Novel approaches for targeted therapy in CTCL

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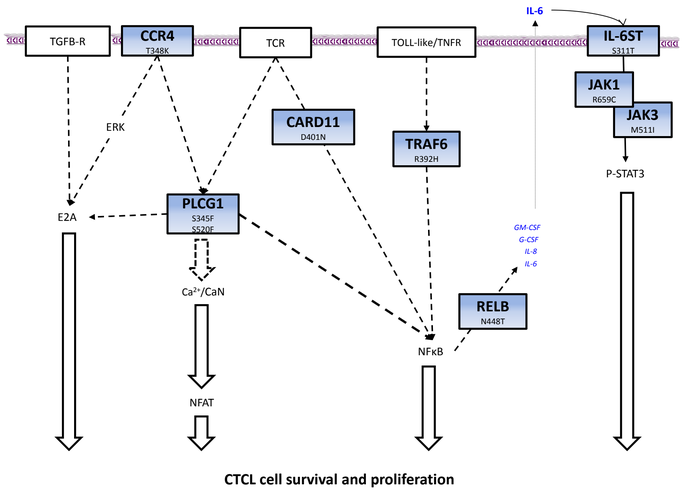
Introduction

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of diseases characterized by the clonal expansion of malignant T-cells in the skin[1]. The two predominant clinical forms of CTCL are Mycosis Fungoides (MF) and Sézary Syndrome (SS). Tumor-stage MF has an unfavorable prognosis with a 10-year survival of approximately 40%, while SS is even more aggressive, with a median survival of around two years[2]. 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) can be considered a driving force of CTCL and that the neoplastic T-cells can acquire Treg and Th17 phenotypes[3].

Using a targeted ultrasequencing analysis focused on 524 genes with known biological relevance in normal T-cells, we have studied the mutational status of paired (non tumoral vs. tumoral) genomic DNA from 11 CTCL patients. As part of our results we found activating mutations recurrently affecting PLCG1 (3/11 patients and also 20% in an independent series analysis). 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 IL-6ST–JAK/STAT pathway genes like IL-6ST, JAK1 and JAK3 were also found mutated in 3/11 patients[4], suggesting that this pathway can also be tested for its clinical implications in this disease. 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 (a CaN inhibitor) and Ruxolitinib (a JAK inhibitor) provoke in neoplastic human CTCL cells. Our results indicate that targeted inhibition of these pathways using already clinically tested drugs can potentially be used to develop new therapeutical strategies to fight this malignancy.



(Modified from Vaqué, JP et al. Submitted)

Mechanistic landscape driving the pathogenesis of CTCL. Schematic representation showing key T-cell pathways harboring somatic mutations as found in our ultrasequencing analysis (mutations are highlighted in blue rectangles)[5]. 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.

Objectives

- Test the **proliferation and survival** responses that **specific pharmacological inhibitors** of PLCG1 and NF-κB-JAK/STAT downstream signaling (Tacrolimus and Ruxolitinib respectively) 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.

Materials and Methods

- Cutaneous T-cell lymphoma cell lines used were HH (MF) and MJ (MF) obtained from ATCC (Rockville, MD, US) and My-La (MF) and HUT-78 (SS) obtained from ECACC (Salisbury, UK).
- Cell proliferation analyses were performed using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) with the appropriate amount of inhibitor, as indicated in the figures, at 0h, 24h and 48h following manufacturer´s instructions. Cell lysates were analyzed using TR717 Microplate Luminometer (Applied Biosystems). All experiments were done in triplicate and all numerical data were expressed as the average of the values ± the standard error of the mean (SEM).
- The induction of apoptosis were evaluated by flow cytometry using FlowCellect Annexin Red Kit (EMD Millipore Corporation, Billerica, USA). All data were detected on aFACS Calibur flow cytometer (BD) and analyzed using CellQuest Pro software (BD).All experiments were done in triplicate and all numerical data were expressed as the average of the values ± the standard error of the mean (SEM).
- Treg/Th17 phenotype was determined by flow cytometry. Cells were stained with monoclonal antibodies: anti-CD4-allophycocyanin (APC)-Cy7 (clone SK3, Becton Dickinson, San Diego, CA). Gated CD4+ cells were fixed and permeabilized using Fix and Permeabilization kit (eBioscience) 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).

Conclusions

- Tacrolimus and Ruxolitinib produced a dose dependent inhibition of My-La, HUT-78, HH and MJ cell proliferation.
- Tacrolimus but not Ruxolinitib markedly decreased cell survival at 24 hours.
- Combined use of Tacrolimus and Ruxolitinib produced a significant greater inhibition of CTCL cell proliferation than each inhibitor alone.
- CTCL cells can simultaneously express phenotypic markers for Treg and Th17 lineages.
- Targeted inhibition of PLCG1 and JAK downstream signaling can disrupt the phenotypic status of CTCL cells.

Perspective

- Can activated PLCG1 and/or JAK downstream signaling lead to a more aggressive disease?
- Can we develop effective therapeutical strategies for CTCL based on patient mutational profiling?
- Can we take clinical advantage of the phenotype disruption in patients treated with targeted therapies?

References

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[3] Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature*. **467**:967-971 (2010)

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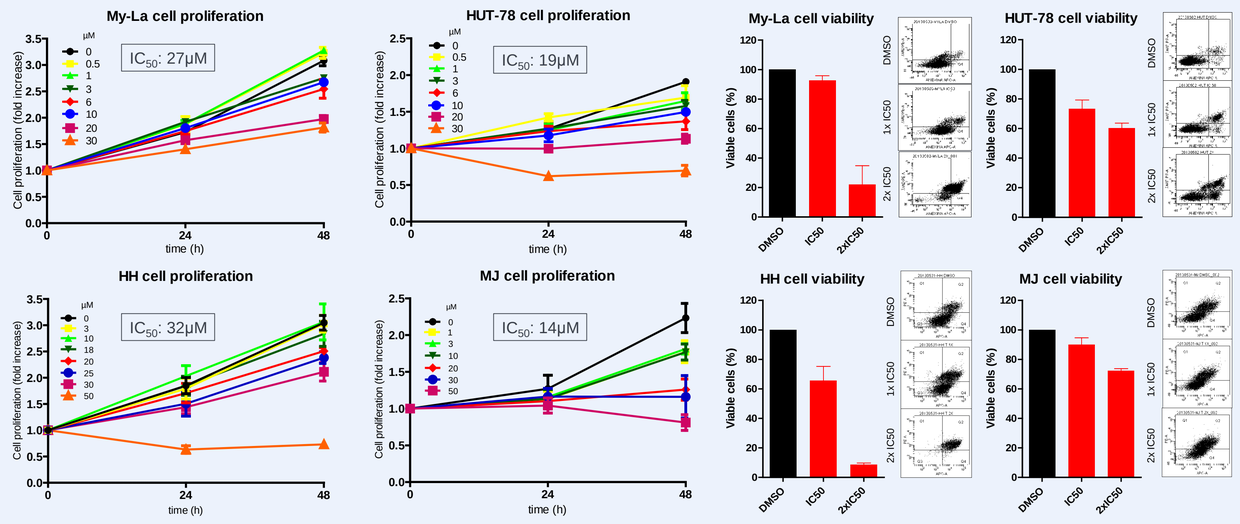
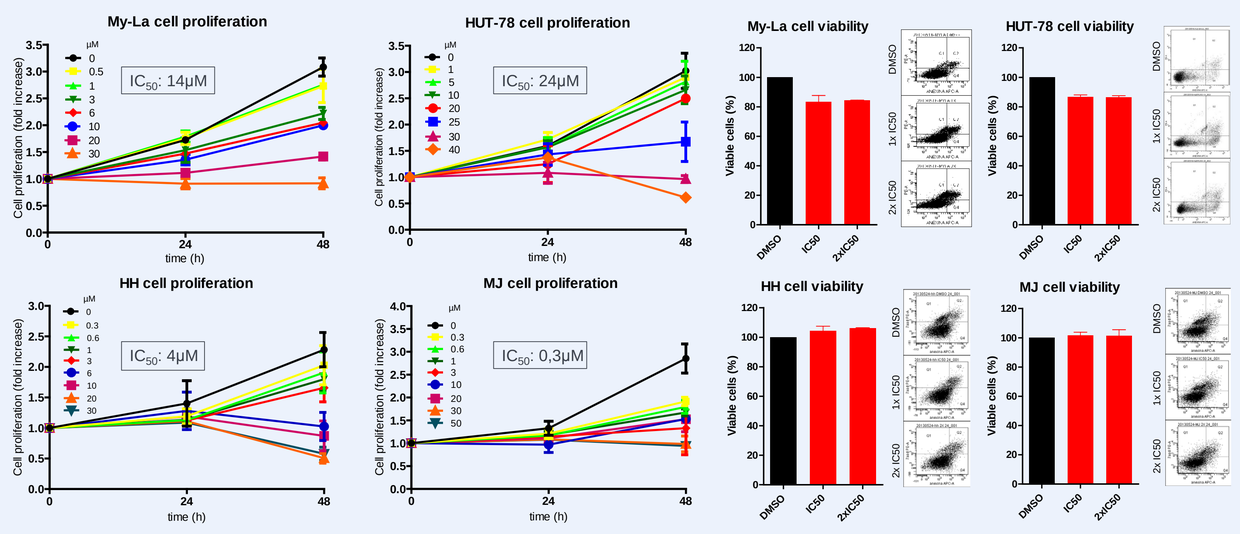
Results 1: Effects of targeted therapy in proliferation and survival of CTCL cells

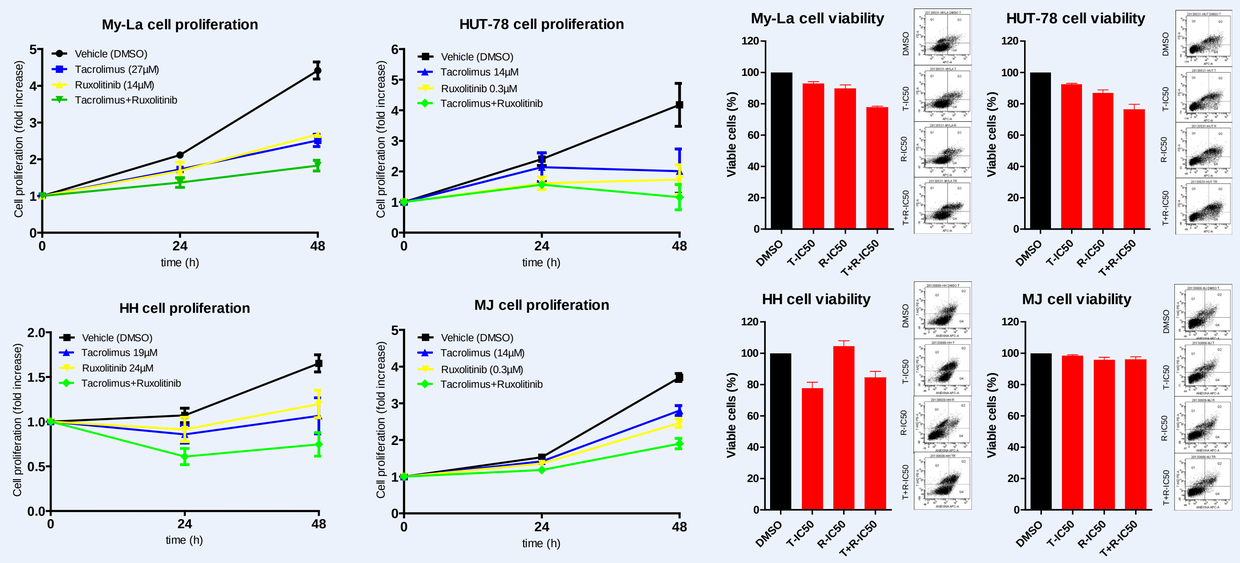
Tacrolimus affects cell proliferation and survival of CTCL cell lines



Ruxolitinib mainly affects CTCL cell proliferation vs. survival

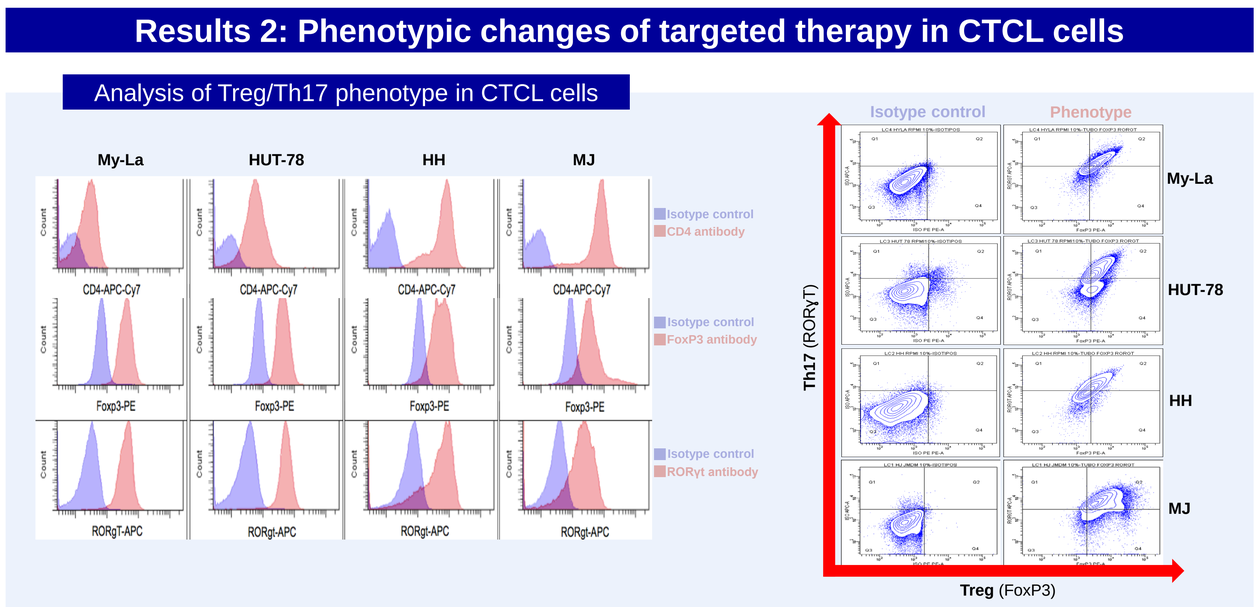


Combined effects of **Tacrolimus** and **Ruxolitinib** in CTCL cell proliferation and survival



Results 2: Phenotypic changes of targeted therapy in CTCL cells

Analysis of Treg/Th17 phenotype in CTCL cells



FoxP3 and **RoRγT** protein expression of CTCL cells treated with Tacrolimus and Ruxolitinib