numbers of $CD8^{+}IFN\text{-}y^{+}$ T cells in L-CTCL responders and non-responders before and after treatment.

	BL	D2-1W	1M	3M-1YR
Responders (n=3)				
1	0.6	16.3	25.8	16.12
11	15.8	n/a	0.2	0.2
18	6.6	3.4	17.5	0.09
Mean±SD	7.7±7.7	9.9±9.1	14.5±13.0	5.5±9.2
Non-Responders (n=3)				
12	0.17	n/a	n/a	0.73
13	0.7	3.6	1.2	n/a
15	0.1	n/a	3.1	1.3
Mean±SD	0.3±0.3	3.6	2.2±1.3	1.0±0.4

The correlation of $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells and clinical response in GVHD patients

All three GVHD patients had decreased numbers of $CD8^+$, $CD69^+$ and $IFN\text{-}\gamma^+$ T cells and were classified as partial responders to ECP. Unfortunately, there were not enough samples to assess the non-responders' $CD3^+CD8^+$ T cells levels and make a comparison.

4.4 Discussion & Conclusions

In this study, we report lower-than-normal baseline levels of $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells in the blood of Sézary Syndrome (SS) patients. This is consistent with previous findings of low levels of $CD3^+CD8^+$ T cells and their inability to produce IFN-γ cytokine in L-CTCL patients [103,126,127]. There are no reported studies on the levels of $CD3^+CD8^+CD69^+$ or $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells in SS patients' blood. However, in GVHD patients, the levels of $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells were higher than normal at baseline which supports increased $CD8^+$ $T_{effector}$ cells in cGVHD patients reported by Grogan et al in 2011 [128]. ECP action on $CD8^+$ T cell subsets in L-CTCL and GVHD patients appear to have opposite effects but how this occurs is unknown.

The major findings of this study are that $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$, and $CD3^+CD8^+INF\text{-}\gamma^+$ T cell numbers increase after ECP treatment in L-CTCL patients but decrease in GVHD patients' blood. Our data suggest that ECP is improving not only the $CD3^+CD8^+$ T cell numbers but also their function as

determined by increased IFN-γ secretion in L-CTCL patients' blood $CD8^+$ T cells after 1 month of treatment. This increase was found in L-CTCL patients who responded to ECP as measured by a decrease in the SS $CD4^+CD26^-$ T cells and by decrease in the skin mSWAT score. Surprisingly, the opposite effect occurred in the blood of GVHD patients. Our results show that in GVHD patients, $CD3^+CD8^+$ T cell numbers are not only reduced but also the function as measured by IFN-γ is reduced by ECP. Since GVHD is mediated by $CD3^+CD8^+$ T cells, it is logical that the reduction of these cells would occur in patient responders to ECP.

These results suggest for the first time that ECP works by different mechanisms in improving L-CTCL and GVHD. This paradox suggests a difference in ECP mechanism of action with different immunological states at baseline. If ECP does not work the same way in these two diseases, it suggests that ECP has differential effects on immunity and works by tailoring to the immune disease state.

A possible mechanism by which ECP is modulating $CD3^+CD8^+$ T cells in L-CTCL patients would be by antigen-presenting cell (APC) taking up UVA/psoralen (ECP) treated cells that are apoptotic. This uptake of apoptotic cells provides a tumor antigen source for activation of $CD3^+CD8^+$ T cells to produce IFN-γ to elicit an anti-tumor immune response for down-regulation of tumor cells in L-CTCL. Since previous studies in GVHD suggest that $CD3^+CD8^+$ T cells are mediators of disease, the decrease of these Th1 producing cells would in turn cause disease regression in L-CTCL. Thus, the mechanism of this

reduction of cells by ECP in GVHD patients may be by regulatory T cell suppression or cell death of $CD3^+CD8^+$ T cells by antigen-primed dendritic cells.

In summary, these results support our hypothesis that $CD3^+CD8^+$ T cells are increased in numbers and activity in L-CTCL patients but are reduced in GVHD patients after ECP treatment. This shows that the ECP mechanism of action is different depending on the disease state. These findings are in a small study cohort and were limited by amount of frozen patient samples available before and after ECP. Although this study cohort was small, the majority of the patients had similar trends. Another limitation of this study is that some of the patients were on other treatments besides ECP. Therefore, it is unclear if the induction of activated, IFN-γ producing $CD3^+CD8^+$ T cells were by ECP or by Bexarotene or IFN-α. Thus, further study in a larger cohort with ECP as a monotherapy in addition to the investigation of CTL activity would be ideal. However, from this study we found for the first time a favorable increase in the function of $CD8^+$ T cells after only 1 month of ECP in L-CTCL patients whereas a favorable decrease in numbers of functional $CD8^+$ T cells in GVHD patients after treatment with ECP. These data suggest that the mechanism of ECP action through $CD8^+$ T cells is different in L-CTCL and GVHD patients.

CHAPTER 5

EFFECT OF EXTRACORPOREAL PHOTOPHERESIS ON DENDRITIC CELLS IN PATIENTS WITH L-CTCL AND GVHD.

5.1 Background and Rationale

Like other immune cells that participate in the innate and adaptive immune response, DCs express distinct cell surface receptors or ligands to sense and respond to environmental stimuli. These cell surface signaling molecules (CSSMs) are vital for their differentiation, maturation, and function [129]. In the normal steady-state condition, DCs express low levels of CSSMs such as co-stimulatory and/or co-inhibitory molecules and MHC Class I and II molecules. This allows the immature DCs to maintain peripheral tolerance to self-antigens. In the presence of danger signals (i.e. antigens and infections), there is an up-regulation of expression of CSSMs resulting in DC maturation and subsequently promoting T cell immunity [130]. The B7 family members including B7-1 (CD80), B7-2 (CD86), and B7-H2 (the inducible costimulator ligand, ICOSL) are major members of CSSMs. They provide T-cell costimulatory/coinhibitory signals upon binding to their receptors CD28, CTLA-4 and ICOS, respectively [131].

DCs are the most important initial source of cytokines governing the development of helper T cell (Th) responses. Two well described human DC subpopulations with different biological functions are: the $CD11c^{+}CD123^{-}$ myeloid DCs (mDCs) and the $CD11c^{-}CD123^{+}$ plasmacytoid DCs (pDCs), which induce different types of Th cell responses based on environmental factors [8]. In

general, mDCs produce IL-12 and polarize naïve T cells toward a Th1 phenotype, whereas pDCs stimulated with IL-3 produce low levels of IL-12 and large amounts of IFN-α and results in a Th2 response. Moreover, ICOSL on pDCs is critical for inducing the immunosuppressive IL-10 cytokine which induces T-regs [8]. The up-regulation of Th1 cytokines and IL-12 is present in chronic GVHD and implicates a Th1-driven immunopathogenesis [132]. Modulation of DC subpopulations and a shift of cytokine profile from Th1 to Th2 in chronic GVHD patients have been observed following treatment with ECP [133]. In contrast, the impaired immunity in L-CTCL has been attributed to the over-production of Th2 cytokines secreted by the malignant T cell clone(s) and down-regulation of Th1 and cytotoxic responses [134]. Moreover, the low baseline mDCs and pDCs found in SS patients are associated with increased tumor burdens and advanced stages of L-CTCL [68]. Others have shown that the clinical and hematological Improvements are associated with a shift from Th2 to IL-12 and Th1 response in SS patients after ECP treatment [108]. We therefore postulated that the paradoxical effects of ECP in L-CTCL and GVHD might be explained if ECP were to modulate DC subpopulations in both diseases.

In this prospective study, we studied the prevalence of mDCs and pDCs in peripheral blood by flow cytometry in 18 L-CTCL patients and 11 GVHD patients during ECP treatment. HLA-DR (MHC class II) and co-stimulatory/co-inhibitory molecules were assessed by flow cytometry and quantitative real-time PCR. We conclude that ECP modulates DC subpopulations' numbers, ratios, and expression of CSSMs. The correction of DC defects and balancing of DCs

towards a more normal status may contribute to the clinical efficacy of ECP in L-CTCL and GVHD.

5.2. Patients & Methods

Please refer to Chapter 7.

5.3. Results

Low numbers of DC subpopulations in GVHD and L-CTCL patients increase after ECP

Since defective blood DCs in L-CTCL and GVHD patients are thought to contribute to impaired immunity [68,135], we first determined the prevalence of $Lin^{-}HLA\text{-}DR^{+}CD11c^{+}$ mDC and $Lin^{-}HLA\text{-}DR^{+}CD123^{+}$ pDC in L-CTCL and GVHD patients at baseline and after ECP treatment.

When compared to the numbers of mDC (0.64±0.15%) and pDC (0.68±0.50%) from normal donors (ND), GVHD patients had significantly lower numbers of both mDCs (0.10±0.11%, $p<0.05$) and pDCs (0.08±0.12%, $p<0.01$) at baseline (**Table 5.1** & **Figure 5.1A)**. In contrast, DC numbers in L-CTCL patients were much more heterogeneous at baseline: 12 of 18 (66.7%) L-CTCL patients had lower than normal mDCs (0.24±0.16%, $p<0.01$) and 10 of 18 (55.6%) had lower than normal pDCs (10/18, 0.13±0.05%, $p<0.01$) (**Table 5.2**). Those L-CTCL patients with low pDCs also had low numbers of mDCs.

One third of L-CTCL patients had normal DC counts (5/18, 27.8%, mDCs; 7/18, 38.9%, pDCs). Our data suggest that loss of mDCs and pDCs is profound in GVHD patients, and commonly occurs in L-CTCL patients.

Patient #17 with a T-reg phenotype, reported elsewhere [136], had high mDC and normal pDC numbers. Clinically his skin looked normal but contained on biopsy atypical lymphocytes with epidermotropism consistent with MF. We hypothesized that the T-reg phenotype may suppress the inflammatory response causing characteristic MF erythema and scaling. Patient #4 with high pDCs and normal count mDCs had the lowest tumor burden (20.6% $CD4^{+}CD26^{-}$ circulating T cells) among all patients.

After ECP treatment, there was a trend towards increasing numbers of mDCs and pDCs over 1-6 month period in 6 GVHD patients (**Figure 5.1B, Figure 5.2B&D**). Similarly, 8 of 12 L-CTCL patients (66.7%) with low mDCs at baseline showed an increase in mDC numbers over 6 months of ECP treatment (**Figure 5.1C, Figure 5.2A**). Four of 5 (80.0%) L-CTCL patients with normal mDC numbers before ECP also showed an increased in mDC numbers with therapy. Patient#17 with a T-reg phenotype had high mDCs before ECP which increased even more after treatment. On the other hand, 7 of 18 (38.9%) L-CTCL patients had increased pDCs and 8 patients had decreased pDCs (44.4%) (**Figure 5.1C, Figure 5.2C**). In half of the L-CTCL patients with low pDC numbers before ECP, pDC numbers increased at 3 and 6 months after ECP (5 of 10, 50.0%). Four of 7 (57.1%) L-CTCL patients with normal baseline pDC numbers had decreased numbers during therapy. Patient #4 with high pDCs before ECP had lower numbers after ECP. Overall, in L-CTCL patients, the average mDC number was significantly increased at 6 months after ECP ($p = 0.0272$) and the average DC number was marginally lower at 3 months after ECP

compared to baseline (p = 0.0944) (**Table 5.3**). Our results suggest that after ECP, in GVHD patients, there is a trend towards increasing numbers of both mDCs and pDCs, whereas in most of L-CTCL patients there was a selective increase of mDCs and heterogeneous changes in the pDC numbers.

Table 5.1. Percentage, ratio, and HLA-DR expressions on mDCs and pDCs in peripheral blood of L-CTCL and GVHD patients

	Percentages (%)		Ratio		HLA-DR expression (MFI)†	
	mDC	pDC	mDC/pDC	pDC/mDC	mDC	pDC
ND (n=3)	0.64 ± 0.15	0.68 ± 0.50	1.34 ± 1.26	1.35 ± 1.08	3956.41 ± 1398.29	1860.10 ± 602.57
GVHD (n=10)	0.1 ± 0.11*	0.08 ± 0.12**	5.66 ± 4.55*	0.46 ± 0.52*	2006.65 ± 1157.27**	1466.11 ± 674.18
L-CTCL (n=18)	0.41 ± 0.32*	0.39 ± 0.48	1.39 ± 0.94	1.05 ± 0.79	1919.62 ± 965.76**	1460.78 ± 609.11

†MFI: mean fluorescence intensity; ND: normal donors;* p<0.05; ** p<0.01

Table 5.2. The numbers, ratios, and HLA-DR expression on mDCs and pDCs in L-CTCL patients

	Percentages (%)		Ratio		HLA-DR expression (MFI)†	
	mDC	pDC	mDC/pDC	pDC/mDC	mDC	pDC
L-CTCL-High						
No. of patients	1	1	3	3	0	0
value	1.33	2.12	3.04±0.58	2.68±0.18	-	-
L-CTCL–Normal						
No. of patients	5	7	15	14	2	9
Value	0.65±0.08	0.50±0.16	1.06±0.57	0.76±0.27	3851.68 ±9.19	2007.42 ±498.81
L-CTCL-Low						
No. of patients	12	10	0	1	16	9
Value	0.24±0.16	0.13±0.05	-	0.27	1678.11 ±702.52	984.16 ±199.66

L-CTCL–High: L-CTCL patients with higher than normal levels; L-CTCL–Normal: L-CTCL patients with normal range levels; L-CTCL–Low: L-CTCL patients with lower than normal levels; †MFI: mean fluorescence intensity

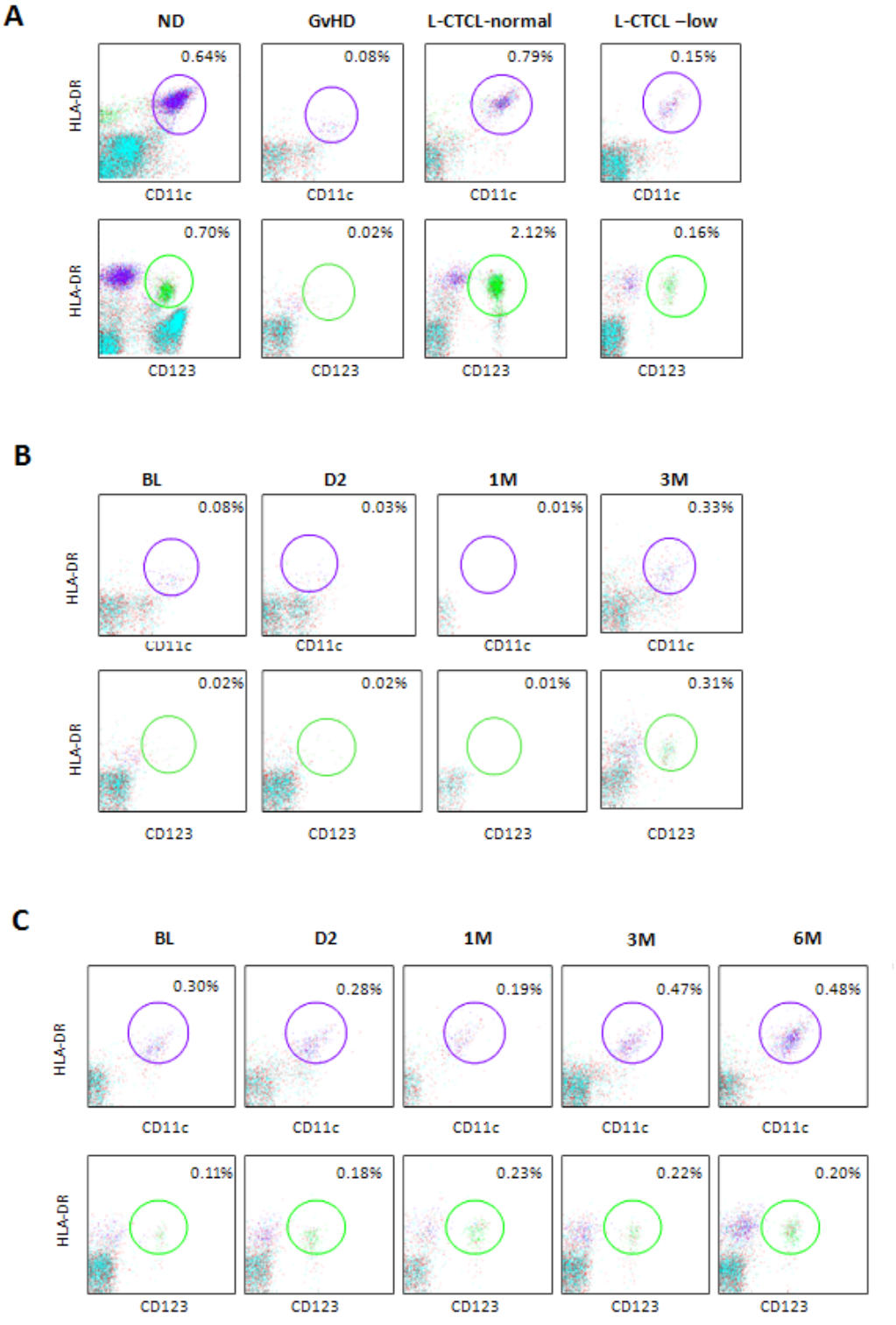


Figure 5.1

Figure 5.1. Flow cytometry analysis of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs in L-CTCL and GVHD patients before and after ECP.
A) Freshly isolated PBMCs from normal donors (ND), L-CTCL patients, and GVHD patients were stained with 4-Color Dendritic Value Bundle kit, and $Lin^-HLA\text{-}DR^+CD11c^+$ mDC populations (upper panel, circled purple portions) and $Lin^-HLA\text{-}DR^+CD123^+$ pDC populations (lower panel, circled green portions) were gated with Becton-Dickinson LSR II (BD Biosciences, San Jose, CA), and analyzed in FCS Express Version 3 (DeNovo software, Los Angeles, CA). The representative flow plots from ND, GVHD patients, and L-CTCL patients with normal (L-CTCL-Normal) and low (L-CTCL-Low) counts were shown.
B) The representative flow plots of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs (upper panel) and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs (lower panel) were presented from GVHD patient #3 at baseline (BL), Day 2, 1M, and 3M after ECP.
C) The representative flow plots of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs (upper panel) and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs (lower panel) were from L-CTCL Patient #11 at BL, Day 2, 1M, 3M, and 6M after ECP.

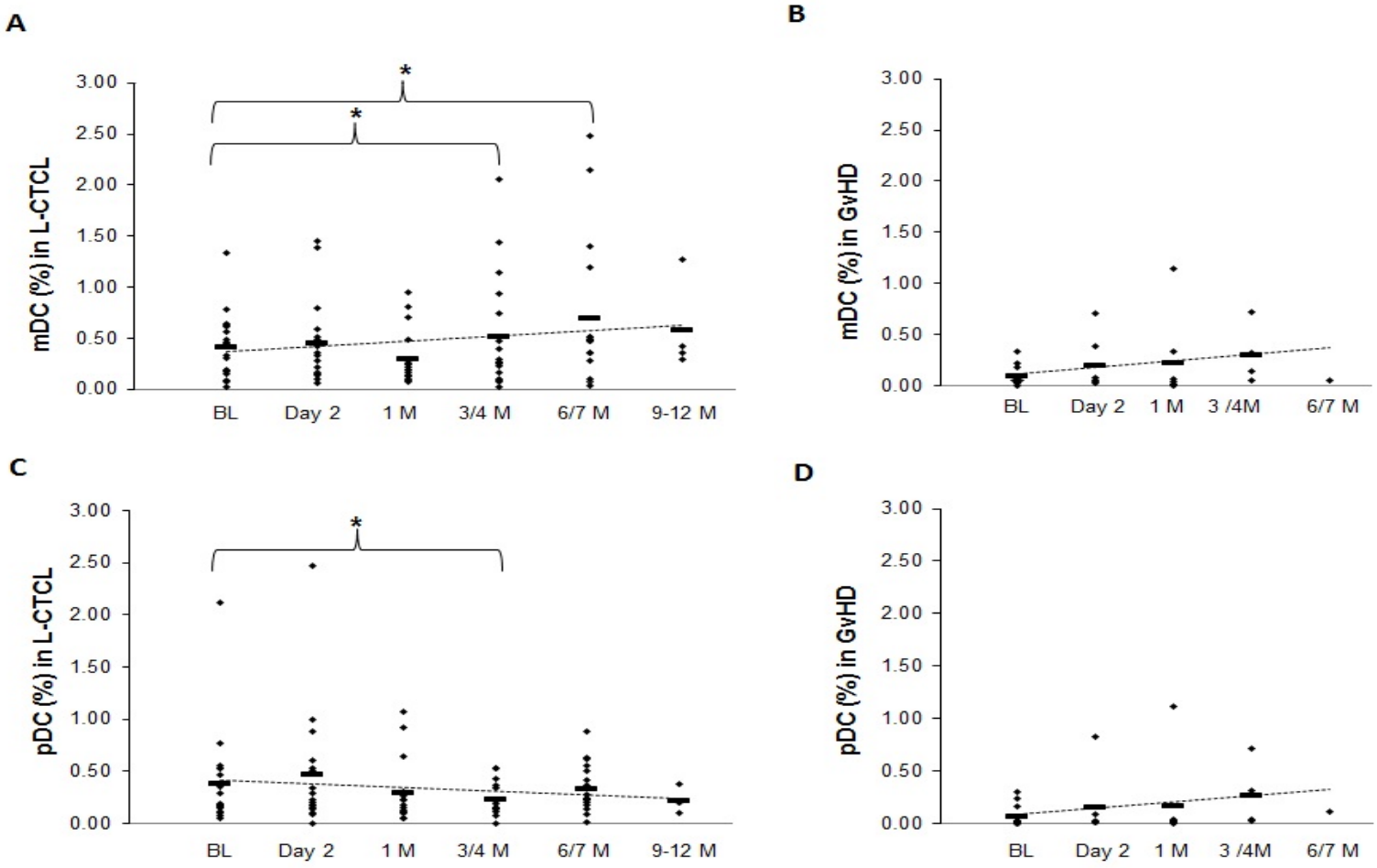


Figure 5.2

Figure 5.2. Changes in numbers of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs in GVHD and L-CTCL patients before and after ECP. Freshly isolated PBMCs from L-CTCL patients and GVHD patients were stained using 4-Color Dendritic Value Bundle kit and analyzed by flow cytometry for $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs.

- **A)** Percentages of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs of PBMCs in L-CTCL patients at BL, Day 2, 1M, 3/4 M, 6/7 M, and 9-12M after ECP.
- **B)** Percentages of $Lin^-HLA\text{-}DR^+CD11c^+$ mDCs of PBMCs in GVHD patients at BL, Day 2, 1M, 3/4 M, and 6/7 M after ECP.
- **C)** Percentages of $Lin^-HLA\text{-}DR^+CD123^+$ pDCs of PBMCs in L-CTCL patients at BL, Day 2, 1M, 3/4 M, 6/7 M, and 9-12M after ECP.
- **D)** Percentages of $Lin^-HLA\text{-}DR^+CD123^+$ pDCs of PBMCs in GVHD patients at BL, Day 2, 1M, 3/4 M, and 6/7 M after ECP. The dotted lines were linear trend lines of change. *Linear mixed model analysis, P value: <0.05.

Table 5.3. Changes in numbers, ratios, and the expressions of HLA-DR on DC subpopulations in all L-CTCL patients treated for 6 months with ECP.

	BL	Day 2	1 M	3/4 M	6 /7M	p-value
mDC(%)	0.41	0.46	0.31	0.53	0.70	**0.0272**
pDC(%)	0.39	0.48	0.30	0.24	0.34	0.0944
mDC/pDC	1.39	1.26	1.41	2.29	2.27	**0.0313**
pDC/mDC	1.05	1.10	1.12	0.80	0.81	0.4600
HLA-DR on mDC	1919.62	2185.08	1939.03	2115.20	1887.15	0.0815
HLA-DR on pDC	1460.78	1403.75	1300.20	1531.65	1348.87	**0.0380***

*: Wilcoxon analysis: the HLA-DR on pDC at 6/7M was significantly lower than baseline in non-responders.

Ratios of mDCs and pDCs in GVHD and L-CTCL patients are favorably altered after ECP

Human mDCs and pDCs induce different types of Th responses depending on environmental factors [8,137]. Skewing of cytokine profiles towards Th2 in L-CTCL and towards Th1 in GVHD are thought to associate partially with the distinct defects of mDCs and pDCs[133], [68]. Therefore, we next determined whether ratios of mDCs and pDCs were affected by ECP.

Compared to ND (1.34±1.26; 1.34±1.08), GVHD patients possessed higher mDC/pDC ratios (5.66±4.55, $p<0.05$) and lower pDC/mDC ratios (0.46±0.52, $p<0.05$, **Table 5.1**). This mDC predominance is consistent with the immunopathogenesis of GVHD as a Th1 mediated process. In contrast, 15 of 18 (83.3%) L-CTCL patients had normal ratios of mDC/pDC (1.06±0.57) and 14 of 18 (77.8%) L-CTCL patients had normal ratios of pDC/mDC (0.76±0.27) (**Table 5.2)**. Three L-CTCL patients had higher than normal mDC/pDC ratios (#11, #15 & #17; 3.04±0.58, $p<0.05$), and 3 patients (#4, #5 & #13) had higher than normal pDC/mDC ratios (2.68±0.18, $p<0.05$). Patient #17 with a T_{reg} phenotype was the only L-CTCL patient manifesting a low pDC/mDC ratio (0.27) and a high mDC/pDC ratio (3.71). Among all L-CTCL patients, responders had relatively higher mDC/pDC ratios on average compared to non-responders at baseline, but the differences were not significant (1.60±0.90 versus 0.98±0.94, $p=0.231$,**Table 5.3**). These data suggest mDC predominance in GVHD at baseline, but relatively balanced DC ratios in most L-CTCL patients despite low DC numbers.

Six GVHD patients with complete samples showed a continuous decrease in the mDC/pDC ratios during therapy (BL: 5.66±4.55; Day 2: 4.41±3.56; 1M: 2.95±3.05; 3/4 M: 2.62±3.06; and 6/7 M: 0.48, **Figure 5.3C**). Conversely, they also had increasing pDC/mDC rations (BL: 0.46±0.52; Day 2: 0.65±0.79; 1M: 0.59±0.36; 3/4 M: 0.72±0.40; and 6/7 M: 2.08, **Figure 5.3D**). This supports the use of ECP as an immunosuppressive therapy inducing Th2 immune response to dampen Th1 mediated GVHD.

In contrast, L-CTCL patients' mDC/pDC ratios increased over the course of therapy with decreased ratios of pDC/mDC, regardless of clinical responses (**Figure 5.3A &B**). These trends continued up to 9~12 months after the initial ECP treatment in 4 patients who remained on therapy. In L-CTCL patients with normal baseline mDC/pDC ratios, the ratio continued to increase in 11 of 15 (73.3%) while the ratio of pDC/mDC decreased. In 2 of 3 patients with high mDC/pDC ratios at baseline, the mDC/pDC ratios decreased over therapy and in 3 of 3 patients with high pDC/mDC ratios at baseline, their ratios decreased after ECP (3/3, 100%). Overall, over the course of ECP therapy the average mDC/pDC ratio in L-CTCL patients significantly increased from baseline to 3/4 months and 6/7 months (P value = 0.0313), and the pDC/mDC ratio decreased, but not significantly (**Table 5.3**). These data suggest that ECP may work differently in L-CTCL versus GVHD by favoring mDC predominance in L-CTCL while shifting mDC to pDC predominance in GVHD (**Figure 5.3E&F)**.

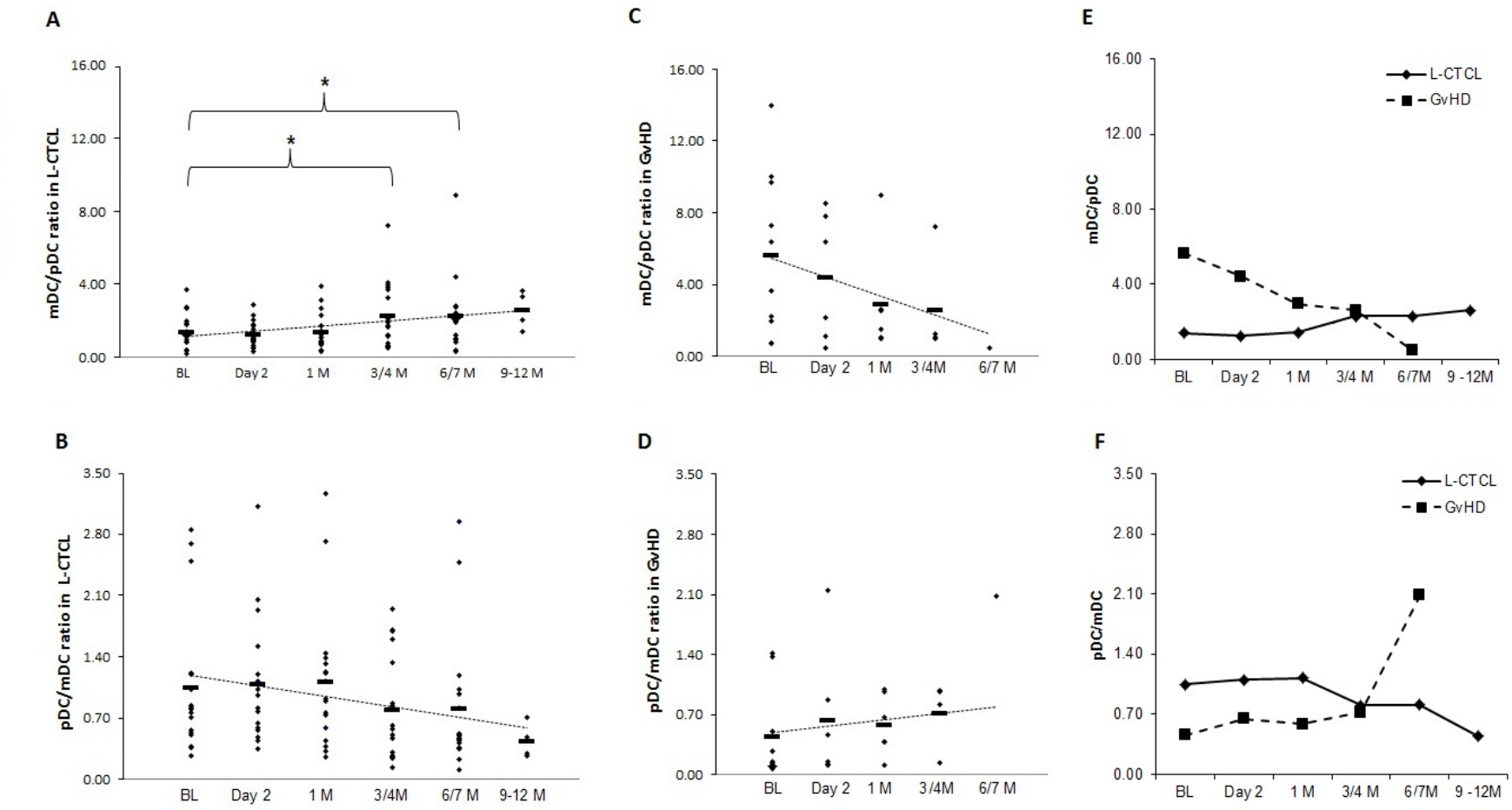


Figure 5.3

Figure 5.3. Changes in the ratios of mDCs and pDCs in GVHD and L-CTCL patients before and after ECP. Freshly isolated PBMCs from L-CTCL patients and GVHD patients were stained using 4-Color Dendritic Value Bundle kit and analyzed by flow cytometry for $Lin^{-}HLA\text{-}DR^{+}CD11c^{+}$ mDCs and $Lin^{-}HLA\text{-}DR^{+}CD123^{+}$ pDCs.

A) The ratio of mDCs versus pDCs (mDC/pDC) in L-CTCL patients at BL, Day 2, 1M, 3/4 M, 6/7 M, and 9-12M after ECP.

B) The ratio of pDCs versus mDCs (pDC/mDC) in L-CTCL patients at BL, Day 2, 1M, 3/4 M, 6/7 M, and 9-12M after ECP.

C) The ratio of mDC/pDC in GVHD patients at BL, Day 2, 1M, 3/4 M, and 6/7 M after ECP.

D) The ratio of pDC/mDC in GVHD patients at BL, Day 2, 1M, 3/4 M, and 6/7 M after ECP. The dotted lines were linear trend lines of changes. *: Linear mixed model analysis, P value: <0.05.

E) The average of mDC/pDC ratio in L-CTCL (-♦-) versus GVHD (-■-) at BL and after ECP. (**F**) The average of pDC/mDC ratio in L-CTCL (-♦-) versus GVHD (-■-) at BL and after ECP.

Low HLA-DR expression on mDCs and pDCs in GVHD and L-CTCL patients is altered after ECP

As mentioned previously, DCs undergo a maturation process through increasing HLA-DR expression [129]. To determine the maturation status, we then evaluated the levels HLA-DR expression by flow cytometry on DCs before and after ECP treatment in GVHD and L-CTCL patients' blood.

The HLA-DR expression on mDCs was significantly lower in both GVHD and L-CTCL patients at baseline (MFI: 2006.65±1157.27 and 1919.62±965.76, **Table 5.1**) than in normal donors (3956.41±1398.29, $p<0.01$). Sixteen of 18 L-CTCL patients had lower than normal HLA-DR expression on mDCs (1678.11±702.02 MFI, $p<0.01$, **Table 5.2**). HLA-DR levels on pDCs in both GVHD and L-CTCL patients were similar to each other and slightly lower (1466.11±674.18 and 1495.74±642.70) than normal donors (1860.10±602.57, $p>0.05$). Half of L-CTCL patients had lower than normal HLA-DR expression in pDC at baseline (984.16.11±199.66 MFI, $p<0.01$). These data suggest that mDCs are immature in most GVHD and L-CTCL patients, and pDCs are immature in half of L-CTCL patients before therapy.

GVHD patients maintained a low expression of HLA-DR on mDCs and pDCs after ECP treatment. In contrast, in L-CTCL patients, HLA-DR expression was up-regulated on mDCs after ECP. Ten of 16 (62.5%) L-CTCL patients with low HLA-DR expression on mDCs had higher levels at 6 months after ECP and 8 of the 10 were responders (80.0%). However, 3 of 6 non-responders had decreased HLA-DR expression on mDCs (3 down, 2 up, 1 no change). At 6

months after ECP, 7 of 9 (77.8%) with low baseline HLA-DR expressions on pDCs also were found to have increased levels with 6 of 7 responders (85.7%). But, 4 of 6 non-responders had decreased expression of HLA-DR on their pDCs. The HLA-DR expression on pDC at 6/7 months in non-responders was significantly lower than baseline (P value = 0.0380, **Table 5.3**). These results suggest that ECP not only modulates the DC numbers, but also regulates expression of HLA-DR which may reflect their maturity and ability to present antigens to T cells.

HLA-DR and costimulatory/coinhibitory molecules mRNA expressions in L-CTCL and GVHD are modulated by ECP

To further assess the maturation status of DCs, we examined mRNA expressions of HLA-DR and costimulatory/coinhibitory molecules in PBMCs isolated from blood of L-CTCL and GVHD patients. As indicated in **Figure 5A**, both L-CTCL and GVHD patients' PBMCs had low mRNA expression of HLA-DR at baseline which was consistent with our flow cytometry findings. Low mRNA levels of ICOS and ICOSL were also detected within PBMCs in both diseases but were much lower in GVHD patients. Expression of CD28, CTLA-4, CD80, and CD86 mRNA were higher than normal in L-CTCL, but lower than normal in GVHD patients. After 6 months of therapy, increased HLA-DR and decreased CTLA-4 mRNA levels were seen in both diseases (**Figure 5.4B & C**). Following ECP therapy, in GVHD patients, ICOSL levels increased by 17-fold, from -33.6 at baseline to -16.3 at 6 month (**Figure 5.4B**). Three L-CTCL patients who responded to ECP had higher levels of CD86 mRNA (responder: 1.12, versus

non-responder: -1.02; rank sum p <0.05) or lower levels of CTLA-4 (responder: 3.86, versus non-responder: 5.67, rank sum p <0.05) at baseline (**Table 5.4**). After ECP, responders also had higher levels of HLA-DR, CD80, and CD86 mRNA compared to non-responders (**Figure 5.4C & D**). These data suggest that ECP may modulate the expression of costimulatory /coinhibitory molecules which may affect DC maturation and function in L-CTCL and GVHD patients, respectively.

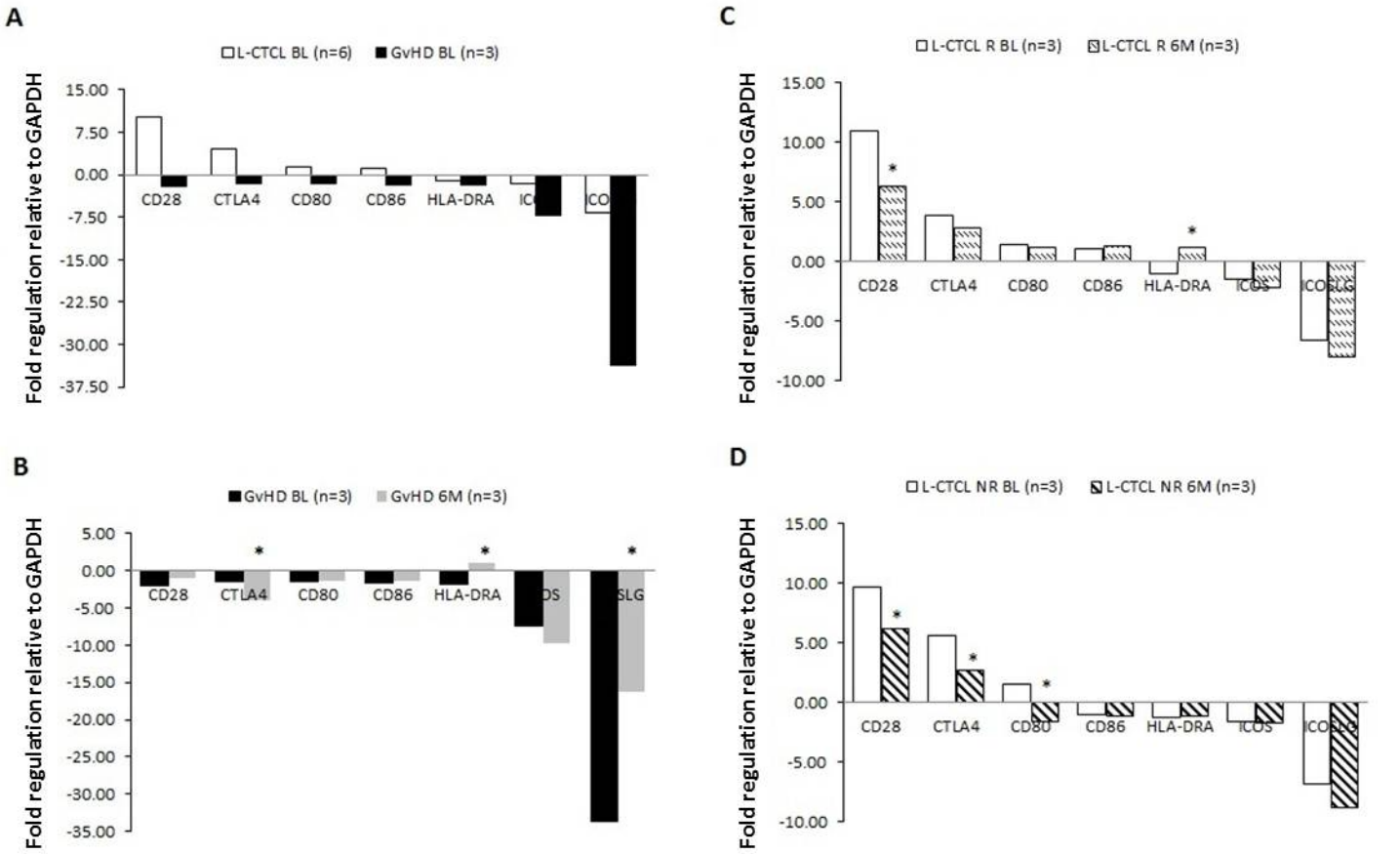


Figure 5.4

Figure 5.4. Expression of HLA-DR and costimulatory/coinhibitory molecule mRNA in GVHD and L-CTCL patients at baseline and 6 months of ECP. Total RNA was extracted from PBMCs, and the first strand cDNA was synthesized with RT^2 First Strand Kit. Relative mRNA levels of HLA-DR, CD28, CD80, CD86, CTLA-4, ICOS, and ICOSL were assessed with RT^2 Profiler Human T-cell Activation PCR Array using ABI Prism 700 Sequence Detection System. The fold changes relative to GAPDH were calculated as the levels of gene expression, and calibrated with normal donors.

- **A)** Relative fold changes at BL in L-CTCL versus GVHD patients.
- **B)** Relative fold changes at BL versus 6M after ECP in GVHD patients.
- **C)** Relative fold changes at BL versus 6 M after ECP in L-CTCL responders (R).
- **D)** Relative fold changes at baseline versus 6 M after ECP in L-CTCL non-responders (NR). *: Rank sum analysis, $p<0.05$.

Table 5.4. Comparison of baseline parameters in L-CTCL responders and non-responders

Parameters	Responders (n=12)	Non-responders (n=6)	P value
Age	66.5 (58.0 – 79.0)	71.0(54.0 – 78.0)	0.332
ECP cycles	9.4 (6.0 -15.0)*	9.2 (6.0 – 12.0)	0.889
BSA	61.9 (5.0 – 89.0)	71.1 (37.5 – 100.0)	0.315
SWAT	65.2 (5.0 – 100.0)	72.2 (47.0 – 100.0)	0.247
CD4+CD26- tumor cells (%)	82.3 (64.2 – 94.7)	66.3 (20.6 – 94.5)	**0.054**
Flow cytometry analysis			
mDC (%)	0.43 ± 0.35	0.39 ± 0.29	0.803
pDC (%)	0.30 ± 0.23	0.56 ± 0.78	0.305
mDC/pDC	1.60 ± 0.90	0.98 ± 0.94	0.231
pDC/mDC	0.89 ± 0.68	1.37 ± 0.97	0.386
HLA-DR in mDC (MFI)	1722.43 ± 917.42	2314.00 ± 1019.59	0.199
HLA-DR in pDC (MFI)	1369.81 ± 621.54	1642.73 ± 593.30	0.231
QT-PCR array (fold change)**			
HLA-DR	-1.01	-1.19	> 0.05
CD28	11.02	9.68	> 0.05
CTLA-4	3.86	5.67	**< 0.05**
CD80	1.43	1.50	> 0.05
CD86	1.12	-1.02	**< 0.05**
ICOS	-1.54	-1.55	> 0.05
ICOSL	-6.56	-6.81	> 0.05

*: Patient#8 is not included

**: relative fold change compared to normal donor. Data from 3 responders and 3 non-responders

5.4 Discussion & Conclusions

The findings of increased mDC numbers, increased mDC/pDC ratios, and up-regulation of CD80, CD86, and HLA-DR expression in two thirds of L-CTCL patients after ECP treatment suggest that ECP treatment is associated with a favorable mDC modulation in patients with L-CTCL. Upregulation of mDC and Th1 cytokines would be expected to improve the Th2 profile of the malignant T-cells. Another possibility is that reduction of malignant cells by apoptosis which would decrease levels of Th2 cytokines could also favorably affect the mDC ratio.

On the other hand, increased pDC/mDC ratios and up-regulation of ICOSL found in three fourths of GVHD patients suggest that ECP treatment in this Th1 mediated disease is associated with a favorable pDC upregulation in patients with GVHD leading to a switch from Th1 to Th2 cytokine profiles. Two thirds of L-CTCL patients and all GVHD patients had some degree of reduced levels of mDC and pDC numbers at baseline that resulted in imbalanced ratios of mDCs and pDCs. Costimulatory/coinhibitory molecules expressed in L-CTCL and GVHD in opposite directions are favorably regulated after ECP in this study. Although L-CTCL and GVHD have opposite immune-pathogenesis, following ECP therapy there are changes of DC numbers, ratios, and expression of costimulatory/coinhibitory molecules in a favorable direction in each disease. We conclude that the normalization of DC defects and balancing of DCs towards a more normal status by ECP may underlie its efficacy in L-CTCL and GVHD patients.

The impaired cellular immunity in L-CTCL patients is attributed to several factors, one of which is reduced DC populations leading to reduction of IL-12/Th1 cytokine secretion and a weakened anti-tumor response. Wyosocka et al. first reported the defect in circulating DCs in SS patients,[68] and found that profound defects of both mDCs and pDCs were correlated to high tumor burdens. Our findings are consistent with their study in that the majority of our L-CTCL patients with > 50% circulating $CD4^{+}CD26^{-}$ tumor cells had decreased mDCs and pDCs at baseline. Only one patient (Pt#4) with an intermediate tumor burden had normal DC counts. It was reported that pDCs synergize with mDCs in the induction of antigen-specific antitumor immune responses in an experimental melanoma mice model [138]. In this study, we found that L-CTCL patients with low numbers of pDCs also had low mDC numbers, and patients who had relatively normal pDCs tended to have normal mDC counts. Our data suggest that a synergistic interaction between mDCs and pDCs may exist in our L-CTCL patients.

After ECP treatment, the numbers of mDCs were increased in most L-CTCL patients but changes in the numbers of pDCs were less pronounced. The mDC numbers were increased significantly at 3 and 6 months after ECP with the highest numbers found at 6 months when clinical responses were most pronounced. The selective increase in mDC numbers after ECP in L-CTCL patients is hypothesized to shift DC subpopulations towards a mDC predominance. These results suggest that ECP has a sustained effect on mDCs. The source of increased mDCs after ECP treatment is unknown. They could

migrate from the bone marrow, or could have an extended life span, or could derive from tissue monocytes.

The maturation status characterized by the expression of CSSMs on DCs is critical for induction of immune responses or immune tolerance [129,130]. After ECP, apoptotic lymphocytes are phagocytosed by macrophages and DCs, and thought to send “danger signals” to the immune system. A transcriptome study of L-CTCL showed that genes associated with DC differentiation, adhesion, maturation, and activation(CD40,CD80, and DC-LAMP) were increased in ECP-treated cells *ex vivo* [139]. Our study consistently showed that CD80, CD86, and HLA-DR expression was up-regulated and CTLA-4 was down-regulated after ECP in L-CTCL patients. Different from their *ex vivo* study, our study was the first to monitor DC kinetic changes *in vivo* in patients’ circulation while they were undergoing ECP over a 6 month period. Change in mDC numbers along with up-regulation of HLA-DR and co-stimulatory molecules may enhance mDC maturation and activity, and further boost anti-tumor immune responses that are thought to be therapeutic for L-CTCL. This type of immune regulation would be expected to exacerbate patients with GVHD.

Previous studies reported that ECP modulates DC populations in patients with GVHD[139-141],[133],[142]. ECP induces a shift from mDCs to pDCs, together with a shift from a predominantly Th1 cytokine profile to a Th2 cytokine profile in GVHD patients [133]. Consistent with these *ex vivo* studies, our study found that ECP did favorably affect DC subpopulations in GVHD patients *in vivo*. Before ECP treatment, there were low numbers of both mDCs and pDCs in

GVHD patients with a severe deficiency in pDC numbers resulting in an mDC predominant status. After ECP treatment, both DC subpopulations were increased in most GVHD patients. Of note, the pDC numbers increased more than mDC numbers resulting in an increased pDC/mDC ratio or an mDC to pDC shift. This agrees with one prior study reported a decreased mDC numbers in GVHD patients after ECP [133]. Differences between our study and theirs may due to our mixed acute and chronic GVHD patient group, *in vivo* assessment, and different combinations of immunosuppressive therapies.

As mentioned before, IL-3 stimulation of pDCs induces a Th2 response, and could also induce T-reg [8]. A recent study reported that monocyte-derived DCs in co-culture with ECP-treated PBMCs produce increased quantities of IL-10, and the co-cultured DCs expressed reduced CD40 and CD86 following stimulation with LPS [142]. Our study did not show much change in CD86 expression, but we did find significant upregulation of ICOSL mRNA expression in PBMCs of GVHD patients. The expression of ICOSL on pDCs is critical for inducing IL-10-producing T_{reg} and immune tolerance [8],[143]. Thus, our results suggest that ECP could induce a shift from mDCs to pDCs in GVHD patients *in vivo*, and this change along with an increase of ICOSL may enhance pDC activity, and further promote a Th2 response and immune tolerance to allo- or auto-reactivity.

The extent of immune stimulation depends not only on the maturation state of DCs, but also on the local cytokine milieu. In a pro-inflammatory environment, DCs undergo maturation and the effector immune responses are

amplified. In contrast, in the absence of suitable inflammation, immune tolerance develops toward the acquired antigen. Clinical and hematological improvements after ECP in SS patients are associated with a shift from Th2 phenotype to an increase of IL-12/Th1 phenotype [108]. A shift of the cytokine profile from Th1 to Th2 by ECP in GVHD have already been observed [133]. Therefore, we hypothesize that in L-CTCL, ECP treatment not only recruits more circulating mDCs, but also creates a pro-inflammatory environment for DCs to mature and be activated. In the presence of the costimulatory molecules, CD80 and CD86, these DCs are capable of presenting tumor antigens from phagocytosed tumor cells in the context of MHC molecules and thus initiating favorable cellular immune responses [144]. In contrast, in GVHD, more pDCs are recruited to the peripheral blood favoring the expansion of Th2 clones and secretion of IL-4 and IL-10. When DCs present antigens to T cells in the absence of the costimulatory molecules, T-cell anergy or tolerance to transplants or auto-tissues may arise [130,133,145]. Our working model is that ECP restores impaired immunity in these two diseases by multiple effects including elimination of the pathologic T-cell populations (Th2 clone in L-CTCL and Th1 clone in GVHD), modulation of the cytokine milieu, and correction of DC defects (**Figure 5.5**).

In this study, we found high variability in DC numbers, ratios, and HLA-DR and costimulatory/coinhibitory molecule expression among L-CTCL patients at baseline, suggesting a heterogeneous, complex immunopathogenesis.

In summary, this translational research study suggests that ECP alone or ECP with immunomodulatory therapy favorably modulate DC subpopulations *in*

vivo in both L-CTCL and GVHD patients. This modulation could underlie and explain the favorable effects of ECP for both diseases. Clinical studies investigating the effect of IL-12 and DC vaccine in L-CTCL patients depend on enhancing DC functions have also been successful [146-148]. Strategies to activate DCs are attractive to enhance insufficient immune responses in patients with infectious diseases and cancers or can be used as well to attenuate excessive immune responses in allergy, autoimmunity and transplantation [149]. This study for the first time assesses DC changes in both L-CTCL and GVHD patients groups in parallel. It provides further insight into how ECP could effectively treat two immunological diseases with nearly opposite immunopathogenesis.

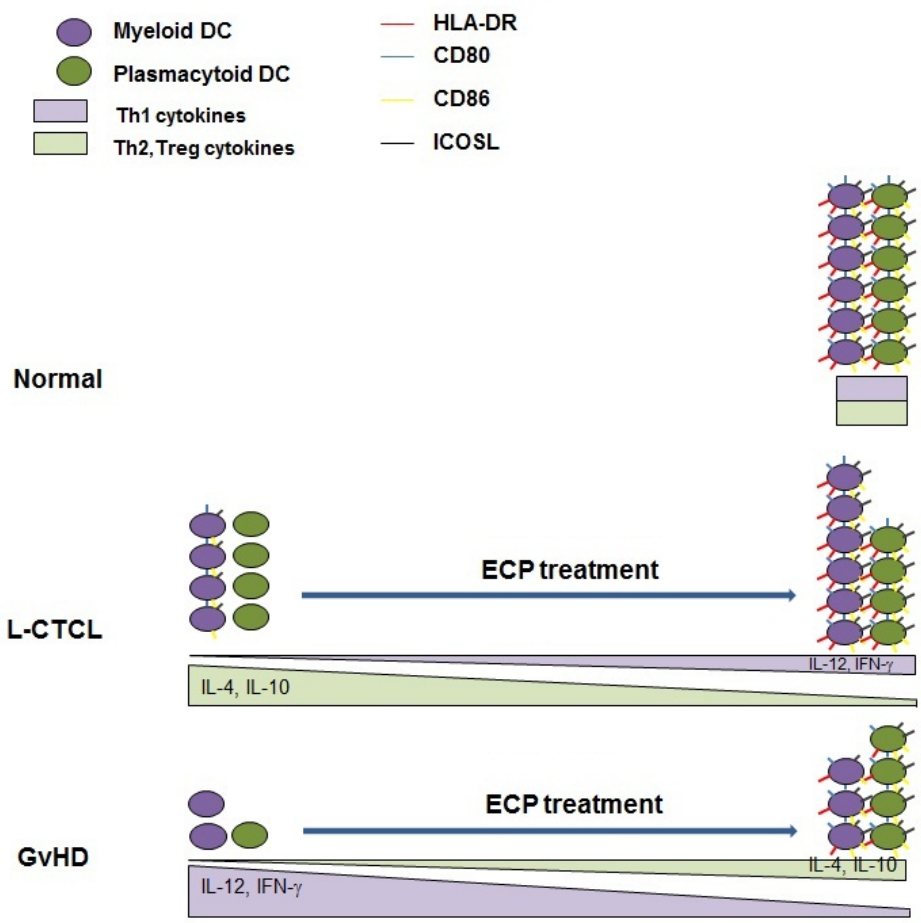


Figure 5.5

Figure 5.5. The working model of immunologic mechanism of ECP action on L-CTCL and GVHD patients. Along with the shift of cytokine milieu; ECP could effectively balance DC subpopulation towards a more normal status in patients with L-CTCL and GVHD. In L-CTCL, ECP treatment not only recruits more circulating mDCs, but also creates a pro-inflammatory environment for DCs to mature and be activated. In the presence of the costimulatory molecules, CD80 and CD86, these DCs are capable of presenting tumor antigens from phagocytosed tumor cells in the context of MHC molecules and thus initiating favorable cellular immune responses. In contrast, in GVHD, more pDCs are recruited to the peripheral blood favoring the expansion of Th2 clones and secretion of IL-4 and IL-10. When DCs present antigens to T cells in the absence of the costimulatory molecules, T-cell anergy or tolerance to transplants or auto-tissues may arise.

CHAPTER 6

SUMMARY & DISCUSSION

6.1 Summary of Chapter 2: Efficacies of extracorporeal photopheresis in patients with L-CTCL and GVHD in this study.

In this study, ECP+BRM was clinically effective in L-CTCL and GVHD patients as measured by regression of disease in skin or blood. We confirmed the published efficacy of ECP for the treatment of patients with both L-CTCL and GVHD. Our overall response rate was 66.7% in 12 of 18 patients with L-CTCL on ECP+BRM and 54.5% in 6 of 11 GVHD patients treated with ECP plus immunosuppressive therapies. Of interest, 4 of 6 (66.7%) L-CTCL patients who received ECP alone and 8 of 12 (66.7%) who received ECP with immunomodulatory therapy achieved the same overall clinical response rate.

6.2 Summary of Chapter 3: Effect of extracorporeal photopheresis on regulatory T cells in L-CTCL

In our study, we found in L-CTCL that $CD4^+CD26^-$ tumor cells correlated with percentages of $CD4^+Foxp3^+$ and $CD4^+$ $Foxp3^+CD25^-$ T cells, suggesting that tumors cell may have the T-reg phenotype. At baseline, seven L-CTCL patients had high $CD4^+Foxp3^+$ T cells (L-CTCL-High, 85.5±20.8%) and 2 patients had low $CD4^+Foxp3^+$ T cells (L-CTCL-Low, 1.4±1.1%). Six of the 7 L-CTCL-High patients had similar percentages of $CD4^+Foxp3^+$ T cells and $CD4^+CD26^-$ tumor T cells. After ECP treatment at 3 months, we found that the percentages of tumor cells as well as percentages of $Foxp3^+$ T cells decreased in L-CTCL-High patients.

The L-CTCL-Low patients initially had low $CD4^+Foxp3^+$ T cells which remained low after treatment. In the GVHD patients, there were 5 patients with high $CD4^+Foxp3^+$ T-regs (GVHD-High, 90.5±5.7%), 1 Low $CD4^+Foxp3^+$ T-regs (GVHD-Low, 0.07%), and 1 normal $CD4^+Foxp3^+$ T-regs (GVHD-normal, 10.3%). In GVHD-High patients, there was a decrease of $CD4^+Foxp3^+$ and $CD4^+Foxp3^+CD25^-$ T cells by increase in $CD4^+Foxp3^+CD25^+$ T cells and 4/5 patients responded.

This data suggest that ECP may work better in L-CTCL patients with a tumor $CD4^+Foxp3^+$ T-reg phenotype compared to patients whose tumor cells are not of the T-reg phenotype. In GVHD patients, the high $CD4^+Foxp3^+$ T cells may be remanent of intial disease. This suggests that Foxp3 is a possible molecule for development of targeted therapies.

6.3 Summary of Chapter 4: Effect of extracorporeal photopheresis on cytotoxic T lymphocytes in L-CTCL and GVHD patients

Since the numbers and percentages of $CD8^+$ T cells are extremely low in L-CTCL patients and cellular cytotoxicity is impaired, $CD8^+$ cytotoxic T lymphocytes (CTLs) are hypothesized to also be low and impaired. As expected, we found abnormally low levels of $CD8^+$, $CD8^+CD69^+$, and $CD8^+IFN\text{-}\gamma^+$ T cells in 6 available L-CTCL patients prior to treatment. After treatment, $CD8^+$, $CD8^+CD69^+$, and $CD8^+IFN\text{-}\gamma^+$ T cell numbers increased. In addition, $CD8^+IFN\text{-}\gamma^+$ T cells increased in number after 1 month of treatment in patients whose

peripheral blood showed decreases in $CD4^+CD26^-$ cells tumor cells, suggesting that cellular cytoxicity was improving.

In GVHD patients, it was expected that $CD8^+$ T cells were high at baseline because GVHD is mediated by $CD8^+$ cytotoxic cells. After treatment, $CD8^+$, $CD8^+CD69^+$, and $CD8^+IFN\text{-}\gamma^+$ T cells decreased. The decreases in the $CD8^+$ T cells show that ECP is favorably down-regulating cellular mediated immunity against host targets in GVHD patients.

6.4 Summary of Chapter 5: Effect of extracorporeal photopheresis on dendritic cells in L-CTCL and GVHD patients

We confirmed the DC defect in L-CTCL and GVHD patients as percentages and numbers of mDCs and pDCs were abnormally low prior to treatment. After treatment, the mDCs and pDCs increased suggesting that the DC compartment was defective but not completely impaired. In addition to the increase in mDCs and pDCs, HLA-DR, and CSSMs also increased on mDCs and pDCs in some L-CTCL and GVHD patients. The increase of HLA-DR and CSSMs suggest that the DCs are maturing and becoming activated. When ratios of mDCs to pDCs were examined after treatment, it was noted that mDCs predominated in L-CTCL patients whereas pDCs predominated in GVHD patients. These findings suggest that ECP may work differently in L-CTCL and GVHD patients. Depending on the environment, DCs differentiate into subsets that elicit appropriate immune responses. ECP may work by modulating the DC compartment towards predominance of mDCs that induce Th1 responses in L-

CTCL patients and pDCs that induce an immunosuppressive Th2 response in GVHD patients, explaining how one treatment may improve both diseases. Further *in vitro* studies to test mDCs and pDCs functions and differentiation in the L-CTCL and GVHD setting are needed.

6.5 Overall Discussion

From this study, we confirmed that ECP is clinically efficacious in L-CTCL and GVHD patients. At the cellular level, mDCs, pDCs, and CTLs were modulated in L-CTCL and GVHD patients after ECP+BRM treatment. In L-CTCL patients, mDCs and CTLs increased whereas in GVHD patients, pDCs increased while CTLs decreased. These findings support our hypothesis and indicate that the mechanism of ECP action is different in L-CTCL and GVHD patients.
An unexpected finding was that L-CTCL patients fell into two separate groups based on $CD4^{+}Foxp3^{+}$ T cells. Six of 9 patients had >80% of $Foxp3^{+}$ T-regs which correlated with the percentages of tumor cells while the other 3 patients had tumors that did not correlate with T-regs. All 6 patients with tumor T-regs responded whereas the other 3 patients without tumor T-regs did not respond. ECP+BRM appears to be more effective in the patients with $Foxp3^{+}$ malignant phenotype cells compared to the patients without the $Foxp3^{+}$ malignant phenotype.

We are the first to identify these cellular changes in patients *in vivo* and compare between L-CTCL patients versus GVHD patients in relation to clinical response. These data impact not only the clinical aspects of L-CTCL and GVHD,

but also translate into a better understanding of L-CTCL and GVHD pathology for advancement of the fields. The increase of mDCs and pDCs in L-CTCL and GVHD, respectively, indicate that the differential DC modulation with a positive clinical response suggest that these DCs are capable of improving disease according to the immune milieu. The identification of these specific DC subsets in these patients shows that the diseased immune system is capable of returning to baseline and provides information on what cellular pathway ECP is beneficially modulating. The induction of mDCs and CTLs with a positive clinical response in L-CTCL patients suggest that ECP is beneficially working on these cells. The enhancement of these cells can possibly lead to complete responses or remission. Addition of biological modifiers such as cytokines, IFN-γ or IL-12, with ECP can target mDCs and/or augment CTLs in L-CTCL patients. Because we observed cellular changes at specific timepoints, addition of biological modifiers at timepoints of change may enhance the later response. For example, we observed that CTLs were immediately increased after 24 hours of ECP and increased the highest at 1 month then decreased at 3 and 6 months of treatment in L-CTCL patients. The early increase of CTLs may need to be sustained at later timepoints to achieve complete responses. Thus, addition of biological modifiers to enhance CTLs at 1 month after initial ECP could be beneficial.

After the 1 month timepoint, CTLs decreased in L-CTCL patients. The decrease of CTLs may be attributed to T-reg suppression. Therefore, to enhance CTLs, T-regs could be down-regulated with anti-Foxp3 therapy or anti-PD1 antibody which has been shown to target T-regs and mask the T-reg

inhibition of CTLs in melanoma patients [150]. Conversely, in GVHD patients, efforts to increase T-regs and decrease CTLs would be beneficial. Addition of anti-CTLA-4 antibody with ECP may expand $Foxp3^+$ T-regs to suppress the unwanted CTLs [151].

Surprisingly, we found a subset of L-CTCL patients with a tumor T-reg population. This finding shifts the paradigm thought that tumors are not T-regs in L-CTCL patients. Tumor T-reg patients were also found to be better responders to ECP compared to patients without a tumor T-reg population.

Since the tumor T-reg patients achieved a better response rate compared to non-T-reg patients, these findings suggest that ECP is a good treatment modality in this subset of patients whereby cells may be more sensitive to ECP. We are the first to identify that tumor T-reg patients are better responders to ECP compared to non-tumor T-reg patients.

The interesting finding that T-regs are tumor cells in a subset of L-CTCL patients can help to better diagnose, treat, and improve quality of life. Since Foxp3 is currently a unique identifier for T-regs, it can be used as a biomarker. Use of Foxp3 in diagnostic tests should be incorporated with standard tests to identify these unique patients to tailor the treatment regime. By utilizing Foxp3 during diagnosis and tracking response by Foxp3, can improve the outcome of these tumor T-reg patients.

To address the intriguing question of “Is the mechanism of ECP same or different in L-CTCL versus GVHD patients, we conclude that the mechanism(s) of ECP action is different in GVHD versus L-CTCL patients. Although ECP

modulated the DCs in both disorders, the favorable increases of mDCs in L-CTCL patients and pDCs in GVHD patients suggest that these DC subsets may be orchestrating opposite T cell responses which is dependent on the disease milieu.

In GVHD patients, the mechanism of ECP action may be through immunoregulation by regression of cytotoxic $CD8^+$ T cells and balancing of immunity. The increase of pDCs after ECP treatment in our study patients suggest that the pDCs may be orchestrating an opposite T cell response or Th2 response to balance out the pathogenic Th1 immunity. The decrease of cytotoxic $CD8^+$ T cells in the GVHD patients supports this mechanism and increases of T-regs which suppress $CD8^+$ T cells may be occurring.

In contrast, in L-CTCL patients, the mechanism of ECP may be through immunostimulation by improved anti-tumor cyotoxicity. The vaccination theory of ECP action proposed that ECP induces maturation of monocyte-derived DCs that injest ECP treated tumor cells, present tumor antigen to $CD8^+$ T cells, and induces cytotoxic T lymphocytes to attack tumor cells. While this mechanism may be occurring in these L-CTCL patients undergoing ECP treatment, we found that the $CD8^+$ lymphocyte numbers and percentages remained low despite the increase after ECP. However, the lack of dramatic increase in $CD8^+$ T cell numbers does not rule out a role for $CD8^+$ T cells in function or cytotoxicity against tumor cells after ECP treatment. Further studies are necessary.

Because we found a unique subset of L-CTCL patients with malignant T-regs and they responded better to ECP+BRM compared to the patient cohort

without tumor T-regs, we hypothesized that the mechanism of action through the regression of malignant T-regs could be not only through the vaccination mechanism, but also T-reg plasticity and increased sensitivity of tumor T-regs to ECP. Since T-regs in the normal state have developmental plasticity whereby they can change into other Th17 or Th cells in the appropriate setting [152], the tumor T-reg decrease may be due to the increase of other Th cell types or the tumor T-regs may be changing into other types of cells. It would be interesting to determine the origin of these tumor T-reg cells. Whether the tumor cells may have evolved into T-regs or the T-regs became malignant could be a subject of future study for therapeutic potential.

Another avenue for potential therapy for L-CTCL patients rely on the characteristics of these tumor T-regs. These tumor T-regs may have more sensitivity towards ECP treatment which may be due to their unique cellular and molecular characteristics. The tumor T-regs may provide a better anti-tumor response because of the increased expression of certain antigens that can mediate a strong anti-tumor cyototoxic attack. The expression of Foxp3 provides a biomarker and possible target for therapy in this subset of L-CTCL patients. Foxp3 can be used to diagnose patients as well as serve as a marker of clinical response to therapies. Exactly what features make tumor T-regs more sensitive to treatment may need to be further investigated.

Thus, we propose that the mechanism of ECP action in L-CTCL and GVHD patients are different and depend on the immune milieu. Although DCs are modulated in both diseases, the T cell responses are different. The

mechanism of ECP action in L-CTCL patients may be through immunostimulation by induction of cytotoxic $CD8^+$ T cells whereas the mechanism of ECP action in GVHD patients may be through immunosuppression by regression of cytotoxic $CD8^+$ T cells and possible induction of T-regs **(Figure 6.1)**. Future studies are needed to monitor T-regs in GVHD patients undergoing treatment, to investigate the cytokine milieu before and after ECP, and to discover specific antigens that may improve responses to ECP for the ultimate goal of improving the lives of patients treated with ECP.

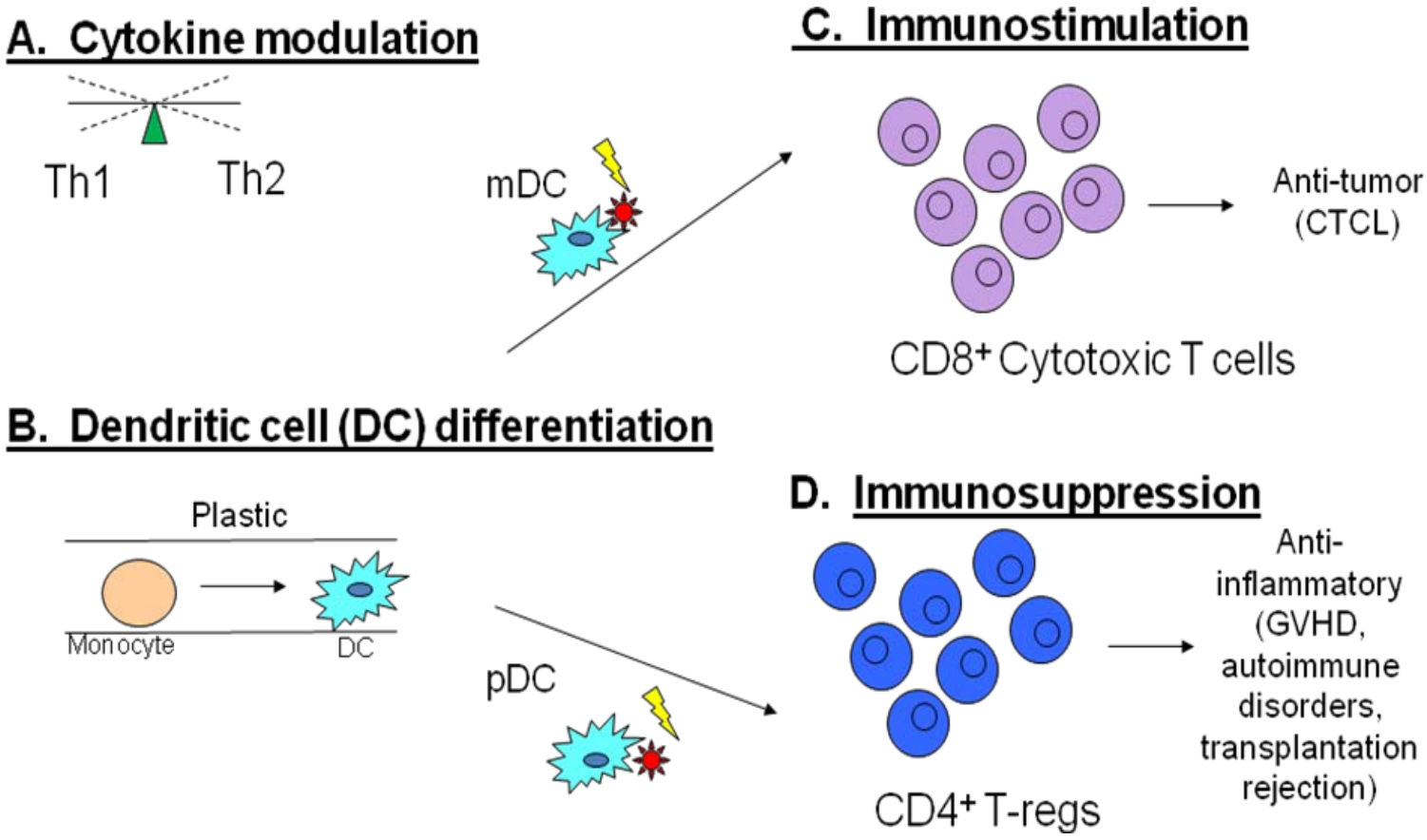


Figure 6.1

Figure 6.1. Proposed immunological mechanisms of ECP in L-CTCL and GVHD patients.

A) ECP can modulate cytokines by shifting the Th1 cytokines dominance to Th2 cytokines in clinically responsive GVHD patients [153]. Improvement of CTCL is associated with shift of Th2 cytokines predominance to Th1 cytokines [108].

B) Dendritic cells are modulated by ECP. DCs can differentiate from monocytes into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) within the plastic treatment apparatus depending on immune milieu. The differences in immune milieu in CTCL and GVHD induce mDCs and pDCs, respectively.

C) MDCs induce an immunostimulatory T cell response through induction of $CD8^+$ cytotoxic T lymphocytes for anti-tumor immunity in CTCL patients.

D) PDCs induce an immunosuppressive T cell response through induction of $CD4^+$ regulatory T cells for anti-inflammatory reactions to ameliorate GVHD, autoimmune disorders, and transplantation rejection.

CHAPTER 7

PATIENTS AND METHODS

7.1 Study design and patients.

Patients with L-CTCL and GVHD starting ECP treatment signed written informed consents to enroll in this study between 2007 and 2010. This study was conducted according to the Declaration of Helsinki Principles and the study protocol was approved by the University of Texas M D Anderson Cancer Center Institutional Review Board. The ISCL/EORTC revised criteria for MF and SS was used for staging and classification.[154] MF/SS patients were recruited based on blood involvement or >50% $CD4^{+}CD26^{-}$ circulating Sézary cells. All patients were starting ECP for the first time and were treated with the UVAR XTS photopheresis system (Therakos, Inc. Raritan, NJ). ECP was administered over 2 consecutive days every 2 to 4 weeks per cycle for L-CTCL patients and weekly for GVHD patients. Fresh blood was collected before ECP (baseline) and after ECP on Day 2 (D2), 1 month (1M), 3 months (3M), and 6 months (6M). Buffy coats from normal donors (ND) were obtained from the Department of Transfusion Medicine at our institution.

7.2 Clinical Responses.

Clinical responses for L-CTCL patients were assessed at 6 months after ECP by one expert clinician (MD). Extent of disease in each body surface area (BSA) was calculated and multiplied by 2 for plaques and multiplied by 4 for tumors to obtain the modified skin-weighted assessment (mSWAT).[154] The mSWAT was performed for each patient at enrollment and every visit. Peripheral

blood $CD4^+CD26^-$ or $CD4^+CD7^-$ T-cells were identified as the circulating tumor cells by flow cytometry [121,148,155,156] The clinical responses for L-CTCL patients were based on the percentage changes of skin scores and circulating tumor cells as described in other clinical trials with modification [154,157-159]. The changes in SWAT were calculated as [(SWAT score from baseline – SWAT score at 6 months)/ SWAT score from baseline × 100]. The tumor cell changes were calculated as [($CD4^+CD26^-$ circulating T cells from baseline – $CD4^+CD26^-$ circulating T cells at 6 months)/ $CD4^+CD26^-$ circulating T cells from baseline × 100] [157-159]. Patients with complete responses (CR) were ones who cleared all skin or blood involvement. Partial responses (PR) were defined as >50% improvement in skin or blood involvement. Minor responses (MR) were defined as 25%-50% skin or blood improvement. Patients with CR, PR and MR were grouped as responders. Non-responders included patients with stable disease (SD) whose skin or blood changes were within 25% from baseline, and patients with progressive disease (PD) who had 25% worsening skin or blood involvements from baseline. The clinical responses for GVHD patients were assessed by an experienced hematologist (AA), and the detailed response criteria for GVHD patients were referred to those used by our institution.[160]

7.3 Blood Samples.

Peripheral mononuclear cells (PBMCs) were isolated by density gradient centrifugation from whole blood. Freshly isolated PBMCs were characterized for myeloid and plasmacytoid dendritic cells by flow cytometry. Slowly

cryopreseved PBMCS were analyzed for $CD4^+Foxp3^+$ T cell subsets and $CD8^+$ T cell subsets by immunofluorescence flow cytometry.

7.4 Immunofluorescence and intracellular flow cytometry analysis of $Foxp3^+$ $CD4^+$ T lymphocyte subsets.

Thawed PBMCs were incubated with CD3-APC/Cy7 (Biolegend), CD4-FITC (BDBiosciences), and CD25-PE/Cy7 (Biolegend) immunofluorescence antibodies on ice for 30 minutes. Cells were washed with FACS Buffer (Miltenyi Biotech) twice prior to intracellular Foxp3 detection. A fixation/permeabilization buffer from the Foxp3 detection kit (eBioscience) was incubated with PBMC for 35 minutes at 4^0 C. Cells were then washed with a 1X perm/wash buffer (eBioscience) prior to incubation with Foxp3-PE antibody (clone PCH101) for 30 minutes at 4^0 C. After incubation, cells were washed twice with 1X perm/wash buffer. Reference counting beads were added to each sample (Spherotech). Cells were run on a Gallios flow cytometer (Beckman Coulter) and analyzed on Kaluza (Beckman Coulter) software as per manufacturer's instructions. Percentages of $CD4^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$, $CD4^+CD25^+Foxp3^+$ T cells were calculated out of $CD4^+$ T cells. Absolute numbers were calculated on the ratio of cell counts over bead counts multiplied by the ratio of known bead numbers and volume.

7.5 Immunofluorescence and intracellular flow cytometry analysis of $CD8^+$ T cell lymphocyte subsets.

Thawed PBMC were activated with Leukocyte Activation Cocktail, with BD GolgiPlug (BDBiosciences) for 3 hours at 37^oC. After activation, the cells were

treated with the Live/Dead Fixable Dead Cell stain kit (Invitrogen) for 30 minutes at room temperature. Cells were then incubated with CD3-FITC (BD Biosciences), CD8-APC (Biolegend) immunofluorescence antibodies for 30 minutes on ice. Cells were washed with FACS Buffer (Miltenyi Biotech) twice prior to intracellular CD69 detection. A BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Bioscience) was used to fix and permeabilize PBMC for 20 minutes at 4^0 C. Cells were then washed with a 1X perm/wash buffer (BD Bioscience) twice prior to incubation with CD69-PerCyPCy 5.5 (Biolegend) antibody for 30 minutes at 4^0 C. After incubation, cells were washed twice with 1X perm/wash buffer. Cells were then washed with a 1X perm/wash buffer (BD Bioscience) twice prior to incubation with IFN-γ –PE (R&D Systems) antibody for 30 minutes at 4^0 C. Reference counting beads (Spherotech) were added prior to flow cytometry. Cells were run on a Gallios flow cytometer (Beckman Coulter) and analyzed on Kaluza (Beckman Coulter) software as per manufacturer's instructions. Percentages of $CD3^+CD8^+$, $CD3^+CD8^+CD69^+$ and $CD3^+CD8^+$ IFN-γ $^+$ T cells were calculated out of $CD3^+CD8^+$ T cells. Absolute numbers were calculated based the ratio of cell counts over bead counts multiplied by the ratio of known bead numbers and volume. Numbers of unactivated cell subsets were compared to activated cell subsets.

7.6 Immunofluorescence flow cytometry analysis of myeloid and plasmacyotid dendritic cells.

$Lin^-HLA\text{-}DR^+CD11c^+$ mDCs and $Lin^-HLA\text{-}DR^+CD123^+$ pDCs were analyzed using 4-Color Dendritic Value Bundle kit (BD Biosciences, San Jose, CA) by immunofluorescence flow cytometry. Freshly isolated PBMCs were stained with the following antibodies: Lineage Cocktail 1-FITC (Lin 1) (anti-CD3, CD14, CD16, CD19, CD20, and CD56), anti-HLA-DR-PerCP, anti-CD123-PE, and anti-CD11c-APC, for 30 minutes on ice. Cells were washed and fixed before subjected to flow cytometry analysis. Mouse IgG1-PE and IgG2a-APC were used as isotype controls. The $Lin^-HLA\text{-}DR^+CD11c^+$ mDC and $Lin^-HLA\text{-}DR^+CD123^+$ pDC portions were gated with Becton-Dickinson LSR II (BD Biosciences, San Jose, CA), and the acquired data were analyzed in FCS Express Version 3 (DeNovo software, Los Angeles, CA). The percentages, ratios, and the mean fluorescence intensity (MFI) of HLA-DR expression of mDCs and pDCs were calculated for all samples.

7.7 Quantitative real-time PCR of Foxp3 mRNA expression.

Total RNA was extracted from PBMCs with Rneasy Mini kit (Qiagen, Valencia, CA). The first strand cDNA was synthesized from 400ng of total RNA with an oligo (dT) 12–18 primer using RT^2 First Strand Kit (Qiagen, Valencia, CA). The relative mRNA levels Foxp3 were assessed using ABI Prism 700 Sequence Detection System (Applied Biosystems Inc. Foster City, CA). Pre-formulated TaqMan primer and probe mixes for Foxp3 (Hs00203958) were used (Applied Biosystems, Foster City, CA). The fold changes relative to the endogenous control gene, glyceraldehyde 3-phosphate dehydrogenase

(GAPDH), were calculated as the levels of gene expression, and calibrated with normal donors.

7.8 Quantitative real-time PCR for expression of CSSMs mRNA.

Total RNA was extracted from PBMCs with RNeasy Mini kit (Qiagen, Valencia, CA). The first strand cDNA was synthesized from 400ng of total RNA with an oligo (dT) 12–18 primer using RT^2 First Strand Kit (Qiagen, Valencia, CA). The relative mRNA levels of HLA-DR, CD28, CD80, CD86, CTLA-4, ICOS, and ICOSL were assessed with RT^2 Profiler Human T-cell Activation PCR Array (SABiosciences, Frederick, MD) using ABI Prism 700 Sequence Detection System (Applied Biosystems Inc. Foster City, CA). SYBR Green-optimized primers and pre-formulated PCR master mix were used. The fold changes relative to GAPDH were calculated as the levels of gene expression, and calibrated with normal donors.

7.9 Statistical analysis of $CD4^+$ and $CD8^+$ T cell subsets.

Each parameter was calculated for mean ± standard deviation. Statistical significance of percentages and numbers of $CD4^+Foxp3^+$, $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$, $CD3^+CD8^+$, $CD8^+CD69^+$, and $CD8^+IFN\text{-}\gamma^+$ between L-CTCL patients and normal donors, between the 2 L-CTCL patients groups, and between baseline and time points after ECP-BRM treatment were determined by the student's T-test and Wilcoxon Rank sum analysis. P-values less than 0.05 were considered as significantly different between the experimental groups. Fold changes of greater or less than 2-fold was significantly different in gene expression. A correlation was determined by Pearson product-moment

correlation coefficient R ≥0.5-1.0 based on the Cohen scale. Statistical analyses were evaluated in Microsoft Office Excel 2007 and PASW Statistics 17.0. Graph Pad Prism and Microsoft Office Excel 2007 were used to design figures.

7.10 Statistical analysis of Dendritic cell subsets.

Each parameter (percentage, ratio, and MFI) was summarized using the mean (standard deviation) and median (range). The levels at baseline and the changes from baseline for each time point were computed. The linear mixed models were used assess the changes (from baseline) as a function of time point and clinical response (PR+MR versus PD+SD). We started with the full model with fixed effect of time point, response status, and their interaction, as well as a random effect of patients. We sequentially eliminated the interaction and the main effect of response in absence of significant effect. Statistical significance of percentages, ratios, and MFI of HLA-DR expression in mDCs and pDCs between L-CTCL patients and GVHD patients, between normal donors and patients, and between baseline and different time points after ECP were assessed. The statistical significance of fold changes of each gene mRNA level between patient response groups and before and after ECP was determined by the student's T-test and Wilcoxon Rank Sum statistical analysis. Differences between experimental groups were considered significant if the p value was less than 0.05. Differences in gene expression were also considered meaningful if the fold regulations were more or less than 2-fold. Statistical analyses were conducted using SAS 9.2 for Windows (Copyright @ 2011 by SAS Institute Inc., Cary, NC) and Microsoft Office Excel 2007.

CHAPTER 8

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CHAPTER 9

VITA

Lisa Harn-Ging Shiue was born in Murphysboro, Illinois on November 11, 1983 to Dr. Wei Luen and Mei-Hu Lin Shiue. After completing high school at Carbondale Community High School in Carbondale, Illinois in 2002, she entered the University of Illinois at Urbana-Champaign (UIUC) in Urbana-Champaign, Illinois. She received the degree of Bachelor of Science with a major in Molecular and Cellular Biology from UIUC in May, 2006. In July of 2006, she entered the Ph.D. program at The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. She began her Ph.D. training under the supervision of Dr. Madeleine Duvic in the Department of Dermatology at the University of Texas M.D. Anderson Cancer Center in June 2007 studying the translational aspects of Immunology and Cutaneous T cell Lymphoma.

CHAPTER 10

PUBLICATIONS

PEER-REVIEWED PUBLICATIONS:

Shiue LH, Ni X, Prieto VG, Jorgensen JL, Curry JL, Goswami M, Sweeney SA, Duvic M. A case of invisible leukemic cutaneous T cell lymphoma with a regulatory T cell clone. International Journal of Dermatology. First published online.

Chung JS, **Shiue LH**, Duvic M, Pandya A, Cruz PD Jr, Ariizumi K. Sezary syndrome cells overexpress syndecan-4 bearing distinct heparan sulfate moieties that suppress T-cell activation by binding DC-HIL and trapping TGF-{beta} on the cell surface. Blood. 2011 Mar 24; 117 (12):3382-90. Epub 2011 Jan 20. PMID: 21252093

Ni X, Richmond HM, Liao XM, Decker WK, **Shiue LH**, Shpall EJ, Duvic M. Induction of T-cell responses against cutaneous T-cell lymphomas ex vivo by autologous dendritic cells transfected with amplified tumor mRNA. J Invest Dermatol. 2008 Nov;128 (11):2631-9. PMID: 18480841

Shiue LH, Alousi AM, Yang BY, Wei C, Hosing C, Duvic M, Ni X. Augmentation of blood dendritic cells by extracorporeal photopheresis in patients with leukemic cutaneous T-cell lymphoma and graft-versus-host disease. J Invest Dermatol. Under revision.

ABSTRACT PUBLICATIONS AND PRESENTATIONS:

H Zhong, **L Shiue**, Y Mekhail, M Goswami, M Duvic, X Ni.
Effect of extracorporeal photopheresis on Th1/Th2/Th17/T-reg cytokines in cutaneous T-cell lymphoma patients. Accepted; J Invest Dermatology 2012. Society of Investigative Dermatology meeting. Oral and Poster presentation.

LH Shiue, M Duvic, M Goswami, A Alousi, C Hosing, X Ni
Effect of extracorporeal photopheresis on dendritic cell populations in patients with advanced stage cutaneous T cell lymphoma and graft versus host disease and its correlation with the clinical response. J Invest Dermatology 2011; #484. 2011 Society of Investigative Dermatology meeting, Phoenix, AZ ; oral and poster presentation.

LH Shiue, M Goswami, A Alousi, M Duvic, X Ni
Effect of extracorporeal photopheresis on dendritic cell populations in patients with advanced stage cutaneous T cell lymphoma and graft versus host disease. 2010 National Predoctoral Clinical Research Training Program Meeting,

Washington University School of Medicine, St. Louis, MO; abstract selected for oral presentation.

X Ni, M Goswami, **L Shiue**, A Dougherty, M Duvic
Increased TWIST expression in advanced stage mycosis Fungoides (MF)/ Sézary Syndrome (SS). J Invest Dermatol 2010; #137. 2010 Society of Investigative Dermatology meeting, Atlanta, GA; Oral and poster presentation.

LH Shiue, M Goswami, A Alousi, M Duvic, X Ni
Effect of extracorporeal photopheresis on dendritic cell populations in patients with Sézary syndrome and graft versus host disease. J Invest Dermatol 2010; #709; 2010 Society of Investigative Dermatology meeting, Atlanta, GA; oral and poster presentation.

LH Shiue, E Aakhus, P Arias-Mendoza, M Duvic, X Ni
Increased levels of CD4+25high regulatory T cells in patients with cutaneous T-cell lymphoma after extracorporeal photopheresis. Accepted-National Student Research Forum, Galveston, TX 4/2009.

J-S Chung, **L Shiue**, M Tomihari, M Duvic, PD Cruz Jr, K Ariizumi. Exploiting the DC-HIL/syndecan-4 pathway to treat cutaneous T cell lymphoma. J Invest Dermatol 2009; 129:S51; #301. Society of Investigative Dermatology meeting, Montreal, Canada, 5/2009, Oral and poster presentation.

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Increased Syndecan-4 expression in malignant T -lymphocytes is associated with progression in Mycosis Fungoides. J Invest Dermatol 2009; 129:S45; #265. Society of Investigative Dermatology meeting, Montreal Canada, 5/2009, Oral and poster presentation.

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W Zhou, **L Shiue**, E Aakhus, M Duvic, X Ni. A novel approach to study tumor-specific CD8+cytotoxic T lymphocytes in cutaneous T-cell lymphoma patients treated with extracorporeal photopheresis. National Student Research Forum, Galveston, TX, 4/2008.

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