Novel approaches for targeted therapy in CTCL

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SUMMARY

Development of targeted therapy for Cutaneous T-Cell Lymphoma (CTCL) patients still requires actionable mutated genes and deregulated pathways to be identified. Although it has been proposed that neoplastic CTCL cells feature increased TCR downstream signaling and also shows a degree of phenotypic plasticity, the mechanistic nature of the pathogenesis of this disease remains essentially unveiled. Our laboratory has recently studied the mutational status of a number of human CTCL lesions, and detected TCR/PLCG1 and JAK/STAT signaling pathways frequently mutated. Taking advantage of these findings, we have explored the biological effects that targeted inhibition, using specific inhibitors of the two aforementioned signaling pathways, exerts in proliferation, survival and phenotype in a panel of human CTCL cell lines. Our results suggest that TCR/PLCG1 and JAK/STAT pathways can control proliferation, survival and phenotype of CTCL cells and hence can serve as potential targets for mono or combinatorial therapy in CTCL patients.

INDEX

CHAPTER 1

Introduction

1.1 Cutaneous T-cell lymphoma

Cutaneous T-cell lymphoma (CTCL hereon) is a heterogeneous group of non-Hodgkin lymphomas characterized by the clonal expansion of malignant T-cells involving primarily the skin (Willemze, Jaffe et al. 2005). While the term CTCL includes a number of rare disease entities, such as $CD30^+$ primary cutaneous anaplastic large cell lymphoma (pcALCL), lymphomatoid papulosis, peripheral T-cell lymphoma (PTCL), and related disorders, the most common subtypes are Mycosis fungoides (MF) and Sézary syndrome (SS) with an incidence rate of 4.1/1,000,000 person-years and male predominance (Bradford, Devesa et al. 2009). These subtypes account for nearly 70-75% of all CTCLs and include a broad variety of clinicopathological conditions, ranging from unique lesions to more generalized skin involvement with extensive tumors and erythroderma in MF while SS is characterized by erythroderma, lymphadenopathy and leukemic involvement with malignant cerebriform lymphocytes (Sézary cells).

1.1.1 Classification of Mycosis fungoides and Sézary syndrome

The prognosis of MF and SS depends on age at presentation, peripheral blood involvement and extra-cutaneous disease. In 2007, a revised staging system of MF and SS by the International Society for Cutaneous Lymphomas (ISCL)/European Organization for Research and Treatment of Cancer (EORTC) was proposed (Olsen, Vonderheid et al. 2007), incorporating stratification of early stage (T1/T2) into patch alone and both patch and plaque disease, as well as detailed histologic and molecular classification of lymph node and peripheral blood involvement leading to a uniform and standardized staging and classification system (Table 1). The ISCL/EORTC has attempted to add some clarity to the situation by suggesting definitions for the skin lesions. Because in patch/plaque disease, histology has been shown to offer an objective means of defining each subtype, be a validated surrogate for the clinical classification of MF lesions, and have prognostic implications, there is a provision in the classification system for characterizing exclusively patch-stage disease with the subscript of "a" (T_{1a} and T_{2a}) versus combined

Table 1. Modified ISCL/EORTC revision to the classification of Mycosis fungoides and Sézary syndrome (Olsen, Vonderheid et al. 2007)

	Skin T	Node N	Visceral M	Blood B
IA	1	0	0	0,1
IB	2	0	0	0,1
II	1,2	1,2	0	0,1
IIB	3	0-2	0	0,1
III	4	0-2	0	0,1
IIIA	4	0-2	0	0
IIIB	4	0-2	0	1
IVA_1	1-4	0-2	0	2
IVA_2	1-4	3	0	0-2
IVB	1-4	0-3	1	0-2

Skin	
T_1	Limited patches,* papules, and/or plaques† covering < 10% of the skin surface. May further stratify into T_{1a} (patch only) vs T_{1b} (plaque ± patch).
T_2	Patches, papules or plaques covering ≥ 10% of the skin surface. May further stratify into T_{2a} (patch only) vs T_{2b} (plaque ± patch).
T_3	One or more tumors‡ (≥ 1-cm diameter)
T_4	Confluence of erythema covering ≥ 80% body surface area
Node	
N_0	No clinically abnormal peripheral lymph nodes§; biopsy not required
N_1	Clinically abnormal peripheral lymph nodes; histopathology Dutch grade 1 or NCI LN_{0-2}
N_{1a}	Clone negative#
N_{1b}	Clone positive#
N_2	Clinically abnormal peripheral lymph nodes; histopathology Dutch grade 2 or NCI LN_3
N_{2a}	Clone negative#
N_{2b}	Clone positive#
N_3	Clinically abnormal peripheral lymph nodes; histopathology Dutch grades 3-4 or NCI LN_4; clone positive or negative
N_x	Clinically abnormal peripheral lymph nodes; no histologic confirmation
Visceral	
M_0	No visceral organ involvement
M_1	Visceral involvement (must have pathology confirmation¶ and organ involved should be specified)
Blood	
B0	Absence of significant blood involvement: ≤ 5% of peripheral blood lymphocytes are atypical (Sézary) cells‖
B_{0a}	Clone negative#
B_{0b}	Clone positive#
B1	Low blood tumor burden: > 5% of peripheral blood lymphocytes are atypical (Sézary) cells but does not meet the criteria of B_2
B_{1a}	Clone negative#
B_{1b}	Clone positive#
B2	High blood tumor burden: ≥ 1000/μL Sézary cells‖ with positive clone#

*For skin, patch indicates any size skin lesion without significant elevation or induration. Presence/absence of hypo- or hyperpigmentation, scale, crusting, and/or poikiloderma should be noted.

†For skin, plaque indicates any size skin lesion that is elevated or indurated. Presence or absence of scale, crusting, and/or poikiloderma should be noted. Histologic features such as folliculotropism or large-cell transformation (> 25% large cells), $CD30^+$ or $CD30^-$, and clinical features such as ulceration are important to document.

‡For skin, tumor indicates at least one 1-cm diameter solid or nodular lesion with evidence of depth and/or vertical growth. Note total number of lesions, total volume of lesions, largest size lesion, and region of body involved. Also note if histologic evidence of large-cell transformation has occurred. Phenotyping for CD30 is encouraged.

§For node, abnormal peripheral lymph node(s) indicates any palpable peripheral node that on physical examination is firm, irregular, clustered, fixed or 1.5 cm or larger in diameter. Node groups examined on physical examination include cervical, supraclavicular, epitrochlear, axillary, and inguinal. Central nodes, which are not generally amenable to pathologic assessment, are not currently considered in the nodal classification unless used to establish N_3 histopathologically.

¶For viscera, spleen and liver may be diagnosed by imaging criteria.

‖For blood, Sézary cells are defined as lymphocytes with hyperconvoluted cerebriform nuclei. If Sézary cells are not able to be used to determine tumor burden for B_2, then one of the following modified ISCL criteria along with a positive clonal rearrangement of the TCR may be used instead: (1) expanded $CD4^+$ or $CD3^+$ cells with CD4/CD8 ratio of 10 or more, (2) expanded $CD4^+$ cells with abnormal immunophenotype including loss of CD7 or CD26.

#A T-cell clone is defined by PCR or Southern blot analysis of the T-cell receptor gene.

patch/plaque disease with the subscript of "b" (T_{1b} and T_{2b}) in order to gather additional longitudinal data on this distinction (Table 1). The original Mycosis Fungoides Cooperative Group (MFCG) skin scoring system for CTCL included a T_0 category for "clinically and/or histopathologically suspicious lesions". While T_0 may be a useful category for tracking disorders with malignant potential, current practice dictates that clinical staging be applied only to cases in which a diagnosis of cancer has been established. Therefore, the T_0 category has been eliminated in the ISCL/EORTC updated staging and classification scheme (Table 1) that requires all staged patients have a definitive diagnosis of MF/SS and/or algorithmic diagnosis of early MF. Agar et al. have validated this new staging system by analyzing the outcome of 1502 MF and SS patients treated at their institution (Agar, Wedgeworth et al. 2010). In this study the median reported follow-up period was 5.9 years (range, 0.4-35.5) years. 71% of the patients had 'limited-stage' disease (Stages IA, IB and IIA) with median overall survival (OS) ranging

between 15.8 to 35.5 years whereas 20% of the patients had 'advanced-stage' disease (stages IIB, III, IV) and their OS was inferior ranging between 1.4-4.7 years. Finally, CTCL diagnosis is currently based mainly on clinico-pathological correlation, occasionally sustained by the presence of T-cell receptor (TCR hereon) monoclonal rearrangements.

1.1.2 Immunopathogenesis of CTCL

1.1.2.1 Normal skin vs. CTCL

In normal skin, reactive or inflammatory cutaneous lymphoid infiltrates are generally composed of a mixture of lymphoid subsets, each displaying its characteristic normal immunophenotype. For T-cells, this involves the expression of mature T-cell markers, such as CD2, CD3, and CD5. There is typically a mixture of the two major subsets, $CD4^+$ and $CD8^+$, with the former predominating (Smoller, Santucci et al. 2003). These T-cells are recruited to the skin following injury. Environmental or infectious damage results in keratinocyte cytokine release and also triggers innate immune responses from skin-resident immune cells, such as dendritic cells (DCs), mast cells, and macrophages. This response may be mediated by pathogen components that are recognized by pattern-recognition receptors, most notably the Toll-like receptors (TLRs), on these cells (Kim, Hess et al. 2005).

The activation of these pathways via upregulation of NF-κB signaling can result in proinflammatory cytokine increased expression and secretion into the cellular microenvironment that can have direct effects on pathogens as a result of provoking additional inflammatory cell recruitment and migration of activated Antigen-presenting cells (APCs) to local skin-draining lymph nodes. Once these APCs meet the appropriate naïve T-cells in the lymph nodes, the T cells become antigen-specific effector/memory cells and acquire the ability to home to the original site of inflammation, the skin. Antigen-specific central memory T-cells are subsequently generated and circulate through lymph nodes to provide a long-term reservoir for specific immune surveillance. These skin effector/memory T-cells express cutaneous lymphocyte antigen (CLA) and normally make up 30% of all circulating memory T cells. This CLA^+ cells also typically co-express the ligand for E-selection (CD62E), CC-chemokine receptor 4 (CCR4), which binds to the skin-manufactured chemokines like CC-chemokine ligand 17 (CCL17) and CC-chemokine ligand 22 (CCL22), among others. All of these interactions are also crucial to the rolling/tethering interaction between the T-cell and the endothelium that is necessary for the T-cell to gain entry into the dermis and epidermis. The balance between appropriate skin defense responses and inappropriate or deregulated responses

appears to be the key to understanding the pathogenesis and treatment of acquired inflammatory skin conditions, which may include MF and SS (Kim, Hess et al. 2005).

The malignant T cells in MF and SS patients have been shown to express the skin-homing receptors CLA and CCR4. Furthermore, MF lesions can express high levels of CCL17 and CCL22 (CCR4 ligands). Other chemokine receptors expressed by skin-infiltrating T-cells found in MF lesions, such as CXC-chemokine receptor 3 (CXCR3) and CXCR4, and surface molecules, such as integrin $\alpha_E\beta_7$, can be activated through their corresponding ligands and may reflect cells of the host immune response, as tumor stage lesions frequently lose the expression of these markers (Kim, Hess et al. 2005). Analyses of cytokine expression in the skin have attempted to identify specific patterns to be associated with early and advanced stage. In early stage MF, the skin has shown normal to increased expression of IFNG, IL-12 and IL-2. As the malignancy progresses to late stage MF, there have shown a loss of these cytokines with a concomitant increase in the expression of other cytokines, such as IL-4, IL-5, IL-10 and IL-13. The patterns seen in the skin have been also seen in analysis of gene expression from peripheral blood of early and late MF/CTCL patients (Chong, Wilson et al. 2008). However, this pattern of differential expression of cytokines has not been yet established in clinic as a vigorous pattern in order to identify the different stages of CTCL.

1.1.2.2 Signaling pathways in CTCL

***TCR*:** T-cell tolerance to self-antigens is crucial for the prevention of autoimmune disease. The affinity of the TCR for self-antigens plays an important role in the survival of developing T cells. The TCR is a multimeric complex including 2 ligand-binding glycoproteins containing variable regions (αβ or γδ TCR heterodimers) that are expressed on the cell surface in association with four CD3 molecules. High-affinity interaction of the TCR with self-antigen-derived peptide-MHC (major histocompatibility) complexes generally results in clonal deletion or inactivation, but it may also result in the upregulation of FoxP3, thus inducing regulatory T-cell (Treg) function (Jordan, Boesteanu et al. 2001). The combination of signals through the TCR, costimulatory molecules and cytokines such as IL-2 and IL-15 can modulate the intrinsic and extrinsic apoptosis pathway in T-cells, and eventually can control central and peripheral T-cell selection. Increased signaling from the TCR, due at least in part by genetic rearrangements, can be considered a driving force of CTCL. It has been shown that CTCL cells can be induced to become Treg cells when DC class II presentation of peptides derived from apoptotic material triggers their TCR. Treg CTCL cells suppress immune responses and secrete IL-10 and TGF-β, cytokines that perpetuate DC immaturity, providing continued opportunity for DC stimulation of CTCL cell growth (Berger and Edelson 2004).

PLCG1 is considered to play a pivotal role at mediating T-cell activities downstream of TCR signaling such as the mechanisms that control cell proliferation and survival as well as regulating T-cell differentiation (Macian 2005) (Abraham, Zhang et al. 2011) (Sundrud and Nolan 2010). PLCG1 is a member of the PI-PLC family of proteins that convert PIP_2 into IP_3 and DAG. It can be activated in T-cells downstream of TCR/CD3 activation, triggering the activation of key lymphomagenesis pathways such as RAS/RAF/ERK, PKC/NF-κB pathways by DAG, or CaM/CaN/NFAT by IP_3/Ca^{2+}. To date, mutations in PLCG1 have been described only sporadically (67 mutations found in 5892 analyzed cases, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database).

***NF-κB - JAK/STAT*:** On the other hand, it is well known that NF-κB activation in lymphocytes results in expression of targeted genes involved in proliferation, production of multiple key T-cell cytokines like CCL17 and CCL22 (CCR4 ligands) or IL-4, IL-6 and IL-10 and survival through the activation of the JAK/STAT pathway. Under normal conditions, NF-κB is sequestered in an inactive state by the IκBs inhibitory molecules in the cytoplasm that can be released by a variety of different stimuli that can activate NF-κB via degradation of the IκBs. NF-κB has been shown to be constitutively activated in several hematopoietic lymphoid malignancies such as large B-cell lymphoma (Davis, Brown et al. 2001), mantle cell lymphomas (Pham, Tamayo et al. 2003), multiple myeloma (Mitsiades, Mitsiades et al. 2002) and CTCL (Sors, Jean-Louis et al. 2006). Downstream of the cytokine production driven by activated NF-κB signaling, it is well known that a number of the receptors for these cytokines (IL-4, IL-6 and IL-10) can activate JAK kinases. JAK proteins activate members of the STAT family through phosphorylation on a single tyrosine. Activated STATs form dimers, translocate to the nucleus, bind to specific response elements and transcriptionally activate these genes. The balance between the degrees of STAT activation appears to be important in normal T-cells to undergo differentiation towards a Th17 vs. Treg phenotype (Yang, Ghoreschi et al. 2011). In this setting IL-6, IL-2 and IL-15 may play a critical role in the stimulation of malignant T-cell phenotypes (Marzec, Halasa et al. 2008).

1.1.2.3 Phenotypic plasticity in CTCL cells

From a biological perspective, it has been shown that malignant $CD4^+$ T cells derived from patients with CTCL can turn into a $CD4^+CD25^+$ Tregs phenotype after stimulation by DCs loaded with apoptotic T cells. (Berger, Tigelaar et al. 2005). The main function of these Tregs is thought to suppress the activity of other immune cells, thus maintaining immunological tolerance. 5-10% of the total $CD4^+$ T cells are Tregs in human peripheral blood. In CTCL patients, has been shown a differential presence depending on the stage of the disease. This Tregs have been found accumulated in early stages of the disease (Gjerdrum, Woetmann et al.

2007) as opposed to advanced stages of CTCL (Tiemessen, Mitchell et al. 2006). This subset are identified by the selective expression of the transcription factor Forkhead box protein 3 (Foxp3) and the high expression of the inhibitory receptor CTLA-4. Moreover CCR4 is a protein that is expressed and plays a role in normal Treg cell activities (Kunkel, Boisvert et al. 2002) and has been detected expressed at high levels in both SS and MF in the very early stages (patch and plaque stages) of the disease. CCR4 expression is limited amongst non-malignant cells as it has not been yet detected in neutrophils, monocytes, B-cells or naïve T-cells, and present on fewer than half of all memory T-cells (Campbell, Haraldsen et al. 1999) hence supporting the role for Treg activity in CTCL.

In this same line of evidence, it has recently been shown that malignant CTCL cells can also possess features of IL-17-producing helper T cells (Th17) (Hoechst, Gamrekelashvili et al. 2011) which in addition to the aforementioned Treg phenotype that can also be detected in this disease, may display a dynamic T-cell phenotypic landscape with yet unexplored activities in the pathogenesis of CTCL (Abraham, Zhang et al. 2011).

From a molecular perspective, both Treg and Th17 phenotypes can be acquired by a combination of TCR-TGF-β signaling in naïve T-cells thus activating FoxP3 (Chen, Jin et al. 2003) that in the conditions may instead promote Th17 differentiation through the activation of JAK/STAT pathway. STAT3 indeed can be an important mediator of plasticity considering that the JAK/STAT pathway can promote the secretion of IL-17 in CTCL cell lines (Krejsgaard, Ralfkiaer et al. 2011) via IL-6 (O'Garra, Stockinger et al. 2008). This can induce the expression of the nuclear factor RORγt, a marker for Th17 cells (Ivanov, McKenzie et al. 2006). In this way, the malignant T-cell phenotype may behave highly flexible as has been observed by a number of groups (Abraham, Zhang et al. 2011) (Eisenstein and Williams 2009) (Hoechst, Gamrekelashvili et al. 2011), due to considerable inputs from the cytokine environment or perhaps to yet unpublished malignant mechanisms like for example those somatic mutations affecting key T-cell signaling pathways that have recently been found by our group in CTCL patients (see Figure 1). Moreover these findings may have detected new targets for therapy with effects in proliferation and survival of CTCL cells but also with potential to control the phenotypic plasticity observed in this disease and that we possibly can exploit to develop novel CTCL therapeutics.

1.1.3 Molecular pathogenesis of CTCL

MF/SS has not been clearly associated with a single or specific chromosomal mutation in either tumor suppressor genes or oncogenes (except for TP53 or RAS). However, CTCL progression

has been associated with several genetic abnormalities. Early cytogenetic studies in CTCL found some alterations that were associated with shorter survival, such as gain of 8q (including MYC) and losses of 6q and 13q (including RB), 17p (TP53), 10 (PTEN, FAS) and 9p21 deletion (including CDKN2A). More advanced lesions have been shown to have p53-expression defects and microsatellite instability and also hypermethylation of mismatch repair genes, and p16/p15 alterations. There have not been extensively mutational analyses of patients with CTCL, excepting those in which the tumor suppressor p53 was frequently found nonfunctional in SS (Lamprecht, Kreher et al. 2012) and mutations found in Ras pathway (KRAS and NRAS genes) (Kiessling, Oberholzer et al. 2011).

1.1.4 Actual therapy of MF and SS

Establishing a definitive diagnosis, accurate staging, risk stratification and selection of appropriate initial therapy still remains critical.

There is a huge variability in the treatment of CTCL patients and although there are some therapeutical guides published, nowadays multiple experimental therapies are being explored in a way that still depends on the hospital´s experience and the preferences and possibilities of the patient. The almost complete lack of randomized clinical assays implies that currently there are no robust high quality scientific data nor any standardized therapy for CTCL.

In early disease stages it is relatively common to use skin targeted therapies (like topically use of corticoids, nitrogenized mustard, BCNU (Carmustine), photo-chemotherapy, radiotherapy, etc.), but as these approaches start to fail a more systemic treatment will be required. Regarding this, early-stage refractory patients may benefit from combination therapies, such as Narrowband UVB (NB-UVB) or psolaren plus UVA (PUVA) with low-dose systemic oral bexarotene or IFN-α. IFN-α directly enhances cell-mediated cytotoxicity, both $CD8^+$ T-cells and NK cells exhibit rapid activation which can lead to enhanced immunomodulation. Bexarotene, a retinoid X receptor-specific compound, has also been determined to have immunomodulatory effects inducing apoptosis within the malignant population of T-cells, particularly when used in combination therapy. However, Sézary cells from approximately one-third of patients demonstrate significant resistance to apoptosis (Budgin, Richardson et al. 2005). Given the role that immunity may exert in this disease, it is preferable to use treatments that will activate or maintain the immune response like for example IFN alone or in combination with retinoids (Etretinate) or rexinoids (Bexarotene). Therapy with mono- or poly-chemotherapy is basically used in those non-responding cases to the aforementioned therapies and also some therapies based on the use of monoclonal antibodies are starting to be explored such as CD25, CD4,

CD30, CD3 and CCR4. Finally, some other targeted therapies against other epigenetic targets such as (HDAC inhibitors), or proteasome inhibitors like bortezomib, are also being explored although as mentioned before they have rendered no conclusive information indicating its effectiveness for the clinic (Wong, Mishra et al. 2011).

In light of these data we propose that clarifying how cytokine-induced intracellular signaling in the tumor microenviroment affects the proliferation, survival and phenotype of the neoplastic CTCL cells together with a better understanding of the molecular pathogenesis of this disease may lead to the development of novel targeted therapeutics able to specifically eliminate the malignant CTCL cells or perhaps induce a phenotypic transition of the malignant T cells into a less aggressive condition.

1.2 Background

In a mutational study performed by the Cancer Genomics group of IFIMAV, there were studied a selection of 524 genes with known biological relevance in normal T-cells by deep targeted sequencing. The genes used in the study are involved in TCR, NF-kB, JAK/STAT and MAPK pathways as well as cytokines, TGF-β and IFN-α pathways, among others. There were analyzed

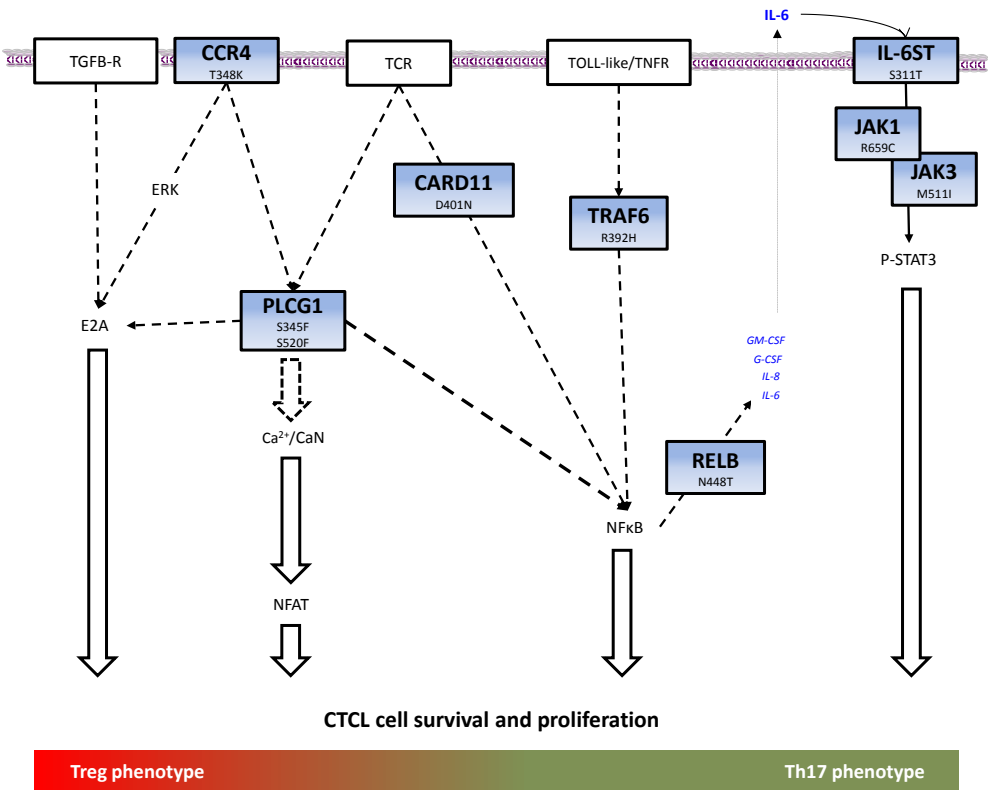

Figure 1. Mechanistic landscape driving the pathogenesis of CTCL. Schematic representation showing key T-cell pathways harboring somatic mutations as found in ultrasequencing analysis performed by our group (mutations are highlighted in blue rectangles). Modified from Vaqué, JP et al. (submitted).

11 non tumoral vs. tumoral DNAs of CTCL patients. Our group found recurrent activating mutations in PLCG1 (in 3/11 patients and also in 20% in an independent series analysis) (see Figure 1). Mutant PLCG1 proteins showed enhanced activity towards NFAT activation, when compared to wild type proteins both in CTCL cell lines and patients. On the other hand the JAK/STAT pathway genes like IL6S/T, JAK1 and JAK3 were also found mutated in 3/11 patients suggesting that this pathway can also be study for its clinical implications in this disease.

These signaling pathways are tightly regulated and can be activated by upstream signals elicited by the interaction at the extracellular membrane of soluble ligands or cell antigens with their cognate receptors, like for example TCR, CCR4, TGFB-R, TLRs and IL-6ST. These have shown to potentiate the activity of transcription factors such as E2A, NFAT, NF-κB and STAT3, which in turn have been shown to participate in essential T-cell activities. TCR-PLCG1 and NF-κB-JAK/STAT signaling pathways have been shown to control CTCL cell proliferation, survival and phenotype mechanisms including the regulation of the expression of T-cells lineage markers such as FoxP3 and RORγt (Sundrud and Nolan 2010) (Abraham, Zhang et al. 2011) (Ivanov, McKenzie et al. 2006).

CHAPTER 2

Hypothesis and Objectives

2.1 Hypothesis

Cutaneous T-cell lymphoma (CTCL) is a relatively uncommon disease with an incidence rate of 4.1/1,000,000 person-years. Although there has been an intensive research trying to elucidate the molecular pathogenesis of this disease, there is still a need to investigate new targeted therapy as the incidence increase.

Our hypothesis is that the results obtained from the mutational study of CTCL can suggest the study and development of new targeted therapies based on the mutations found in PLCG1 and JAK/STAT signaling pathways.

2.2 Objectives

The main goal of this Thesis Project is to explore the biological effects that the use of PLCG1 and JAK/STAT pathways inhibitors provokes in human neoplastic CTCL cell lines since both signaling pathways have been shown to control proliferative, survival and phenotypic activities in normal T cells. This is divided in the following specific objectives:

- Test the proliferation and survival responses that specific pharmacological inhibitors of PLCG1 and JAK/STAT downstream signaling (Tacrolimus and Ruxolitinib respectively) exert in a panel of CTCL cell lines (My-La, HUT-78, HH and MJ).

- Test the proliferation and survival responses that the combined used of Tacrolimus and Ruxolitinib exert in a panel of CTCL cell lines (My-La, HUT-78, HH and MJ).

- Explore the expression of specific lineage markers for Treg (FoxP3) and Th17 (RORγt) T cell phenotypes in a panel of CTCL cell lines (My-La, HUT-78, HH and MJ).

- Analyze the phenotypic effects that pharmacological inhibition of PLCG1 and JAK signaling exerts in CTCL cells.

CHAPTER 3

Materials and methods

3.1 Cell Lines

Four human cutaneous T-cell lymphoma (CTCL) cell lines were used. HH (aggressive CTCL) and MJ (mycosis fungoides) were obtained from the American Type Cell Collection (ATCC, Rockville, MD, USA); My-La (mycosis fungoides) and HuT-78 (Sézary's syndrome) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

All cell lines used are non-adherent cells and grow up in suspension. These cells were cultured at 37°C in a humidified sterile atmosphere of 95% air and 5% CO_2, using Iscove's Modified Dulbecco's Media (IMDM) for MJ cell line, and Roswell Park Memorial Institute 1640 (RPMI) for the rest of cell lines supplemented with 10% heat-inactivated fetal bovine serum (FBS), glucose (4.5 g/L), *L*-glutamine (292 mg/L), streptomycin sulphate (10 mg/L) and potassium penicillin (10000 U/L).

Cell lines were maintained frozen in FBS with 10% of dimethyl sulfoxide (DMSO). 2 mL CryoTubes™ were filled with the cell suspension and placed in a -80º freezer. Frozen cells were rapidly transferred to a liquid nitrogen container (-196°C) and stored.

3.2 Compounds

Tacrolimus (FK 506) and Ruxolitinib (INCB018424) were purchased from Selleck Chemicals. All drugs were dissolved in DMSO to stock concentrations of 20mM or 10mM and stored at -20º. Serial dilutions were freshly made in RPMI 1640 or IMDM.

3.3 Cell Viability Assay

To determinate the effects of the inhibitors in cell proliferation, 10^4 cells/mL were seeded in black 96-well plates and incubated in the presence or absence of the different inhibitors at the indicated amounts in each graphic. After 0, 24 and 72 h of treatment cell viability was calculated as the intracellular ATP content using CellTiter-Glo® Luminescent Cell Viability

Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. At the indicated times, 100μL of reagent was added to each well. The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present and was determined by TR717 Microplate Luminometer (Applied Biosystems). The amount of ATP is directly proportional to the number of living cells present in culture. All experiments were done in triplicate and all numerical data were expressed as the average of the values ± the standard deviation (SD).

IC_{50} values were obtained using the GraphPad Prism software 5 (GraphPad Software Inc, La Jolla, CA, USA)

3.4 Apoptosis assay

To analyse the effects of the inhibitors to the survival of the cells, cells were seeded at 10^5 cells/mL and treated with the vehicle (DMSO), the IC_{50} and the double of the IC_{50} concentrations at 24 h. The induction of apoptosis was evaluated by flow cytometry using FlowCellect Annexin Red Kit (EMD Millipore Corporation, Billerica, USA). Annexin V is a phospholipid-binding protein that specifically binds to phosphatidylserine (PS) which is normally located in the inner membrane of viable cells. The translocation of PS to the exposed membrane surface is an early event in apoptosis. Thus Annexin V is able to bind only those cells exposing PS and recognize the early apoptotic cells. All data were detected on a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ, USA) and analyzed using Cell Quest Pro software (BD, Franklin Lakes, NJ, USA). All experiments were done in triplicate and all numerical data were expressed as the average of the values ± the standard deviation (SD).

3.5 Treg/Th17 phenotype assay

To determine the Treg/Th17 phenotype, 2.5 x 10^5 cells/mL were seeded and treated or not with the inhibitors. After 24 h of treatment, 5 x 10^5 cells of each cell line were stained with monoclonal antibodies: anti-CD4-allophycocyanin (APC)-Cy7 (clone SK3, Becton Dickinson, San Diego, CA), and incubating for 30 minutes in dark. Gated $CD4^+$ cells were fixed and permeabilized using Fix and Permeabilization kit (eBioscience, San Diego, CA, USA) following manufacturer's instructions. Monoclonal antibodies to detect intracellular antigens were: anti-Foxp3-PE (clone PCH101, eBiosciences, San Diego, CA), anti-RORgt-APC (clone AFKJS-9, eBioscience, San Diego, CA), and IgG2a isotype controls conjugated with PE and APC. The cells were acquired by flow cytometer (FACS Canto-II, Becton Dickinson).

CHAPTER 4

Results

4.1 Effects of Tacrolimus in proliferation and survival of CTCL cell lines

Previous results from our laboratory, detected PLCG1 recurrently mutated in 3 out of 11 CTCL patients that were analyzed by targeted ultrasequencing and moreover it was also detected in 20% of an independent series analysis of 42 CTCL patients (a summary of these results is in Figure 1). These mutations showed enhanced mutant PLCG1 protein signaling towards NFAT activation when compared to wild type proteins both in cell lines exogenously expressing PLCG1 wild type and mutant proteins as well as in patient samples where this mutation was detected. Considering that PLCG1 is thought to play a pivotal role at mediating T-cell activities downstream TCR signaling (Macian 2005) (Abraham, Zhang et al. 2011) (Sundrud and Nolan 2010) and that increased signaling from the TCR can be considered a driving force of the CTCL pathogenesis, we have decided to explore the inhibition of this pathway using specific and clinically tested PLCG1 downstream signaling inhibitor such as Tacrolimus.

Tacrolimus (FK 506) binds to immunophilin FK506 binding protein 12, interfering with a $Ca2^{+}$-sensitive T-cell signal transduction pathway thus inactivating the phosphatase activity of calcineurin (CaN), a phosphatase effector downstream of PLCG1 signaling which can dephosphorylate and activate NFAT, a transcription factor that when in its active state is located in the cellular nucleus. Thus the impact of inhibiting PLCG1 downstream signaling in the proliferation and survival of My-La, HUT-78, HH and MJ CTCL cell lines was analyzed using the specific pharmacological inhibitor Tacrolimus.

4.1.1 Proliferation

To test the effect that inhibition of PLCG1 downstream signaling provokes in CTCL cell lines growing under normal conditions, we decided to incubate with increasing concentrations of Tacrolimus or DMSO for 0, 24 and 48 h. The results (Figure 2) show that increasing concentrations of this inhibitor affected the proliferation of CTCL cells in a concentration dependent manner. Using these data we calculated an IC_{50} value at 48h for each cell line as follows: My-La (27μM), HUT-78 (19μM), HH (32μM) and MJ (14μM). The IC_{50} value indicates the concentration needed to inhibit a biological or biochemical function by half.

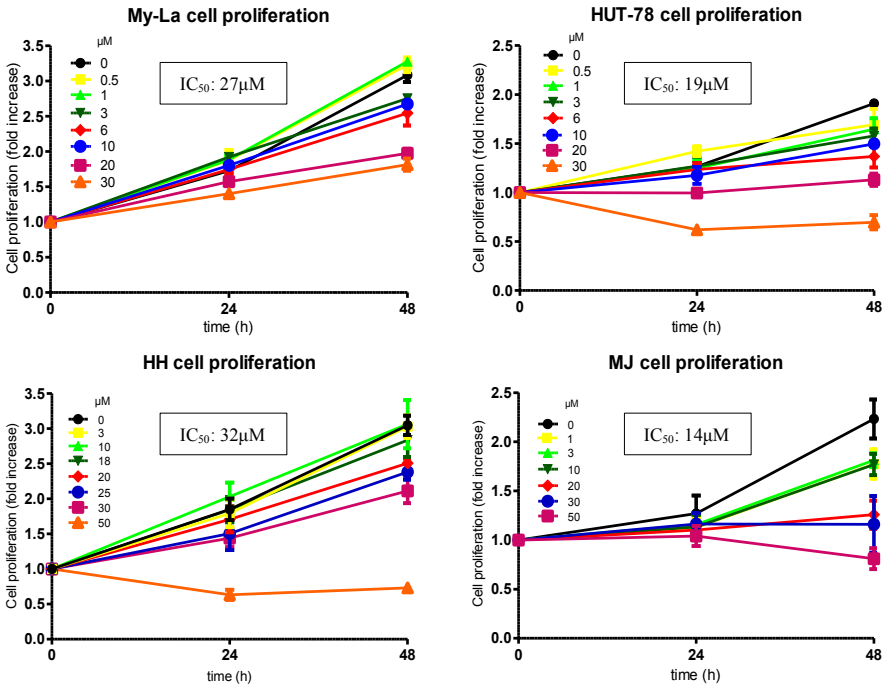

Figure 2. Effects of the Calcineurin specific inhibitor Tacrolimus in proliferation of My-La, HUT-78, HH and MJ CTCL cell lines at 0, 24 and 48 h at the indicated concentrations. The IC_{50} values, indicated in boxes, were calculated using GraphPad Prism software. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

4.1.2 Survival

To test the effects of Tacrolimus in survival, the CTCL cell panel was incubated under normal

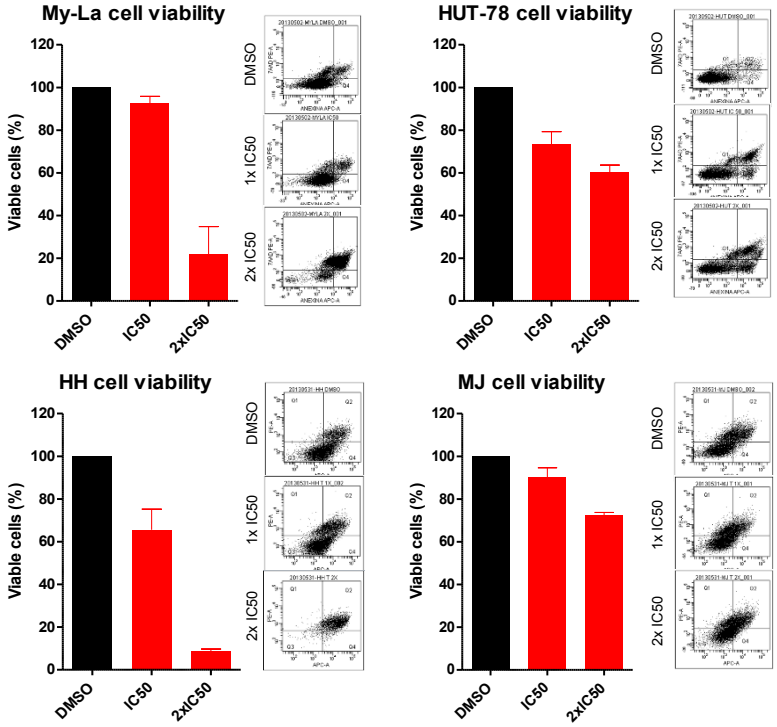

Figure 3. Effects of the Calcineurin specific inhibitor Tacrolimus in survival of My-La, HUT-78, HH and MJ CTCL cell lines at 24 h at IC_{50} and $2xIC_{50}$ concentrations. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

proliferative conditions with DMSO (vehicle) or Tacrolimus for 24h. Using 1X or 2XIC_{50} concentrations as calculated in Figure 2. As shown in Figure 3, treatment of CTCL cells with this inhibitor significantly reduced the viability of the cells by inducing apoptosis in a concentration dependent manner at 24h. Higher apoptosis levels were observed at higher concentrations especially in My-La and HH cells versus HUT-78 and MJ cells.

4.2 Effects of Ruxolitinib in proliferation and survival of CTCL cell lines

Genes belonging to the JAK/STAT signaling pathway were also found mutated in the aforementioned study performed by our group (see Figure 1). More specifically IL6S/T, JAK1 and JAK3 were found mutated in 3 of 11 CTCL patients. Taking into account that this pathway has been shown to regulate proliferation and survival mechanisms in many cell lines and tissues, we decided to explore the biological effects that the inhibition of this pathway using a specific JAK inhibitor like Ruxolitinib. Ruxolitinib (INCB018424) is a small-molecule ATP mimetic that inhibits JAK1, JAK2 and JAK3 kinase activity, which has been used in clinic for the treatment of myelofibrosis (Mesa, Gotlib et al. 2013).

The impact of inhibition of JAK/STAT pathway in the proliferation and survival of My-La, HUT-78, HH and MJ CTCL cell lines was analyzed using the specific pharmacological inhibitor Ruxolitinib.

4.2.1 Proliferation

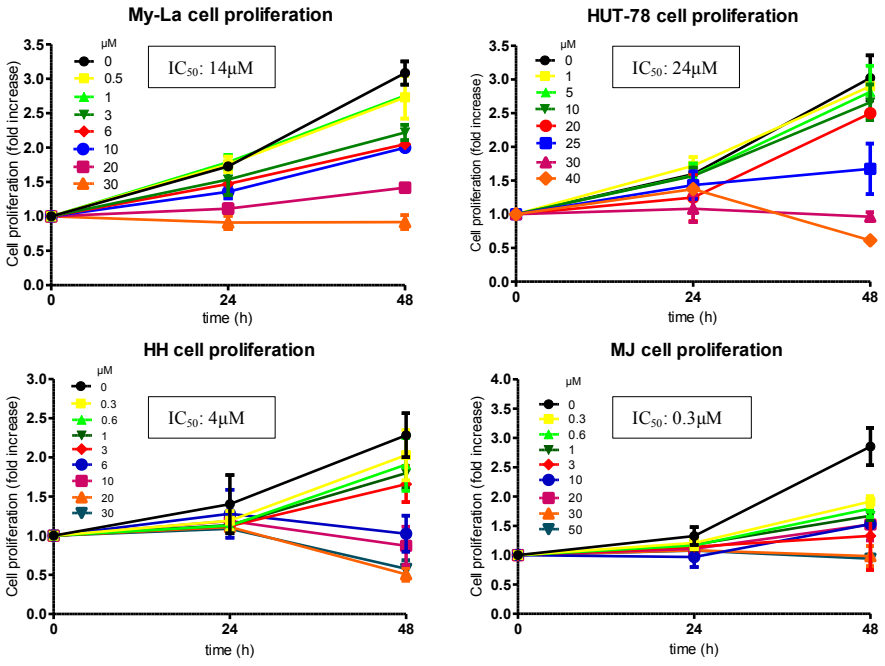

Figure 4. Effects of the JAK specific inhibitor Ruxolitinib in proliferation of My-La, HUT-78, HH and MJ CTCL cell lines at 0, 24 and 48h at the indicated concentrations. The IC_{50} values, indicated in boxes, were calculated using GraphPad Prism software. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

To test the effect that inhibition of JAK downstream signaling provokes in CTCL cell lines growing under normal conditions, we decided to incubate with increasing concentrations of Ruxolitinib or DMSO for 0, 24 and 48 h. The results (Figure 4) show that increasing concentrations of this inhibitor affect the proliferation of CTCL cells and obtained an IC_{50} value at 48h for each cell line as follows: My-La (14µM), HUT-78 (24µM), HH (4µM) and MJ (0.3µM).

4.2.2 Survival

To test the effects of Ruxolitinib in survival, the CTCL cell panel was incubated under normal proliferative conditions with DMSO (vehicle) or Ruxolitinib for 24h. Using 1X or $2XIC_{50}$ concentrations as calculated in Figure 4. As shown in Figure 5, treatment of CTCL cells with this inhibitor did not induced apoptosis of HH and MJ cells at 24 h both at 1X and $2XIC_{50}$ concentrations while My-La and HUT-78 cells survival was lightly affected. These results suggest that JAK signaling may contribute modestly to the anti-apoptotic mechanisms associated to CTCL cells.

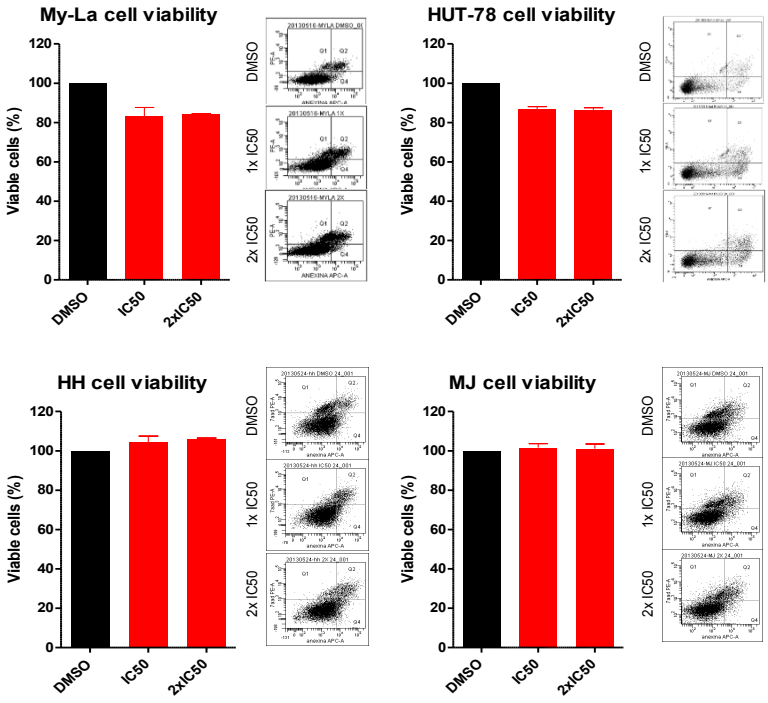

Figure 5. Effects of the JAK specific inhibitor ruxolitinib in survival of My-La, HUT-78, HH and MJ CTCL cell lines at 24 h at IC_{50} and 2x IC_{50} concentrations. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

4.3 Combined effects of Tacrolimus and Ruxolitinib in proliferation and survival of CTCL cell lines

Considering that both TCR-PLCG1 and JAK/STAT pathways have been shown to control important mechanisms in normal T-cells such as proliferation and survival together with our results (see above) when used separately, we decided to test the biological effects that the combined use of Tacrolimus and Ruxolitinib exert in proliferation and survival of our panel of CTCL cell lines.

4.3.1 Proliferation

Once the IC_{50} values where calculated for each inhibitor, CTCL cell lines were treated at 0, 24 and 48 h with Tacrolimus and Ruxolitinib per separate and in combination at IC_{50} concentrations. As shown is Figure 6, combined use of Tacrolimus and Ruxolitinib produced a significant greater inhibition of CTCL cell proliferation than each inhibitor alone except of HUT-78, where the combined treatment was not significant.

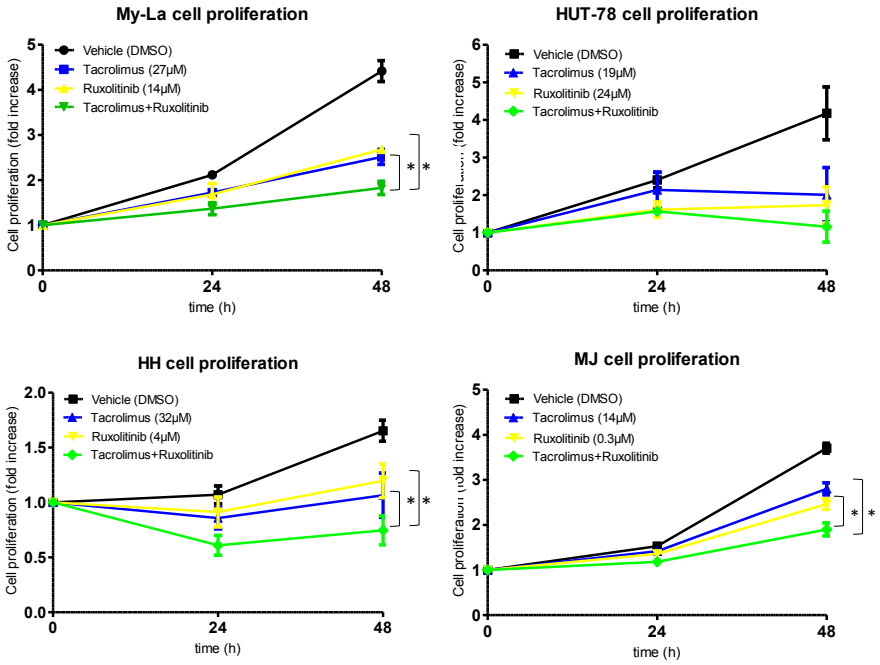

Figure 6. Effects of the combined treatment (tacrolimus and ruxolitinib) in proliferation of My-La, HUT-78, HH and MJ CTCL cell lines at 0, 24 and 48 h at IC_{50} concentrations. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

4.3.2 Survival

The effects of combined treatment of Tacrolimus and Ruxolitinib alone and in combination were analysed in CTCL cells survival at 24 h. Cell lines were treated with DMSO, Tacrolimus

(T-IC50), Ruxolitinib (R-IC50) and Tacrolimus plus Ruxolitinib (T+R-IC50), all of them at IC_{50} concentrations (Figure 7).

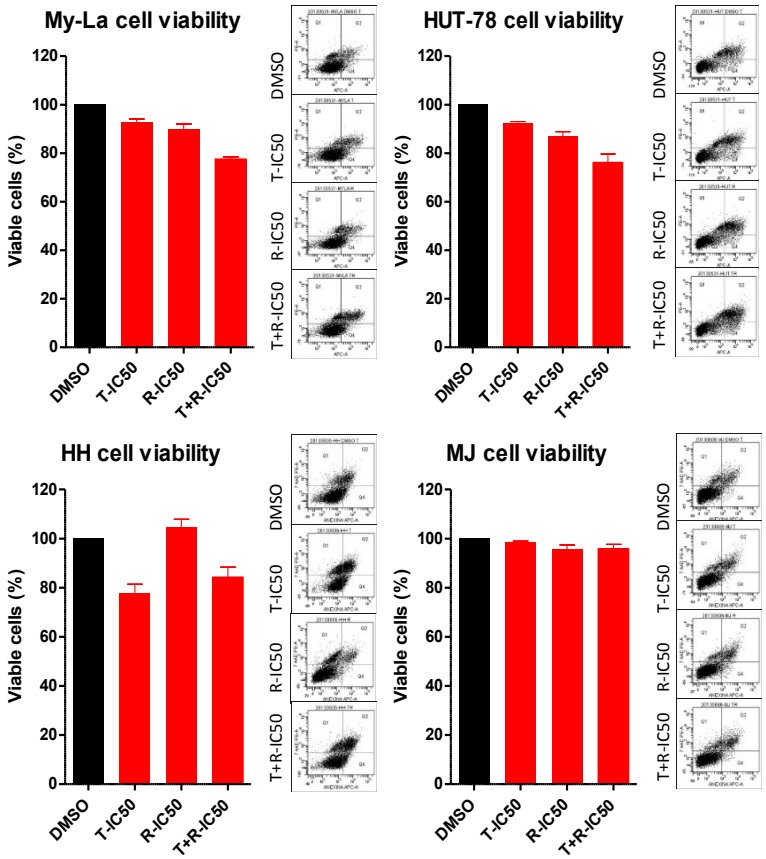

Figure 7. Effects of the Calcineurin and JAK specific inhibitors, Tacrolimus and Ruxolitinib respectively, in My-La, HUT-78, HH and MJ CTCL cell lines survival at 24 h at IC_{50} and $2xIC_{50}$ concentrations. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD

In My-La and HUT-78 cell lines, the combined treatment produced a slightly better effect on cell viability than each inhibitor used alone, while in MJ cell line there is no effect of both combined treatment and each inhibitor alone on cell survival. This results point at TCR/PLCG1 at participating in both proliferation and survival activities in CTCL cells whereas JAK/STAT seems to participate only in the proliferation mechanisms of the neoplastic cells.

4.4 Analysis of Treg/Th17 phenotype in CTCL cells

Considering that have been described that neoplastic T-cells can acquire Treg and/or Th17 phenotypes at different stages of the disease (Tiemessen, M.M. et al. 2006) and that there is a CTCL phenotypic plasticity described already by a number of groups (Abraham, Zhang et al.

2011) (Eisenstein and Williams 2009) (Hoechst, Gamrekelashvili et al. 2011), we have decided to study these phenotypes in the four CTCL cell lines.

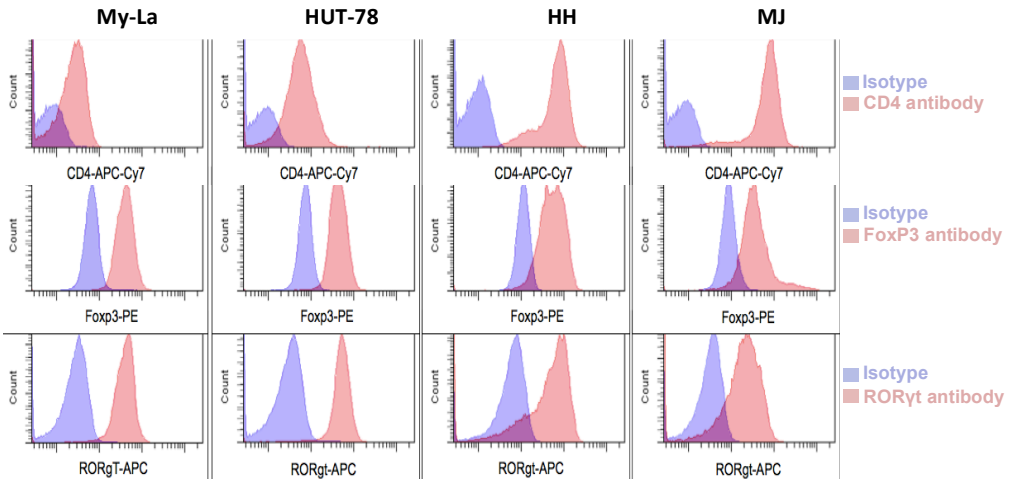

Figure 8. FACS analysis of molecular markers to define regulatory T cells and Thelper-17 in different CTCL cell lines. The blue peak represents the isotype control and the red peak the CD4, FoxP3 or RORγt antibody.

To this end we decided to analyze by FACS CTCL cells stained with monoclonal CD4, FoxP3 (Treg marker) and RORγt (Th17 marker) antibodies separately (Figure 8). The blue peak indicates the isotype control, and the red peak the monoclonal antibody. Surprisingly all the cell lines expressed CD4, FoxP3 and RORγt in this setting but considering that all CTCL cell lines were positive for the two phenotype markers in a population of cells, we decided to ask whether CTCL cells could express both markers simultaneously by gating CD4+ cells and then by checking the expression of FoxP3 (x axis) and RORγt (y axis) in a comparable manner. Strikingly we found that most cells in all CTCL cell lines were double positive for the expression of Treg and Th17 markers (Figure 9).

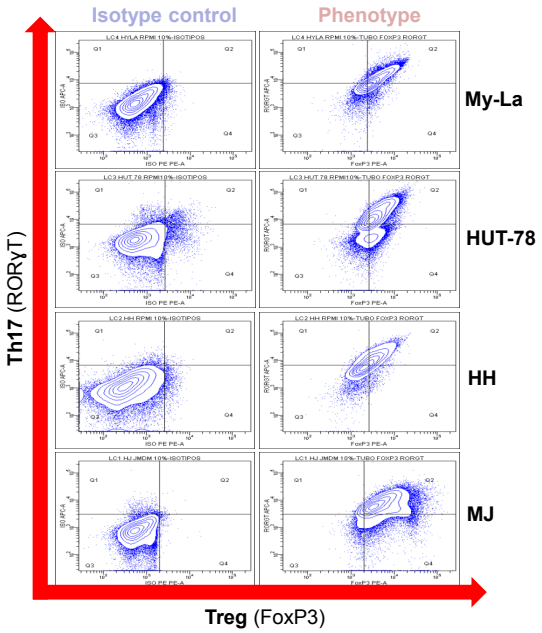

Figure 9. Density plot showing intracellular staining of master gene of Tregs and Th17 cells, anti-Foxp3 (x axis) and anti-RORγt (y axis) respectively, in four different CTCL cell lines.

4.5 FoxP3 and RoRγt protein expression of CTCL cells treated with Tacrolimus and Ruxolitinib

Since have been shown that Treg and Th17 phenotypes can be acquired by TCR and JAK/STAT signaling pathways in naïve T-cells thus activating Foxp3 and RORγt respectively, and that T-cell phenotype may behave highly dynamically as observed by a number of groups (Abraham, Zhang et al. 2011) (Eisenstein and Williams 2009) (Hoechst, Gamrekelashvili et al. 2011), we have decided to test the expression of the Treg and Th17 lineage markers (FoxP3 and RORγt respectively) after treatment with Tacrolimus and Ruxolitinib.

4.5.1 CTCL cells treated with Tacrolimus

Cells were incubated with DMSO and Tacrolimus at IC_{50} and $2XIC_{50}$ concentrations for 24 h and then CD4+ cells were checked for the expression of FoxP3 and RORγt.

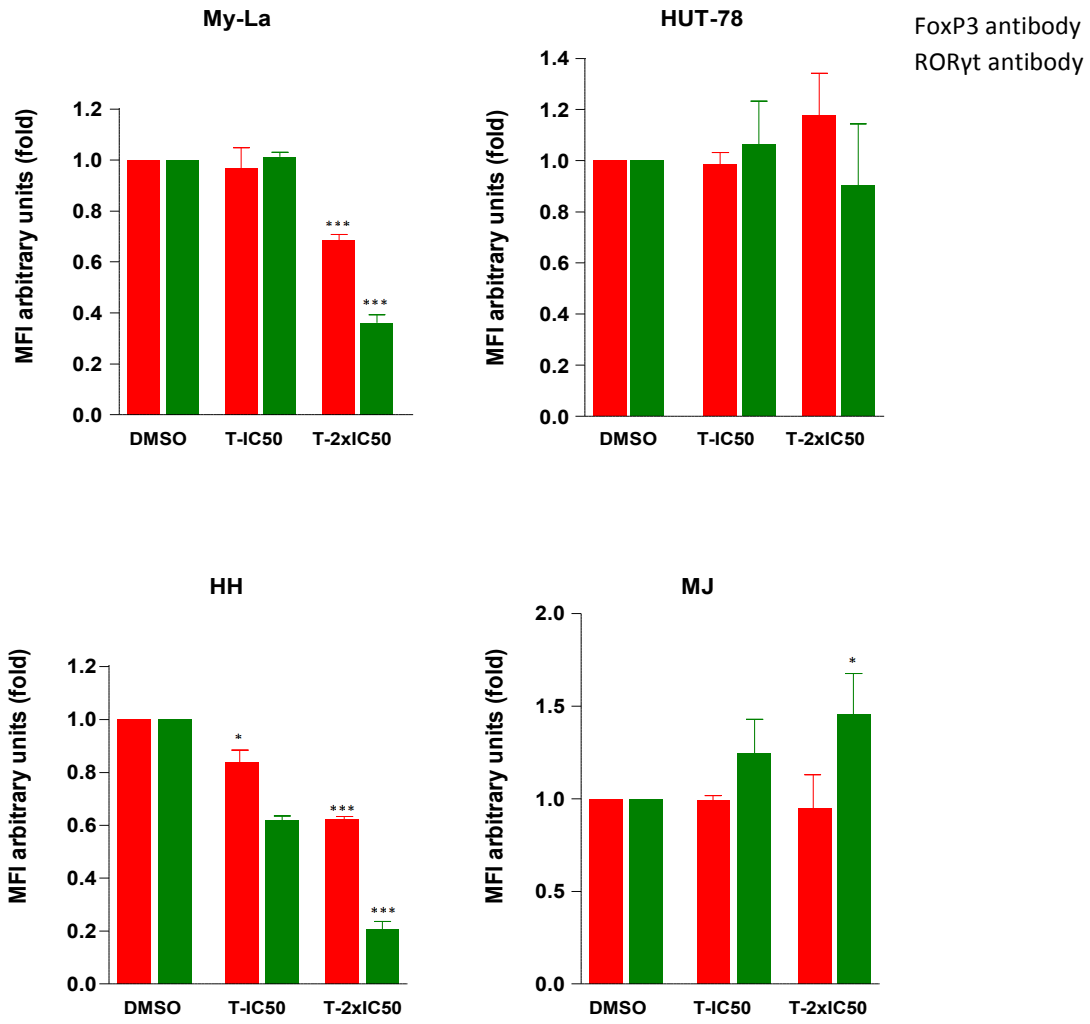

Figure 10. The mean fluorescent intensity (MFI) arbitrary units of lineage markers (FoxP3 in red and RORγt in green) of a panel of CTCL cells treated with DMSO and Tacrolimus at IC50 and 2XIC50 concentrations at 24 h. Each condition was assessed in triplicates. The graphs show the average of the triplicates ± SD. Asterisks indicate a statistical significant difference compared to control (DMSO).

As shown in Figure 10, Tacrolimus provoked an unbalance in the expression of the Treg and Th17 markers in all CTCL cell lines. In My-La and HH cell lines, Tacrolimus produce a significantly decrease in the expression of the lineage markers, whereas in HUT-78 and MJ the inhibitor does not significantly change the expression of FoxP3 and RORγt markers.

4.5.2 CTCL cells treated with Ruxolitinib

My-La and HUT-78 cells were treated with DMSO and Ruxolitinib at IC_{50} and $2XIC_{50}$ concentrations at 24 h and then stained with FoxP3 and RORγt monoclonal antibodies.

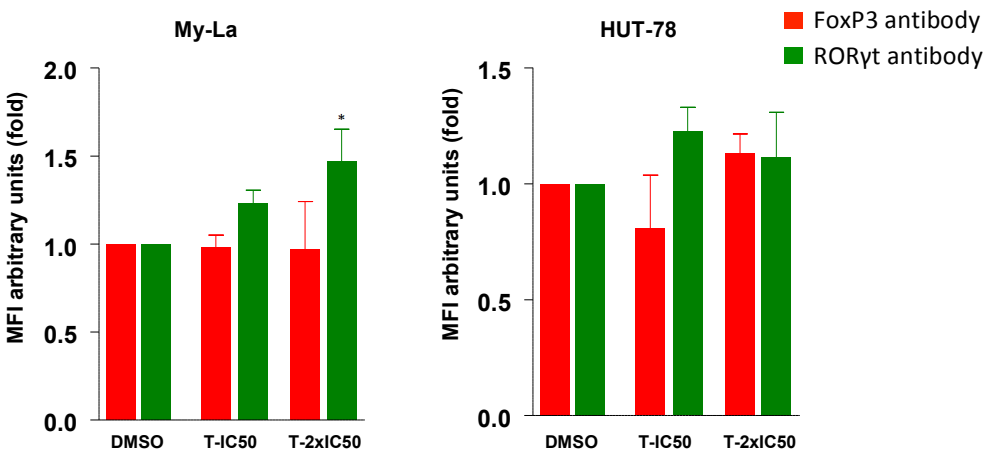

Figure 11. The mean fluorescent intensity (MFI) arbitrary units of lineage markers (FoxP3 in red and RORγt in green) in a My-La and HUT-78 CTCL cells treated with DMSO and Tacrolimus at IC50 and 2XIC50 concentrations at 24 h. Each condition was assessed in triplicates. The graphs shows the average of the triplicates ± SD. Asterisk indicate a statistical significant difference compared to control (DMSO).

The effect of Ruxolitinib in My-La and HUT-78 cells is represented in Figure 11. Ruxolitinib does not provoke a significant effect in the expression of Treg and Th17 markers in HUT-78 but it does when the cells are treated with Ruxolitinib at $2XIC_{50}$ concentrations for RORγt.

CHAPTER 5

Discussion

5.1 CTCL landscape

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of diseases characterized by the clonal expansion of malignant T-cells in the skin (Willemze, Jaffe et al. 2005). Despite the molecular pathogenesis of CTCL is still basically unknown, some data including gene expression profiling studies have shown that increased signaling from the T-cell receptor (TCR), due to at least in part by genetic rearrangements, can be considered a driving force of CTCL. This absence of knowledge about the molecular pathogenesis of CTCL may be an important reason why there is a current lack of a standard therapy for CTCL patients and provides us with an opportunity to explore in depth its molecular pathology to develop new targeted therapy.

In order to elucidate the mechanistic landscape driving the pathogenesis of CTCL, our group has performed a mutational analysis of 524 genes with known biological relevance in normal T-cells. As part of the results, we found activating mutations recurrently affecting TCR signaling pathway genes (PLCG1) and in JAK/STAT pathway genes such as IL6S/T, JAK1 and JAK3 (see Figure 1). Since both signaling pathways have been shown to control proliferative, survival and phenotypic activities in normal T-cells, we have decided to explore the biological effects that the use of Tacrolimus (FK506, an inhibitors of calcineurin a phosphatase effector downstream of PLCG1 signaling) and Ruxolitinib (a JAK inhibitor) provoke in a panel of neoplastic human CTCL cells. With the inhibition of these two pathways we aim at exploring new therapeutical strategies to fight this malignancy.

5.2 Identification of new targeted therapy for CTCL

We have analyzed the pharmacological and targeted inhibition of TCR/PLCG1 and JAK/STAT signaling pathways in a panel of CTCL cell lines. Our results indicate that the specific inhibition of PLCG1 downstream signaling has an inhibitory effect in proliferation (Figure 2) and survival activities of CTCL cells (Figure 3). This correlates well with the fact that deregulated TRC signaling has been pointed to be a driving force of this malignancy and considering that PLCG1

is considered a mayor effector of TCR-mediated signaling and also with our findings where PLCG1 has been found recurrently mutated in 20% of a series of CTCL patients (Vaqué JP et al. submitted for publication). On the other hand, the impact of targeted inhibition of JAK/STAT pathway was more pronounced in proliferation (Figure 4) than in survival (Figure 5). The combined used of these two inhibitors was significantly more effective than each inhibitor used alone in three of the CTCL cell lines. To our knowledge these results are the first evidences for the use of a combinatorial targeted therapy developed on the basis of a mutational analysis in CTCL, which in the future may serve as a lead to explore into detail about a potential utilization of this therapeutical approach in the clinic.

A number of groups (Abraham, Zhang et al. 2011) (Eisenstein and Williams 2009) (Hoechst, Gamrekelashvili et al. 2011) have described CTCL lesions showing a degree of phenotypic plasticity where neoplastic cells can acquire characteristics of Treg and Th17 lineages during the course of the disease. In this line of evidence it has also been proposed that early CTCL lesions can present a high Treg activity that is not present at more advanced stages (Tiemessen, Mitchell et al. 2006) and that activated STAT3 (that can lead to RORγt expression (Ivanov, McKenzie et al. 2006)) and Th17 activity can be detected in aggressive forms of this disease (Krejsgaard, Ralfkiaer et al. 2011). The acquisition of these Treg and Th17 phenotypes could be explained by those somatic mutations found by our group affecting TCR/PLCG1 and JAK/STAT pathways (Figure 1) which have been widely shown to regulate the expression of FoxP3 and RORγt respectively.

Surprisingly, our results evidence a novel hybrid Treg/Th17 phenotype in our panel of CTCL cell lines, as assessed by the simultaneous protein expression of specific lineage markers like FoxP3 and RORγt respectively that we could alter by using specific inhibitors of the two aforementioned signaling pathways. Although these are still primary results, it will be important to confirm these phenotypes in cells directly obtained from CTCL patients and also explore how to take clinical advantage of these phenotypic characteristics in patients. As for this we could alter the biological activities by means of the putative Treg/Th17 acquired activities of the neoplastic cells and their interactions with the tumoral microenvironment in the CTCL lesions by interfering specifically with TCR/PLCG1 and/or JAK/STAT downstream signaling using Tacrolimus and/or Ruxolitinib respectively.

CHAPTER 6

Conclusions

1. Targeted TCR/PLCG1 and JAK/STAT signaling pathway inhibitors, Tacrolimus and Ruxolitinib respectively produced a dose dependent inhibition of My-La, HUT-78, HH and MJ cell proliferation.

2. Tacrolimus but not Ruxolitinib markedly decreased cell survival at 24 hours.

3. Combined use of Tacrolimus and Ruxolitinib produced a significant greater inhibition of MY-La, HH and MJ cell proliferation than each inhibitor alone.

4. MY-LA, HUT-78, HH and MJ cells can simultaneously express phenotypic markers for Treg and Th17 lineages (FoxP3 and RORγt respectively).

5. Targeted inhibition of PLCG1 and JAK downstream signaling can disrupt the phenotypic status of CTCL cells.

CHAPTER 7

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