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Supplementing Immunity: Investigating the Effect of Vitamin D Supplementation on Human Cancer T-Cell Proliferation and IL-2 Secretion

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ABSTRACT

In the realm of immune-related research on natural products to protect against growing public health concerns, the influence of market-available vitamin D supplements, with lipid additives, on cellular behavior, remains a mystery. This project looks at the relationship between supplemental vitamin D serum with lipid ingredients, and cell death of human cancerous T cells (type of white blood cell). This study aims to unveil potential modulatory influences of this vitamin D, with lipid additive, on cellular responses, contributing crucial insights to the broader understanding of supplements' public health implications. The effect of varying concentrations of vitamin D (independent variable) is measured on the stimulated cells by measuring the resulting optical density values (dependent variable), which represents the viability of the cells. Cancerous T cells stimulated with certain compounds (PMA and Ionomycin) secrete a protein, IL-2, representing T cell growth. To further assess cell viability, this protein's secretion is measured in wells with given optical densities, and interpolation with a standard curve. It is hypothesized that the presence of varying vitamin D, and therefore lipid additive, concentrations would significantly affect the T cells' viability. Specifically, a potential linear relationship, with higher concentrations resulting in higher cell viability and more IL-2 secretion. Post-experimentation, ANOVA analysis for concentration-specific analysis of cell viability returned a significant $p = 0.000000018$, concluding that the concentrations of vitamin D used as experimental conditions significantly influenced cellular response. Cell viability analyzed in correlation with IL-2 secretion, a concentration dependent inhibitory effect on PMA+IO induced IL-2 secretion is found.

Key Terms

1. T cell- subtype of a white blood cell, part of the body's immune system.
2. Interleukin-2 (IL-2)- a type of cytokine, signaling molecule, and protein part of the body's immune system.
3. Vitamin D- A fat soluble vitamin essential for healthy bones, immune system, and other cellular functions. It can be obtained from certain foods, supplements, or sunlight.

Introduction

The purpose of this project is to systematically explore the response of cancerous T cells to varying concentrations of a commercially available vitamin D supplement formulated with an olive oil lipid carrier. Specifically, aiming to investigate how the supplement formulation influences both cellular viability and interleukin-2 (IL-2) secretion: crucial aspects of T cell function and immune regulation.

The pervasive issue of vitamin D deficiency, affecting approximately 42% of the U.S. population, underscores a significant concern for public health (Forrest, Stuhldreher, 2011). Our skin's ability to synthesize vitamin D is tied to the wavelength of UV-B rays immitted from the sun; lack of sun resulting in improper vitamin D levels. Specifically, diminished exposure to sunlight and the altered solar angle during, for example, the winter, significantly hampers the body's capacity to produce sufficient vitamin D. Consequently, research continues to unveil negative correlations between low vitamin D levels and various health issues, from causing an imbalance in calcium/phosphorus regulation, to bone and metabolic health (Holick, Chen, 2008). Sufficient levels of vitamin D have been further associated with decreased risks of cancers, including colorectal and bladder cancer (McCullough, et al., 2019). As awareness of these health implications has grown, the market has responded with the development of vitamin D supplements designed to address deficiencies and promote overall well-being.

Exploring the variety of vitamin D products available, reveals that these products exhibit variability not only in their vitamin D content, but also their formulations. A deeper investigation into the effects of the formulation contents used in the supplements is necessary to determine the influence of the constituents on cellular systems. Notably, the various carriers (which protect, stabilize, and support consistent delivery) play a pivotal role in influencing the bioavailability and efficacy of these supplements. In essence, creating more effective supplements.

Lipid carriers, in particular, are used to increase absorption and the overall potential effect of vitamin D. While there is evidence stating that lipids may contribute to immune modulating effects themselves, such as the secretion of a set of immune proteins in mammals given olive oil diets, the effects in combination with vitamin D are unknown (Puertollano, et. al., 2007).

Building on this understanding of carriers, this research project aims to explore the effect of varying concentrations of a commercially available vitamin D supplement formulated with one of these lipid additives, olive oil, in JURKAT cells. Serving as the model system, JURKAT cells are a cancerous transformed human T lymphocyte cell line, originally derived from a 14-year-old boy with T cell leukemia. While a strong correlation between vitamin D and cancer risk has been reported, the specific effects of vitamin D on human T cells remain relatively unclear. To quantitatively explore the potential effect, the cell's secretion of a protein will be used. Known for its upregulation of proliferation and activity of T lymphocytes (T cells), IL-2 is a crucial cytokine, or signaling protein, in the immune system. Additionally, cellular proliferation will be assessed to evaluate the overall impact of the supplement on cell growth and activity. By simultaneously examining both IL-2 secretion and cellular proliferation, this study aims to uncover the nuanced relationship between varying concentrations of the vitamin D supplement and their effects on JURKAT cells, thereby examining the potential mechanisms underlying immune modulation and cancer cell behavior. This could guide the optimization of strategies for supplementation of vitamin D deficient patients and individuals at a higher cancer risk.

It is hypothesized that the incorporation of varying concentrations of a commercially available vitamin D supplement formulated with olive oil as a lipid additive will elicit distinct modulatory effects on cell proliferation as well as interleukin-2 (IL-2) secretion. Specifically, regarding cell proliferation, varying concentrations of the vitamin D supplement will likely affect cells differently, hypothesizing that higher concentrations will result in higher cell viability. It is anticipated that increasing concentrations of the supplement will lead to a dose-dependent increase in cell viability, reflecting the potential stimulatory effects of vitamin D on cellular proliferation. Evidence suggests that lipid additives influence the effectiveness and uptake of vitamin D. Therefore, the hypothesis could be formulated that this lipid specific market-available supplement may play a distinct impact on IL-2 secretion in the cells with each varying concentration.

Methodologies

Broken up into two major parts: two experiments (XTT cell proliferation assay, and ELISA IL-2 measurement assay)

Methodology (Part 1: XTT Assay- Measuring Cell Viability Post Vitamin D treatment)

Includes steps for cell culture preparation, as well as the assay itself.

Cell Culture Preparation

JURKAT cells originally from the American Type Culture Collection must be removed from nitrogen tank storage.

To begin with culture preparation, thaw JURKAT cells in 37°C water bath. Transfer thawed cells into warmed RPMI40 media with 10% FBS and 2MM L-Gln. Centrifuge cells at speed 750 rpm for five minutes. Decant cells and resuspend cells in warmed RPMI media. Over the next few days, the cells are counted under a microscope using a hemocytometer (see Figure 1 and 2 below) and trypan blue dyeing technique to assess viability and cell concentration.

For intended cell viability as well as protein secretion assays, the cell line JURKAT E6.1 must be split to the concentration of $1X10^5$ c/mL, as well as $4X10^5$ c/mL (two XTT, or cell viability, assay plates are created to compare/contrast noise readings of data).

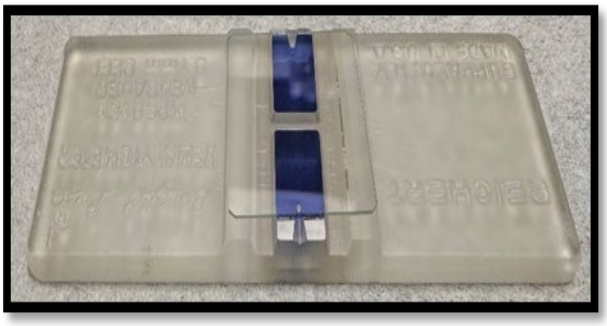

Figure 1. Hemocytometer used for cell counting.

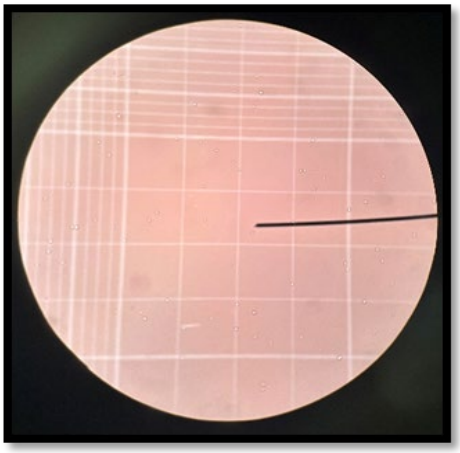

Figure 2. One 4X4 grid corner on hemocytometer, with cells, under microscope.

Assay

For this experiment measuring the effect of the varying concentrations of vitamin D supplement on cell viability was achieved through an XTT assay. An XTT assay is a colorimetric assay used to quantify JURKAT cell viability based on the metabolic activity of the cells which is hypothesized to alter based on cell treatment which is varying vitamin D concentrations.

This assay requires the plating of the cells with phorbol 12-myristate 13-acetate (PMA) and Ionomycin- compounds which induce the production of cytokines (IL-2) and other immune response mediators. Optimization of PMA is essential to ensure the conditions used in the assay are optimal for stimulating T cell activation and subsequent cytokine secretion. Therefore, the XTT method begins with the standardization of PMA, using six different compound concentrations for testing and the creation of an optimization curve. Begin by splitting JURKAT cells to concentration required for this optimization test- $1x10^5$ c/mL. Prepare six PMA dilution calculations. The concentrations of PMA were 0.1, 1, 2.5, 5, 10, and 20 ng/mL. See Table 1 below for PMA optimization results, and Figure 3 for PMA optimization data visualization.

Table 1. Average Absorbance (Optical Density 475-660) from PMA compound standardization.

Tx Conc	Absorbance (OD475-660)	Std Dev
Media	0.019	0.002
Cells	0.641	0.082
Iono	0.784	0.106
C6	0.518	0.049
C5	0.502	0.040
C4	0.509	0.068
C3	0.501	0.058
C2	0.524	0.061
C1	0.227	0.040

Note: Higher Absorbance (OD475-660) indicates more cell activity, or more cells being alive.

C2 (10ug/mL PMA) chosen as optimal PMA concentration for required XTT experiment, as can be seen by the sharp drop in Absorbance (OD475-660) value meaning PMA above the concentration of C2 has a cell death inducing effect.

Next, a vitamin D supplement concentrations range is formulated. Using 1 ug/mL as the dosage, a log scale with a few interval points is created to be the 5 different concentrations being tested on JURKAT T cells. With each well in the plate set to have 200 ug/mL, the divisions of cells, PMA/IO, and treatment are divided up. In each well, 100 uL of cells (at $1X10^5$), 25 uL of PMA, 25 uL of Ionamycin, and 50 uL of treatment (vitamin D supplement at specific concentration).

To prepare serial dilutions of vitamin D3, PCR tubes or conical tubes are labeled sequentially from P1 to P5. See Table 2 below to note each label with its designated concentration. In the first step, the purchased vitamin D3 is diluted to create P1 (the first, most concentrated variation). Assuming the initial concentration of the vitamin serum is 990 µg/mL as indicated on the vitamin bottle, 808.08 µL of the vitamin D3 solution is extracted. Next, P2 is prepared by combining 1000 µL of P1 with 1000 µL of media. Similarly, P3 is created

by mixing 200 µL of P1 with 1800 µL of media. P4 is then obtained by adding 20 µL of P1 to 1980 µL of media, while P5 is prepared by combining 2 µL of P1 with 1998 µL of media. These serial dilutions allow for the generation of a range of concentrations of the vitamin D3 solution for subsequent experimental use.

Note: *the creation of these vitamin D concentrations requires an additional step of vortexing inside the hood. A decision that was made based on the oil separation of the serum due to its oil consistency and ingredient.*

Table 2. Conical Title and Corresponding Vitamin D Supplement Concentration

Concentration Title	**P1**	**P2**	**P3**	**P4**	**P5**
Vitamin D Concentration (ug/mL)	100	50	10	1	0.1

The final experimental groups to be plated are the control groups. To ensure comprehensive evaluation, several controls were included in the plate setup. These included a vitamin D3 concentration without PMA and IO, serving to assess the vitamin D3-specific effects. Additionally, a positive control with MG132 confirmed external cellular responsiveness to stimuli. Controls with PMA and IO validated T cell activation, while IO alone assessed its isolated effects. Finally, control with cells and media alone accounted for nonspecific effects. These controls collectively confirm the reliability and validity of the experimental results.

With all treatment and control solutions prepared, plate them according to figure 3 below. Accounting for six replicates of each of the 5 vitamin D concentrations, and six replicates of each of the 5 controls. In this, practicing border well exclusion will minimize edge effects such as evaporation or inconsistent temperature distribution.

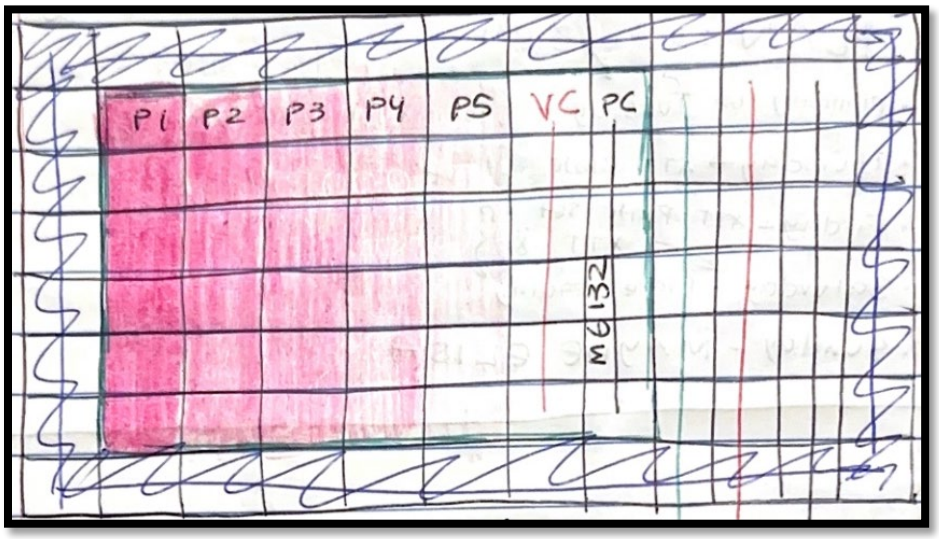

Figure 4. XTT Assay 96-well Plate Set-Up: Vitamin D concentrations arranged on left of plate, highest concentration (P1) at left, and lowest concentration (P5) at right. Complete right hand side of plate (VC, PC, and last three columns) are the controls to compare cell viability and IL-2 reading against.

Stimulation of the cells with these serum and compound varieties requires multi-channel pipettes, as well as troughs to pour each dilution or liquid into for plating.

This experiment was ran with two plates, one with cells at $1X10^5$ c/w and a second with cells at $4X10^5$ c/w. Quantities of supplements as well as controls are consistent throughout both plates.

Prior to the plates being read, liquid supernatants from the cell plates are removed and re-plated for the next ELISA. Plates of supernatants (total of two, each from plates with the two different cell concentrations) are kept in fridge till ELISA assay.

For the plates with remaining cells, set up plate reading template on SoftMax_Pro for them to be read. Absorbance is read off, OD475–OD650, using a microplate reader. Final quantitative values are given in Optical Density and analyzed thoroughly using software such as Excel. Higher optical density value correlates to more metabolic activity in the cells, essentially more cells being alive.

Methodology (Part 2: ELISA Assay)

ELISA (enzyme-linked immunosorbent assay) was conducted to quantify interleukin-2 (IL-2) secretion in JURKAT cells. ELISA kit from bio-techne was utilized in specific scenario, and kit instructions were followed. First, create buffer between 4.7-4.9 using pH strips. Coat plate with coating buffer day before performing ELISA assay.

Samples of supernatants are then added and incubated (after coating buffer) to allow IL-2 to bind to plate. After washing and blotting, detection antibody specific to IL-2 is added, followed by a secondary antibody. To induce color change for plate reading, substrate solution is added. Color change is measured on microplate reader. The resulting absorbance values used to determine IL-2 concentrations in the samples, interpolated from a standard curve generated using known IL-2 samples. This allows for the quantification of IL-2 secretion in response to varying concentrations of vitamin D supplementation in the cells.

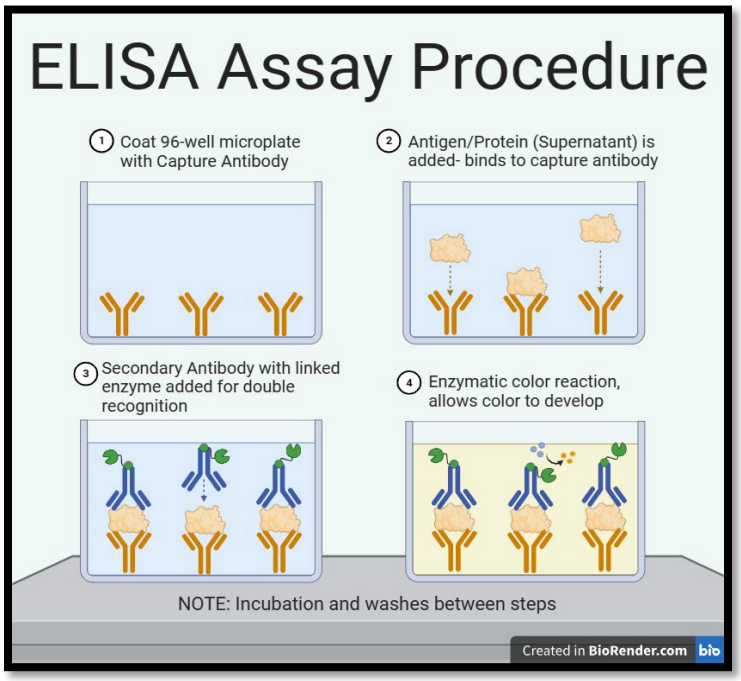

Figure 5. ELISA Assay Procedure: Procedure for testing IL-2 secretion quantitatively using ELISA assay. Step (1), coating microplate with antibody for capturing antigen. Step (2) displaying the capture of antigen due to recognition site. Step (3) introducing secondary antibody for antigen to be recognized by two domains. Step (4) adding substrate to solution to test color variance.

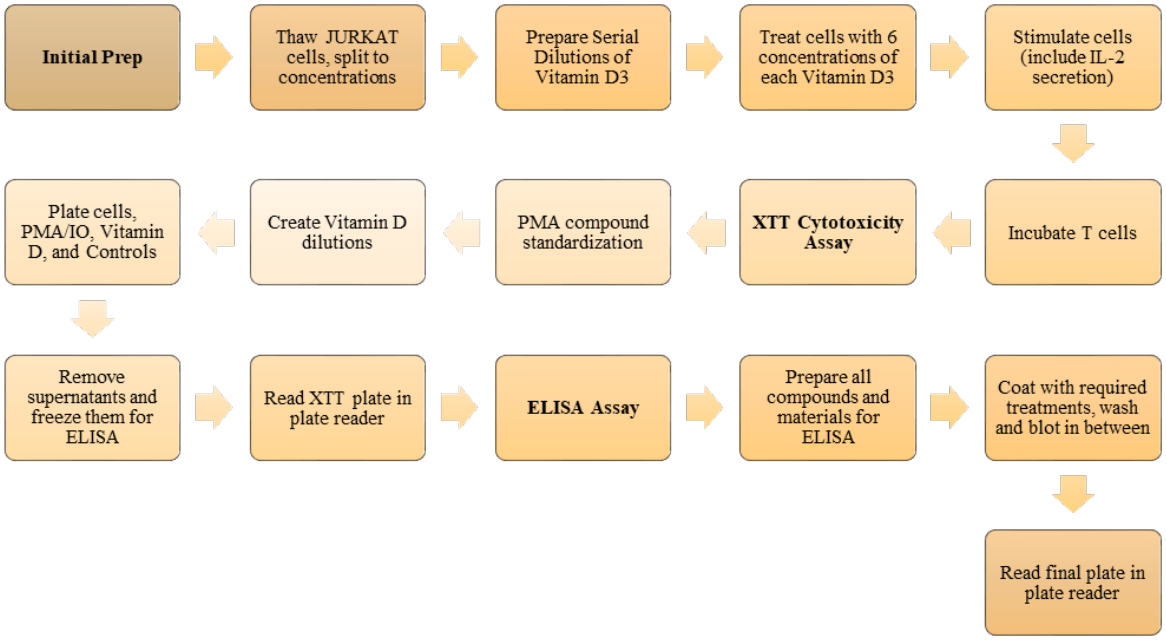

Figure 6. Whole procedure generalized

Figure 5 is an overview of total procedure. Beginning with preparation of cells, preparation of vitamin D3 for testing, onto treatment, stimulation, incubation, and analysis using XTT and ELISA assays.

Data Analysis

Data analysis was conducted using Microsoft Excel, where graphical representations were generated for both the XTT assay, as well as the ELISA experiment. Excel's graphing functionalities were used to visualize trends and patterns in the data. Gaining insight on how the treatment groups (which were a range of concentrations) affected the JURKAT T cells differently. Additionally, a statistical analysis was performed using analysis of variance (ANOVA) to assess the significance of difference observed among the experimental groups. This would help confirm that the vitamin D varying in concentration did in fact play a specific role on the cells (dependent to that concentration, or not). The ANOVAs on both the XTT as well as the ELISA yielded statistically significant results, indicating that the observed variations in cell viability (XTT) and interleukin-2 (IL-2) expression levels (ELISA) among different treatment groups were not due to chance.

ELISA ANOVA

Source of Variation	*SS*	*df*	*MS*	***P-value***	*F crit*
Between Groups	1691637.5	5	338327	**1.93804E-29**	2.53355
Within Groups	16061.4862	30	535.383		

XTT ANOVA

Source of Variation	*SS*	*df*	*F*	***P-value***	*F crit*
Between Groups	0.569677472	5	18.83956	**1.86E-08**	2.533555
Within Groups	0.181430167	30			

Results

In analyzing results, positive control, MG-132, a known anti-inflammatory and cytotoxic agent resulted in statistically significant inhibition of cell viability, showing the XTT assay system being able to accurately measure

the effect of vitamin D and stimulants on cell viability (Figure 7). Increasing concentrations of vitamin D3 treatment did not show a correlational trend in modulation of cell proliferation (Figure 7). All treatment groups less than 50 ug/mL did not have a significant effect on cell viability. Only the highest concentration tested resulted in a statistically significant reduction in cell viability, compared to PMA+IO alone (control group), suggesting a potential cytotoxic effect. A one-way analysis of variance (ANOVA) revealed statistically significant differences between the various concentrations of vitamin D on cell viability.

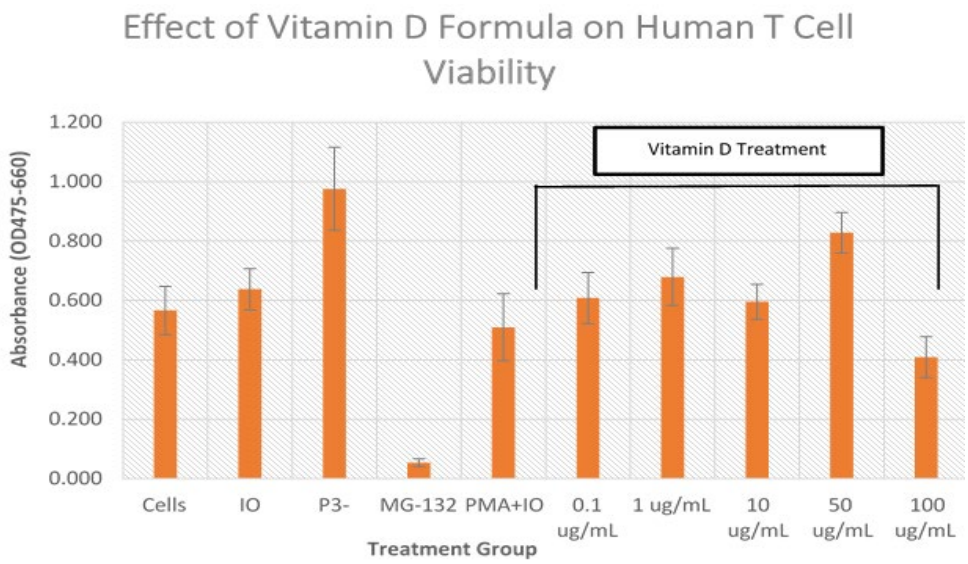

Figure 7. Cell Viability Data from XTT Assay Visualized. Data results from XTT Assay. P3- is concentration 10 ug/mL added to cells alone (no PMA/IO). MG-132 is positive control for cell killing. PMA + IO stimulate IL-2 secretion in T cells.

Notably, examination of the data points on Figure 8 (scatter plot), reveal that data points were fairly close together, indicating consistency and reproducibility in the experiment results across the plate. This further strengthens the reliability of the findings and suggests that the results are replicable.

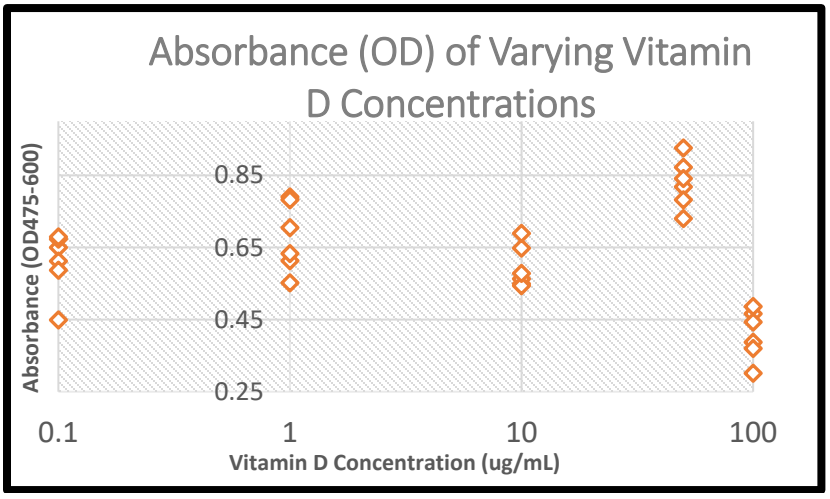

Figure 8. Scatter Plot of Absorbance Data Points in XTT Assay (Distribution Visualization). Distribution of Absorbance readings in plate- pointing out any potential outliers (limited).

Based on the ELISA standard curve (Figure 9) generated to interpolate IL-2 secretion by cells treated with vitamin D, a concentration-dependent inhibitory effect on PMA + IO-induced IL-2 secretion was observed (Figure 10).

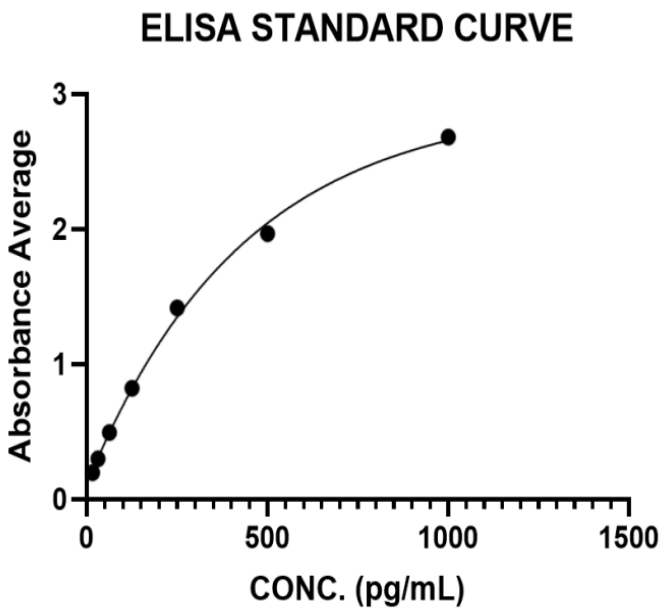

Figure 9. ELISA Standard Curve. Standard curve generated for IL-2 concentration measurements. Graph depicts the relationship between known IL-2 concentrations and their corresponding absorbance values, obtained through ELISA assay. This standard curve will be used to interpolate and accurately quantify IL-2 values in treatment groups (visualized in Figure 9).

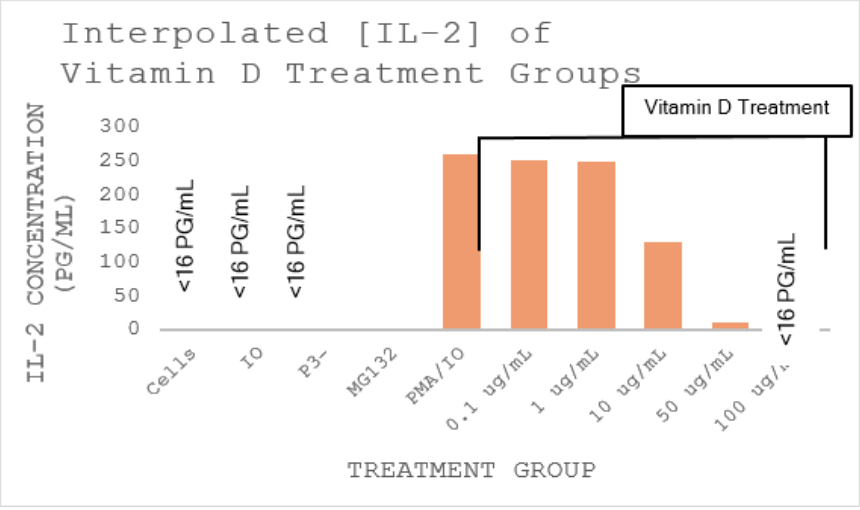

Figure 10. IL-2 concentrations from all T cell treatments groups, interpolated from IL-2 standards (Figure 9), are visualized. As the concentration of treatment (vitamin D) increases, there is a corresponding decrease in IL-2 concentration.

Discussion

XTT cytotoxicity assay was conducted on two JURKAT cell concentrations, $1x10^5$ c/mL (data not shown) and $4x10^5$ c/mL. Control P3- (10 ug/mL concentration of vitamin D) at 4E5 c/mL cell concentration, with no stimulant added, seemed to increase cell proliferation, above that of the cells alone (control). However, this was not observed at the XTT assay done on cells at $1x10^5$ c/mL concentration. Thus, this increase seen with vitamin D alone at the higher cell concentration may be an assay artifact and would need to be retested. Further regarding the XTT assay, only at the highest vitamin D concentration and only at the higher cell concentration ($4x10^5$ c/mL) does cell viability decrease lower than that of the PMA+IO control.

The absence of a linear relationship between concentration and cell proliferation in the XTT assay results highlights the complexity of the response of JURKAT cells to varying concentrations of the vitamin D3 supplement. Despite this, the significant differences observed among the concentrations emphasize the potency of the supplement in modulating cell viability. The proximity of data points on the graph confirms the consistency and reproducibility of the experimental results, enhancing the reliability of the findings.

The non-linear relationship observed suggests that the effects of the vitamin D3 supplement on cell viability may be influenced by factors beyond simple concentration-dependent mechanisms. Further investigation into the underlying mechanisms driving the observed effects, such as potential saturation points, may provide valuable insights into the dose-response relationship.

The concentration dependent IL-2 inhibitory effect of vitamin D could potentially be due to a cytotoxic effect of the highest vitamin D concentration. However, analysis of concentration 50 ug/mL vitamin D resulted in high cell viability and reduced IL-2 secretion. This IL-2 inhibitory effect of vitamin D thus appears to be occurring independently of any cytotoxic effect.

XTT and ELISA results align with existing evidence suggesting vitamin D's inhibition of the NFkB pathway. This sheds light on the potential therapeutic implications of vitamin D supplementation in modulating cellular responses, particularly in the context of cancer treatment. In this study's case, while the observed death of cancer cells may be considered beneficial, it also highlights the need to further understand the mechanisms underlying vitamin D's effects as a supplement, on cellular pathways.

Additionally, comparing and contrasting cellular affects when testing additional vitamin D supplements with varying lipid additives could be essential to identify the most effective formulations, optimizing therapeutic strategies, and understanding the mechanisms influencing JURKAT T cell death and IL-2 secretion

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References

(2023). Global Vitamin D Market Insights Forecasts to 2030. *Spherical Insights*. https://www.sphericalinsights.com/reports/vitamin-d-market.

Bowles, J. (2021). Overwhelming Proof that Vitamin D3 Deficiency Causes Most Human Diseases. https://jefftbowles.com/vitamin-d3-deficiency-causes-most-human-disease/.

Budhathoki, S., et. al. (2018). Plasma 25-hydroxyvitamin D concentration and subsequent risk of total and site specific cancers in Japanese population: large case-cohort study within Japan Public Health Center-based Prospective Study cohort. *The BMJ*. 10.1136/bmj.k671.

Forrest, K., and Stuhldreher, W. (2011). Prevalence and correlates of vitamin D deficiency in US adults. *Nutrition Research.* 31(1). https://doi.org/10.1016/j.nutres.2010.12.001.

Holick, M., and Chen, T. (2008). Vitamin D Deficiency: A worldwide problem with health consequences. *The American Journal of Clinical Nutrition*. 87(4). https://doi.org/10.1093/ajcn/87.4.1080S.

McCourt, A., Mulrooney, S., O'Neil, G., O'Riordan, E., and O'Sullivan, A. (2023). Serum 25-hydroxyvitamin D response to vitamin D supplementation using different lipid delivery systems in middle-aged and older adults: a randomised controlled trial. *British Journal of Nutrition*. https://doi.org/10.1017/S0007114523000636.

McCullough, M., et al. (2019). Circulating Vitamin D and Colorectal Cancer Risk: An International Pooling Project of 17 Cohorts. *Journal of the National Cancer Institute*. Circulating Vitamin D and Colorectal Cancer Risk: An International Pooling Project of 17 Cohorts - PMC (nih.gov)

Moyer, V. (2014). Vitamin, Mineral, and Multivitamin Supplements for the Primary Prevention of Cardiovascular Disease and Cancer: U.S. Preventive Services Task Force Recommendation Statement. *Annals of Internal Medicine*. https://doi.org/10.7326/M14-0198.

Puertollano, M., et al. (2007). Significance of Olive Oil in the Host Immune Resistance to Infection. *Cambridge University Press*. Significance of olive oil in the host immune resistance to infection | British Journal of Nutrition | Cambridge Core

Sasagawa, M., Cech, N., Gray, D., Elmer, G., Wenner, C. (2006). Echinacea alkylamides inhibit interleukin-2 production by Jurkat T cells. *International Immunopharmacology*. doi:10.1016/j.intimp.2006.02.003.