5-11-2023

Retinoids and their Clinical Benefits to Treating Cutaneous T-Cell Lymphoma

Baker Kendrick

SENIOR THESIS APPROVAL

This Honors thesis entitled

“Retinoids and their Clinical Benefits to Treating Cutaneous T-Cell Lymphoma”

written by

Baker Kendrick

and submitted in partial fulfillment of
the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

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Dr. Tim Knight, thesis director

_____

Dr. Lance Bridges, second reader

_____

Dr. Brian McKinney, third reader

_____

Dr. Barbara Pemberton, Honors Program director

Date

May 7, 2023

Introduction

Cutaneous T-Cell Lymphoma (CTCL) is a deadly form on non-Hodgkin lymphoma that primarily affects the skin. Non-Hodgkin lymphoma means that the lymphoma does not have the presence of Reed-Sternberg lymphocytes. A lymphoma, also known as lymphatic cancer, affects the lymphoid tissues, which plays a major role in the immune system. Lymphoid organs include the lymph nodes, thymus, spleen, and bone marrow. Lymphatic fluid, also known as lymph, travels through the blood stream to transport lymphocytes, a type of white blood cell, to and from lymphatic organs. Due to a high concentration of lymphocytes in the skin, lymphoma primarily affects cutaneous areas.

There are many classifications and presentations of CTCL. The most common form is mycosis fungoides. Mycosis fungoides is presented as pink itchy rash made up of rapidly dividing T-cells that have made their way from to blood into the skin. These cells target the skin due to their expression of the skin-homing receptor cutaneous lymphoid antigen. These are primarily CD4+ memory T cells. CD4+ memory T cells are a form of helper T cell, which are tasked with marking foreign cells for later destruction by CD8+ cells, also known as cytotoxic, or "killer" T cells. Currently there is very little understanding of the disease etiology or origin.

There is currently one FDA approved retinoid for CTCL treatment, Bexarotene (Targretin®). Bexarotene is among vitamin A derivatives that affect many processes. These include but are not limited to cellular differentiation, apoptosis, and proliferation. Retinoids operate by binding to retinoic acid receptors (RAR) and retinoid X receptors (RXR). Among these RAR and RXR receptors are three subtypes of each (-α, -β, and -γ). Ligand activation of these subtypes typically result in the dissociation of corepressors that control proliferation and

differentiation. In simpler terms, binding of ligands to these receptor proteins will induce cellular reproduction and specialization. Unfortunately, Bexarotene has been found to cause serious side effects such as hyperlipidemia, hypercholesterolemia, and hypothyroidism. These side effects can be found in roughly one-in-five users, and it has a particularly high prevalence in African American males. Hyperlipidemia results in a higher concentration on lipids in the blood, which can result in the restriction of blood flow. Hypercholesterolemia is an excess in blood cholesterol, excluding triglycerides, which can lead to heart disease. Hypothyroidism is the underactivity of the thyroid gland, which produces thyroid hormone. Imbalance in thyroid hormone can result in abnormal heart rate, body temperature, and metabolism.

Understanding the molecular function of retinoids and how they affect CTCL will be beneficial in future attempts to synthesize drugs with a higher specificity, hopefully eliminated the metabolic side effects that affect its patients.

Retinoids have long been used as an anti-infective agent, and it is known from previous research that non-malignant T-cells use retinoid binding to induce the differentiation into mucosa-associated lymphoid tissues (MALT). It is also known that exposure to Bexarotene induced the expression of integrin protein β7 and the chemokine receptor CCR9. These proteins facilitate lymphocyte homing to the digestive tract. It is possible that exposure to Bexarotene can “trick” a cancerous cell into “thinking” that is belongs in the digestive tract. A gut lymphocyte found in the skin would likely not be able to survive its environment, making it a plausible cause for apoptosis and CTCL treatment. The goal of this project is to determine if retinoids cause a level of cellular differentiation by determine the expression of two known T cell transcription factors (FOXP3 and ROR-γ) within CTCL lines.

In this experiment, experiments were run to collect data to determine: baseline levels of FOXP3 in human CTCL cells vs. healthy cells, rates of cell death when treated with different isoforms of RXRs and RARs at varying concentrations, the degree to which cells can recover after retinoids have been removed from the media, synergism when treated with pairs of different isoforms, and baseline levels of FOXP compared to ROR-γ and T-Bet in human CTCL lines.

Materials and Methods

2.1 Cell culture

SeAx and MyLa cell lines were provided by Dr. Robert Gniadecki (University of Copenhagen). The RPMI8866 B-cell line was given by J.C. Wilkins (University of Manitoba). The MOLT-4 cell line was the kind gift of Dr. Ted Bertrand (UAB, Birmingham, AL). The T cell lines, Jurkat, CCRFCEM, HuT78, MJ, HuT102, HH were obtained from ATCC (Manassas, VA). All lines were maintained in RPMI 1640 media containing 10% FBS, 1% sodium pyruvate, and 1% penicillin/ streptomycin at 37°C in a 5% CO_2 atmosphere. SeAx and HuT102 lines were supplemented with 10 U/mL IL-2 from Sigma (St. Louis, MO) to aid growth.

2.2 Retinoids

Naturally occurring retinoids (ATRA, 9-*cis* RA, 13-*cis* RA) and Bexarotene were purchased from Sigma (St. Louis, MO). The pan-RXR agonist UAB30 was a generous gift from Dr. Clinton J. Grubbs (UAB, Birmingham, AL). All nuclear receptor agonists and antagonists were obtained from TOCRIS bioscience. The isoform selective agonists employed were AM580 for RAR-α, CD2314 for RAR-β, and BMS961 for RAR-γ. These agents were administered at the established $2\times EC_{50}$ levels to

minimize activation of other receptor isotypes. The RAR isoform selective antagonists were ER50891 and BMS195614 for RAR-α, LE135 for RAR-β, MM11253 for RAR-γ, and the RAR-β,-γ dual antagonist CD2665. UVI3003 and CD3254, were also products of TOCRIS. Concentrations of CD3254 were based upon established work. All retinoids were dissolved in DMSO and handled in a manner that restricted light exposure.

2.3 Flow Cytometry

Cell permeabilization, fixation, and staining with the APC conjugated anti-FOXP3 monoclonal antibody was performed according to the manufacturer's recommendations from Life Technologies with scaling modification. Briefly, cells were harvested with HEPES Tyrodes solution (5 mM HEPES, pH 7.4, 150 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 0.2 mg/ml BSA, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$) and enumerated with Guava ViaCount® Assay. A total of 500,000 cells were then spun down at 1000 x g for 5 minutes, supernatant was discarded, and 200 mL of the FOXP3 fixation/permeabilization solution was applied for 30 minutes at room temperature in the dark. After fixation, 200 mL permeabilization buffer was added, cells were pelleted and washed with an additional 200 mL of permeabilization buffer to ensure removal of fixative before antibody addition. Pellets were resuspended with 100 mL of permeabilization buffer. 5 mL of the APC conjugated anti-FOXP3 antibody was added for 30 minutes at room temperature. 500 mL of the permeabilization buffer was added to each sample to wash unbound or disrupt nonspecifically bound antibody. Cells were centrifuged and suspended in 500 mL of HEPES Tyrodes for analysis by Guava easyCyte flow cytometer.

2.4 Statistical analyses

All analyses were performed using GraphPad Prism 8.4 (La Jolla, CA). A student's *t* test was employed to establish significance when comparing experimental conditions to vehicle alone. Where appropriate, a one-way ANOVA with a Tukey's *post hoc* test was utilized for multiple comparisons. Significance is denoted with asterisks (*P<0.05, **P<0.01). All data reflect a representative experiment done in triplicate, and all error bars represent the standard deviation of technical triplicates in a single experiment.

Experimental

The following section contains a detailed account of the experiments undergone during the summer of 2022. Experiments included at *Cell Culture Dosing of CTCL Cell Lines for Intracellular Analysis* and *Antibody Intracellular Protein Analysis*

Cell Culture Dosing of CTCL Cell Lines for Intracellular Analysis

Beginning the project involves dosing of multiple CTCL cell lines. These cell lines, named MJ, Hut78, and Myla, are the lines used to conduct intracellular protein analysis following dosing. The MJ, Hut78, and Myla cell lines have been extracted from CTCL patients and given code names to preserve patient confidentiality (perhaps the "MJ" line came from a patient named Mary Jane, for example). Cell culture is needed to grow the CTCL cells in a sterile environment as well as dose. No testing was conducted on any noncancerous cell lines. Cells were initially dosed into T75 flasks (75ml). T75 flasks take roughly 3 days to become confluent enough to split into T25 flasks (25 ml).

For splitting and maintaining cell lines in media:

1. Pour liquid from T75 flask into a centrifuge conical. Spin down at 1000rmp for 5min.
2. Remove supernatant – media.
3. Resuspend in 10mL of fresh warm media.
4. Place 1mL into each T25 Flask with 9mL of fresh warm media.
5. Place 3mL of leftover cells into a T75 flask with 25mL of media as a maintenance flask.

I. Make Required Media: a. *RPMI Media* -

i. 500mL *RPMI Media 1640 1x Premade Solution w/L-Glutamine + 25mM HEPES*

ii. 50mL Fetal Bovine Serum. Fetal Bovine Serum (FBS) is a serum that has been separated out of fetal blood. FBS has very low level of antibodies and contains more growth factors than adult bovine serum. The naive immune system reduces the risk of an immune response but gives the growth factors needed for cell growth.

iii. 5.5mL Sodium Pyruvate. Sodium pyruvate is a supplement added to improve cell survival in culture. Pyruvate is an intermediate in the glycolytic pathway. This improves the cell's ability to metabolize glucose in the media to produce energy.

iv. 5.5mL Penicillin Streptomycin. This is an antibiotic used to prevent bacterial contamination. Antibiotics target bacterial microbes, not human cell lines.

I. Take confluent T75 flask of each different cell line used and split 1:10 – Putting 1mL of resuspended cells into 3 separate T25 flasks (per cell line) each with 9mL of RPMI Media.

There is about 25mL in the T75 to begin. This is then spun down, and the supernatant (media) discarded. The cell pellet is then resuspended in 10mL of media, and 1mL is added to each T25.

II. Dose cells in T25 with retinoids- 3 Conditions:

V (Vehicle) – Dose with 1uL DMSO for control

ATRA (All Trans Retinoic Acid) – Dose with 100nM ATRA

Bex (Bexarotene) – Dose with 1uM Bex

III. Gently swirl to mix in retinoids, ensuring care not to get media on filter flask top.

IV. Incubate for 72 Hours in 5% CO2 Incubator at 37 degrees Celsius. Each line MUST be incubated for the same length of time to ensure there are no variables in conditions between cell lines.

V. Start Antibody Intracellular Analysis.

Antibody Intracellular Protein Analysis Protocol

The purpose of Antibody Intracellular Protein Analysis is to find what the CTCL cells are differentiating into – T-Bet, ROR-γ, or FOXP3 by testing the amounts of receptor protein in each cell line.

VI. Make required buffers:

a. *FOXP3 Fixation/Permeabilization Working Solution (Working Solution)-*

i. 3mL FOXP3 Fixation/Permeabilization Diluent

ii. 1mL FOXP3 Fixation/Permeabilization Concentrate

b. 1x Permeabilization Buffer –

iii. 1mL 10x Permeabilization Buffer

iv. 9mL DI Water

- Parts A-C make up the fixation and permeabilization solution. This will fix the cells in place while also permeabilizing the cell so that the antibody may enter the cell without lysing the cell and losing its contents.

VII. Pour flasks into their respectively labeled 14mL centrifuge tubes. Spin in centrifuge at 1000 x g for 5 minutes. This step removes the media containing free retinoids to allow the cells to be properly analyzed.

VIII. Discard test tube supernatant and resuspend pellet in 380µL of Hepes Tyrodes. Fill resuspended tube to 10mL with Hepes Tyrodes. Place in centrifuge to spin at 1000 x g for 5 minutes. At this step, the cells are in the pellet. Hepes Tyrodes is a buffer solution used for cell washing, enabling the cells to be analyzed without it affecting or changing the analysis results.

IX. Discard test tube supernatant waste into waste container and gauge how much Hepes Tyrodes to resuspend pellet in. This is determined by trial-and-error. This is because a cell count must be done on Guava-Via Count to determine how much volume to add of resuspended cells to have even number of cells (500,000) for the assay. If too much is resuspended, it is too diluted for the flow to count. If too little is resuspended, it is too concentrated for the flow to accurately count. This is gauged by analyzing cell pellet

size and estimating how much. If too much is diluted, cells will be re-spun down to re-attempt with less liquid following a re-count. If it is diluted too little, more will be added following a re-count.

X. Acquire correct number of vials needed for the number of samples obtained. Transfer 380µL of Guava Via-Count into each vial. Transfer 20µL of each resuspended pellet to respective vials.

XI. Place in Guava Count Instrument to Via Count. Via Count determines the count of total live cells and dead cells so volume required to maintain 500,000 cells may be determined.

XII. Acquire correct amount of microfuge tubes needed for the samples obtained, including both a V- and a V+ for the Vehicle. Add 500,000 cells to labeled tubes by using the calculating:

XIII. Spin down at 1000 x g for 5 minutes. Pipet out supernatant surrounding pellet, ensuring not to pipet out the pellet.

XIV. Spin down at 1000 x g for 5 minutes. Pipet out supernatant surrounding pellet

XV. Resuspend in 200µL of working solution buffer. Allow to sit in the dark for 30 minutes (antibodies are light-sensitive).

XVI. Centrifuge at 1000 x g for 5 minutes. Pipet out supernatant surrounding pellet.

Resuspend in 200µL of 1x Permeabilization Buffer. This removes the working buffer so that any unbound antibody can be washed away.

XVII. Repeat Step X.

XVIII. Pipet out supernatant surrounding pellet. Resuspend in $100\mu L$ of 1x Permeabilization Buffer. Inject $5\mu L$ of FOXP3 into each sample (except for V-). Allow to sit in the dark for 30 minutes. This permeabilizes the cell in case FOXP3 is an intracellular receptor.

XIX. Add $500\mu L$ of 1x Permeabilization Buffer. Centrifuge at 1000 x g for 5 minutes. Pipette out supernatant surrounding pellet. Resuspend stained cells in $500\mu L$ of Hepes Tyrodes. Hepes Tyrodes serves as the buffer used to resuspend cells for analysis.

XX. Analyze samples by flow cytometer (if doing triplicates, place $150\mu L$ in 3 wells per samples). R-R-Log Channel APC Conjugated is the specific laser channel used on flow cytometry.

1. Results

3.1 FOXP3 expression is enriched in CTCL cell lines compared to non-CTCL immune lineages.

Because the heterogeneity of CTCL is very complex, the original goal was to determine the expression of the T-Reg-specific transcription factor FOXP3 within established CTCL cell lines. All six CTCL lineages analyzed exhibited detectable levels of FOXP3 with anti-FOXP3 stained cells averaging a 5-fold higher signal than unstained cells (Figure 1A). In contrast, a B cell line, RPMI 8866, which is not expected to express Treg markers, exhibited a nominal shift in fluorescent signal, a 1.5-fold increase over unstained cells (Figure 1B). Of note, non-CTCL T cell lines (e.g. Jurkat, Molt-4, and CCRF-CEM) exhibited signals comparable to the RPMI 8866 B cell line

indicating FOXP3 staining was enriched within the CTCL etiology compared to other common lymphoma lines analyzed.

3.2 Retinoid exposure decreases FOXP3 expression within CTCL cell lines

Next, it was determined if retinoid exposure alters expression of the FOXP3 T-Reg marker within CTCL cells. Bexarotene significantly decreased the expression of FOXP3 in a dose dependent manner within the MJ cell line when compared to the control retinoid UVI3003, an RXR antagonist. UAB30, a separate pan-RXR agonist with proven efficacy in multiple cancer settings including CTCL[22], also decreased FOXP3 expression (Figure 2A). Then MJ exposure to naturally occurring retinoids was assessed, i.e. retinoic acids, which function as pan-RAR agonists and are also used therapeutically. As with the synthetic retinoids, naturally occurring retinoids decreased the expression of FOXP3 in MJ cells in a dose-dependent manner (Figure 2B). Interestingly, the novel differentiation effects of synthetic or natural retinoids were not permanent or conferred to progeny. After an initial retinoid exposure, cells were washed and sub-cultured for an additional 96 hours in media lacking retinoids. Irrespective of the retinoid utilized, FOXP3 expression was significantly lower when the retinoid was present (Figure 2C). Removal of the pan-RXR agonists resulted in recovery of FOXP3 expression to levels obtained with vehicle treated cells. While removal of ATRA, a pan-RAR agonist, resulted in a statistically significant increase in FOXP3 expression within 96 hours, FOXP3 levels did not fully return to vehicle levels. As much of the data gathered utilized MJ cells only, the group assessed the effect

of retinoids on five additional human CTCL cell lines. All CTCL lines exhibited significantly decreased FOXP3 expression in response to retinoid exposure (Figure 2D).

3.3 RXR/RAR receptor synergism potentiates retinoid dependent FOXP3 expression changes

As separate activation of either RXR or RAR decreased FOXP3 expression in CTCL cells, it was determined if concomitant activation of both receptors would prompt expression changes through receptor synergism. Synergism allows abbreviated exposure times and lower ligand concentrations to achieve comparable cellular responses[23,24]. Indeed, dosing cells with nanomolar levels of Bexarotene or ATRA yielded no difference in FOXP3 expression when compared to vehicle treated cells. However, when the same nanomolar doses of Bexarotene and ATRA were added to MJ cells simultaneously, FOXP3 expression decreased significantly, indicating that RXR/RAR nuclear receptor synergism drives a decrease in FOXP3 expression in CTCL cells (Figure 3A).

The next goal was to attribute the observed changes in FOXP3 expression to the activity of specific nuclear receptor isoforms. Application of RAR isoform-selective agonists to MJ cells demonstrated that the RAR-α isoform clearly drives the changes in FOXP3 expression observed (Figure 3B). While RAR-β and RAR-γ isoforms contributed to statistically significant changes in FOXP3 expression, this response was only observed at the longest exposure time point of 72 hours. In contrast, the RAR-α agonist generated significant decreases at all exposure times examined, including after only 24 hours of exposure. To validate the findings regarding RAR-α activity, an antagonist approach was employed. Selective antagonists to RAR-β or RAR-γ isoforms

failed to prevent ATRA-mediated decreases in FOXP3 expression. Blocking RAR-α receptor activation with two distinct RAR-α selective antagonists, ER50891 or BMS195614, effectively inhibited FOXP3 decreases in CTCL cells treated with ATRA (Figure 3C). Efforts to identify RXR isoforms are greatly frustrated due to lack of reagent availability[25,26]. However, CD3254, a commercially available RXR-α selective agonist, allowed us to address this aspect to some extent. As with RAR-α activation, RXR-α selective activation resulted in a dose-dependent decrease in FOXP3 expression (Figure 3D).

3.4 FOXP3/T-Bet/RORγ expression in 4 human CTCL lines

After experiments 1-3, the assumption that FOXP3 is the best receptor protein for CTCL diagnosis came into question. FOXP3 is the hallmark transcription factor of T-regulatory cells. It was then determined if ROR-γ (transcription factor in TH17 cells), or T-Bet (transcription factor in TH1 cells) would be a more optimal indicator than FOXP3 for CTCL identification. The baseline levels of ROR-γ, FOXP3, and T-Bet were measured in 4 CTCL lines. The same procedure used to measure baseline FOXP3 levels in experiment 3.1 was used here, but all antibodies for all three transcription factors were added. Results indicated that ROR-γ levels were relatively high in all four cell lines in contrast to FOXP3 and T-Bet.

4. Discussion

The paradigm of retinoid-induced differentiation as a basis of therapeutic intervention embodies a milestone in the treatment of acute promyelocytic leukemia (APL) [27]. The major success of this strategy in APL prompted consideration of the possibility that retinoids might also impact the differentiation state of CTCL. To this end, the expression profile of the hallmark Treg marker FOXP3 in CTCL cells following treatment with clinically relevant retinoids was assessed.

Though FOXP3 T cells have been recovered from skin biopsies of patients with CTCL, the expression level of FOXP3 within CTCL cells residing in the skin niche remains to be definitively established. Studies reporting FOXP3 expression in the context of CTCL patients is complicated with varied detection of FOXP3 expression in biopsies, skin infiltrates, and peripheral blood lymphocytes (PBL). Select studies report minimal FOXP3 staining in PBLs and neoplastic infiltrates of CTCL patients but propose the expression is sufficiently significant that FOXP3 staining may serve as a distinguishing biomarker between CTCL variants or stages[28-30]. Additional work demonstrates the clear presence of FOXP3 within CTCL, yet variants associated with greater tumor burden exhibit a lower level of FOXP3 expression[31]. Knol and colleagues observed a greater abundance of Tregs within the PBL fraction of a CTCL cohort compared to healthy donors[32]. Importantly, Knol *et al*. were one of the only groups to consider the effects of retinoid-based strategies on the FOXP3 dynamic but reported no alteration in the distinct Treg proportions between the groups after 6 months of Bexarotene treatment. Part of this ambiguity is due to the heterogenous nature of CTCL coupled with a lack of clear markers for identification and isolation of malignant T cells from skin. Due to these confounding issues, numerous CTCL-derived cell lines were utilized to demonstrate that FOXP3 expression in CTCL cells is variable, but routinely detectable, and changes upon retinoid exposure.

The current work further demonstrates RAR-α function to be critical in prompting changes in FOXP3 expression. Linking RAR-α activity to gut tropism, growth arrest, apoptosis and now Treg differentiation status, establishes that this RAR isoform dictates retinoid responsiveness within CTCL lineages[15,37,38]. Collective evidence indicates that targeting RAR-α could yield the increased efficacy long desired for retinoids. For instance, the unparalleled success of retinoids in APL stems from the identification of an aberrant RAR-α fusion protein that promotes myeloid neoplasia[39,40]. Furthermore, recent studies have mapped and functionally characterized an RAR-α mutation within a patient with peripheral T-cell lymphoma[37]. In contrast to RAR isoforms, defining the RXR isoform contribution to health and disease has been problematic. RXR isoform selective agents are not as readily available or characterized; a barrier stemming from the extensive structural homology of the RXR subtypes[25,26]. However, this landscape is rapidly changing, and a commercially available RXR-α selective retinoid, CD3254, alone prompted changes in FOXP3 expression (Figure 3D). Most notable, the changes in FOXP3 expression were prompted with concomitant RAR/RXR activation at retinoid concentrations that failed to generate detectable changes in FOXP3 expression when RAR or RXR were solely targeted. A clearer understanding of the molecular events that transduce retinoid therapies into clinically desirable responses will enable fine-tuning those therapies and restricting their metabolic sequelae.

It was not until late in the project that ROR-γ was determined to be a potentially better indicator of CTCL. Results also suggest that CTCL originates from TH17 cells instead of the previously theorized T-regulatory cells. It is also possible that CTCL induces differentiation of a TH1 or T-reg cell into TH17 cells. Next steps for this project will include similar experiments to

those previously conducted pertaining to retinoids and how they affect CTCL. Instead of testing for FOXP3 expression, experiments will be run to determine how retinoids affect ROR-γ expression in CTCL lines.

It remains to be determined if the retinoid-induced differentiation of CTCL forces a phenotype that is incompatible with cutaneous localization and predisposes the cells to apoptosis. Future studies must determine if the CTCL cell populations that decrease ROR-γ levels are the same as those exhibiting alteration in migratory tendencies through integrin and chemokine receptor expression. It is tempting to speculate that retinoids supplant a cutaneous ROR-γ+ expression profile for an ROR-γ- gut tropic one. However, this thought is incongruent with the well-known role of natural retinoids to induce ROR-γ expression and TH17 differentiation in nonmalignant primary T cells [41-43]. Alternatively, retinoid-forced differentiation of cells with a terminally differentiated phenotype, like CTCL, may trigger apoptosis. Future studies will determine if this newly identified consequence of retinoid exposure to alter T cell differentiation markers in CTCL has clinical ramifications reminiscent of APL.

Figures

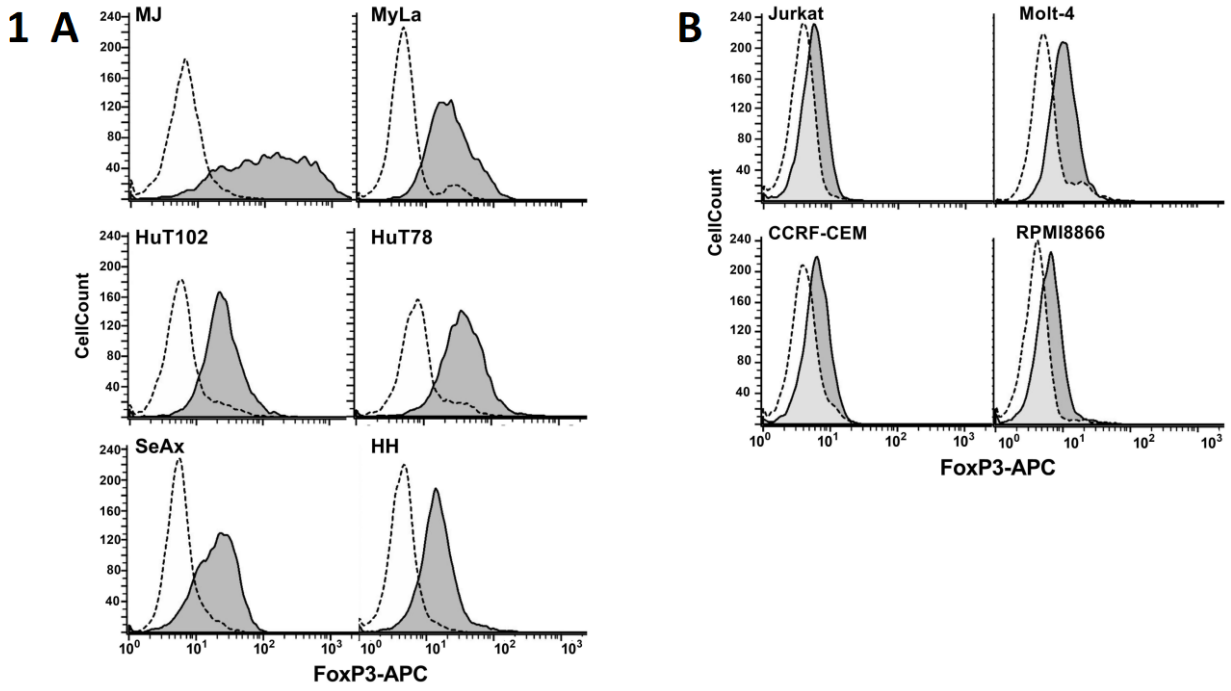

Figure 1. Basal expression of FOXP3 in human CTCL cell lines. (A) Flow-cytometric analysis of FOXP3 expression in multiple human CTCL cell lines. Fluorescent intensity of permeabilized, fixed cells lacking APC-conjugated anti-FOXP3 antibody (unfilled histograms with dashed lines) are plotted against samples containing the antibody (shaded histograms with solid line). (B) Analysis described in panel (A) was repeated with human immune cell lineages not of CTCL etiology.

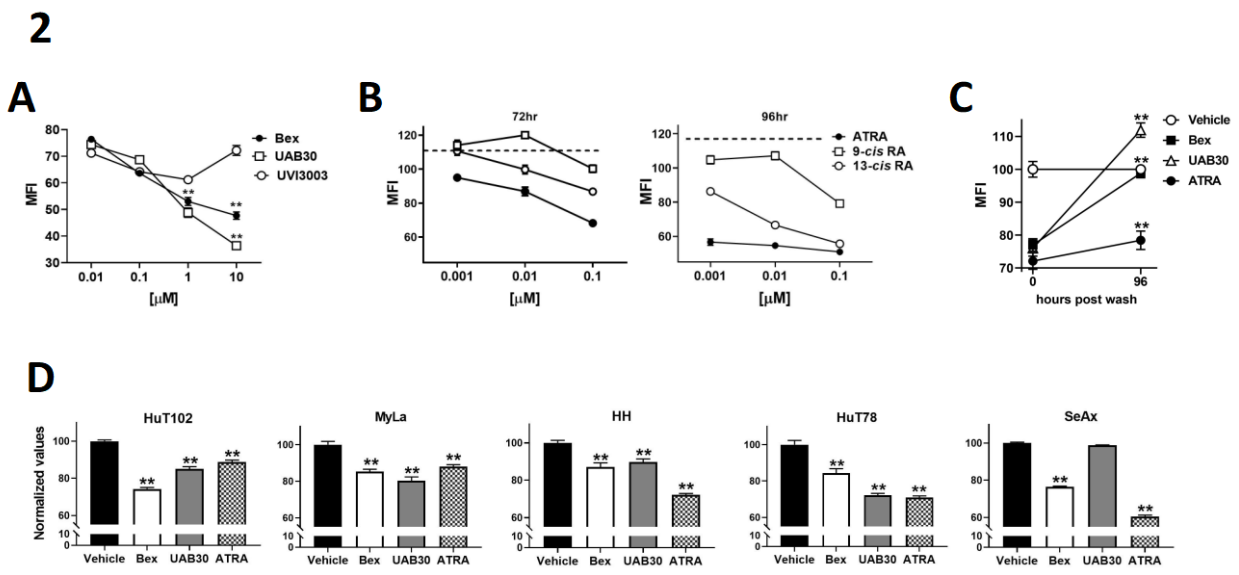

Figure 2. Therapeutic retinoids decrease FOXP3 expression in a dose-dependent manner. (A) MJ cells were cultured in the presence of varying concentrations of the pan-RXR agonists Bexarotene (Bex) or UAB30 for 72 hrs. FOXP3 expression was then determined and displayed as mean fluorescent intensity (MFI). The retinoid UVI3003, an RXR antagonist, was utilized as a negative control. (B) FOXP3 expression was determined in MJ cells exposed to increasing doses of natural retinoids for 72 or 96 hrs. Dotted line represent level of FOXP3 expression obtained with an equimolar concentration of vehicle (DMSO). (C) MJ cells were cultured with 1 mM Bexarotene, 1 mM UAB30, 100 nM ATRA, or vehicle (DMSO) for 72 hrs. After the initial exposure, the cells were washed and sub-cultured in media lacking retinoids for 96 hrs before FOXP3 expression was assessed. (D) Human CTCL cell lines were treated with 1 mM Bexarotene, 1 mM UAB30, 100 nM ATRA, or DMSO (vehicle) for 96 hrs. Data were normalized to MFI expression obtained with cells treated with vehicle alone.

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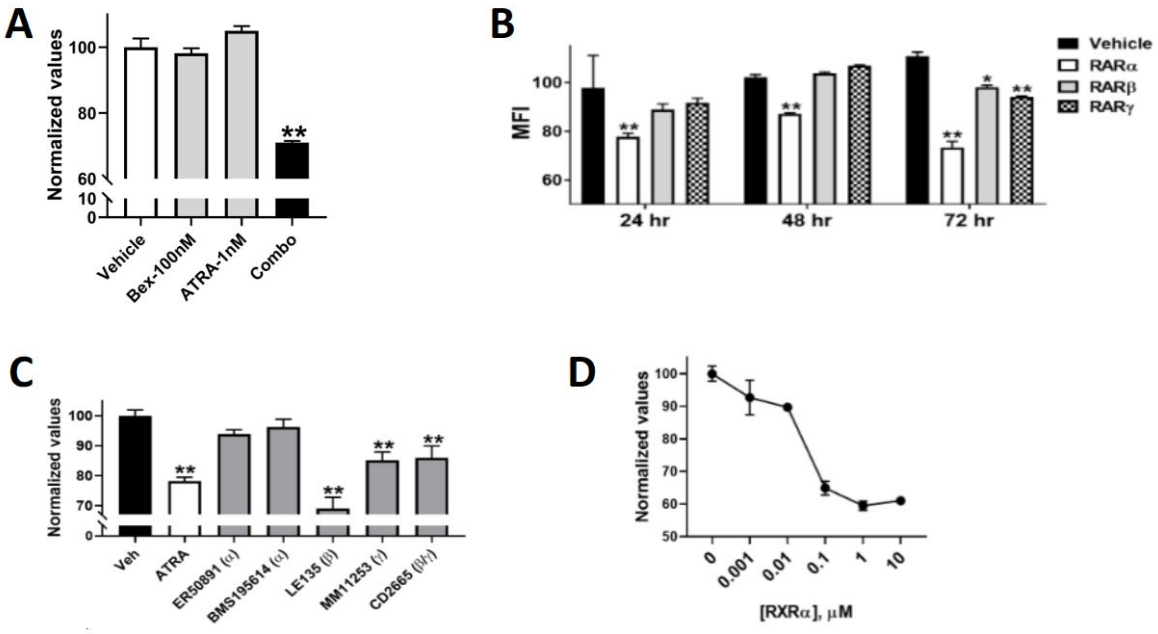

Figure 3. Changes in FOXP3 expression are driven by RAR/RXR nuclear receptor synergism. (A) MJ cells were cultured for 48 hrs in media containing vehicle (DMSO), 100 nM Bexarotene, 1 nM ATRA, or a combination thereof. FOXP3 expression levels were normalized to values obtained with the vehicle condition. (B) MJ cells were treated with DMSO (vehicle) or 2×EC_{50} of a RAR selective isoform agonist for the designated time. (C) Cells were initially dosed with 0.5 mM RAR isoform selective antagonist for 24 hrs and subsequently treated with 200 nM ATRA for an additional 24 hrs before FOXP3 expression was analyzed. All data were normalized with expression levels obtained when cells were maintained with the vehicle DMSO alone for the entire 48 hrs. (D) The expression of FOXP3 was evaluated in MJ cells dosed with varying concentrations of the RXRa selective agonist, CD3254, for 72 hrs.

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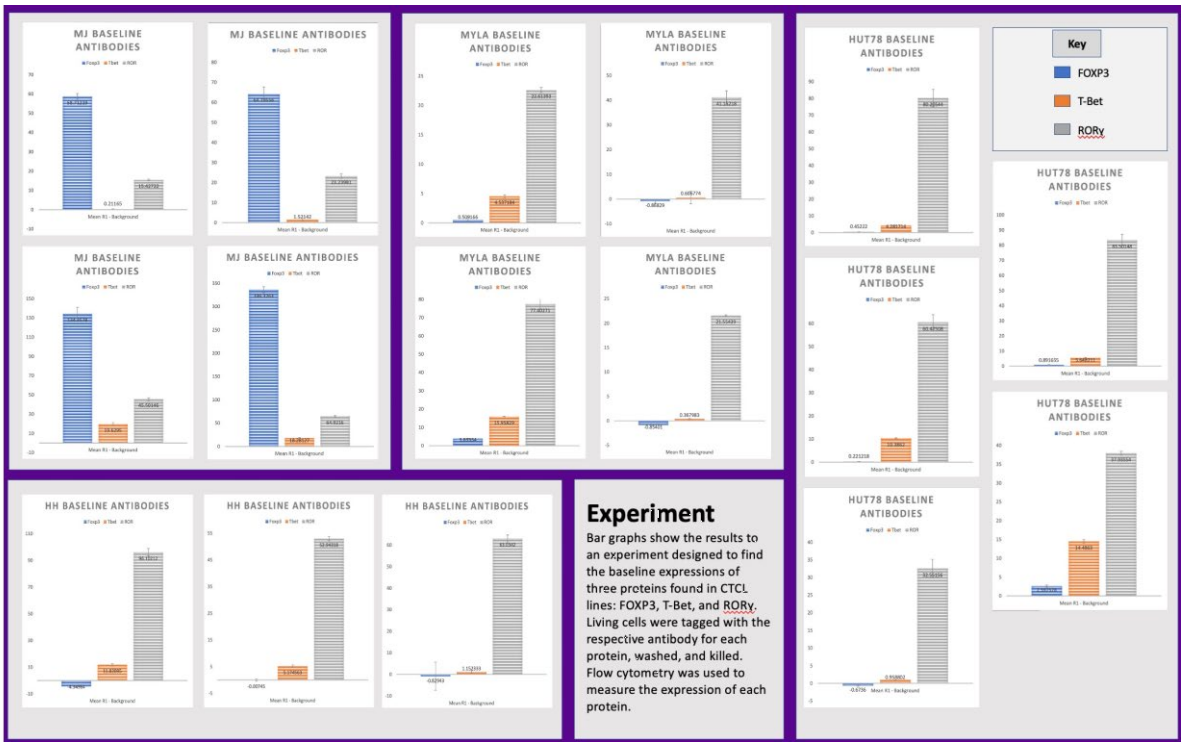

Figure 4. Basal expressions of FOXP3, T-Bet, and ROR-γ in human CTCL lines. Flow-cytometric analysis of FOXP3, T-Bet, and ROR-γ expression in multiple human CTCL cell lines. Fluorescent intensity of permeabilized, fixed cells lacking APC-conjugated anti-FOXP3, anti-T-Bet, and anti-ROR-γ antibodies are plotted. Analysis was repeated in 4 human CTCL lines: MJ, Myla, HUT78, and HH. The Y-axis values represent Mean Fluorescence Intensity (MFI).

Acknowledgements

This thesis was made possible by the Department of Biochemistry, the Department of Molecular and Cell Sciences, the Department of Immunology, and the Department of Microbiology and Pharmacology at Arkansas College of Osteopathic Medicine (ARCOM) in Fort Smith, Arkansas. Much of this paper has been previously contributed to by many authors, including Andrea M. Powers, Samuel L. Stahly, Reid Cline, Lauren A. Griffin, Bryce Sarcar, Alexis N. Danekas, Nabila A. Siddiqui, Lucas R. Johnson, Sykes Martin, Lance C. Bridges and Abby L. Geis. The original paper (*Retinoid Exposure Decreases FOXP3 Expression in Cutaneous T Cell Lymphoma Lines*) served as a template for additional work and research conducted in the summer of 2023.

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