Thyroid Hormone Mediation of T cell Proliferation and Survival; Implications for Hypothyroidism and Hyperthyroidism.

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Abstract

Thyroid Hormone Mediation of T cell Proliferation and Survival; Implications for Hypothyroidism and Hyperthyroidism.

Tanya Babiuk-Henry

Hypothyroidism and hyperthyroidism affect 5.9% of Americans over the age of 12. It is not known how dysregulation of thyroid hormones such as triiodothyronine (T3), impacts the immune system.

A laboratory model system was used to study the interactions between T3 and T cells. Jurkat T cells, a leukemic lymphocyte cell line, were cultured in fetal bovine serum (FBS) restricted growth media containing four different levels of T3. In order to assess T3 activity on proliferation and survival of Jurkat T cells, cell staining and flow cytometry-based techniques were used. Adding 10 µg/mL of T3 to culture media increased cell survival rates over a 20 day period ($p<0.05$) and allowed the cells to utilise palmitic acid (PA) as an alternative energy source. Adding T3 10 µg/mL significantly increased T cell proliferation over 12 days. While T3 was essential to T cell survival, it did not significantly affect T cell apoptosis and necrosis rates over 12 days. When 2 µM of PA and 10 µg/mL of T3 were added to the cultured cells, T cells survived better than with PA alone ($p<0.05$).

The data collected demonstrates that T3 promotes both the survival and proliferation rate of T cells cultured in 1% FBS media due to enhanced use of free fatty acids as fuel. This work has implications for how hypothyroidism and hyperthyroidism, impacts the proper functioning of the immune system. Low T3 levels could decrease T cell survival and proliferation, while high T3 levels may result in changes in T cell substrate utilisation.

Keywords

Thyroid hormones, triiodothyronine, T cell survival, T cell metabolism, fatty acids

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Abbreviations

7AAD: 7-Aminoactinomycin D

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

AUC: Area under the curve

CFSE: Carboxyfluorescein succinimidyl ester

CPT1: Carnitine palmitoyltransferase I

FBS: Fetal bovine serum

FSC: Forward scatter

NS: Not significant

PA: Palmitic acid

PBS: Phosphate buffered saline

PI: Propidium iodide

RPMI: Roswell Park Memorial Institute

SSC: Side scatter

T3 : Triiodothyronine

T4 : Thyroxine

TR : Trend

T-test: Student's t-distribution

Special symbols

µ: micro

1.0 Introduction

1.1 Hormones and the endocrine system

Human beings adapt to their environment throughout changing external conditions. In order to function properly, the body must detect deviations from optimal conditions and respond accordingly to return to normal ranges using chemical signals. Thus, the body has developed a finely tuned ability to maintain stable optimal functioning of all the body's systems known as homeostasis.

The endocrine system has the essential role of regulating and stabilising the internal environment of the body (Maia, Goemann, Meyer, & Wajner, 2011). The regulation of cell metabolism, growth, and development is largely controlled by the thyroid hormones (Rhoades & Bell, 2013). Thyroid hormones are simple amine-based hormones formed in the thyroid gland, a palpable butterfly -shaped structure situated in front of the trachea (Hall & Guyton, 2011). The thyroid gland assembles and releases the biologically active hormone triiodothyronine (T3), alongside the T3 precursor hormone thyroxine (T4) (Bianco & Kim, 2006; Olivares et al., 2012). Deiodinase type 1 is essential for the conversion of T4 into T3, while type 3 deiodinase activity converts T4 and T3 into their inactive form which can then be eliminated from the blood (Bianco & Kim, 2006; Maia et al., 2011). The presence of type 3 deiodinase activity is the reason, T3 has a half-life of 24 hours in vivo. T3 has a half-life of 12 to 15 hours when added to cultured rat neuroglial cells, as these cells actively convert thyroid hormones through deiodinase activity (Courtin, Chantoux, & Francon, 1986). The half-life of T3 and uptake of T3 in Jurkat T cell culture has not been previously described.

T3 regulates tissue metabolism and gene expression by binding to target cell's nuclear thyroid hormones receptors (Sinha et al., 2012). The thyroid hormones receptors are ligand-inducible transcription factors, which once bound to T3, will activate gene transcription (Das et al., 2006; Yen, 2001), which in turn affects protein synthesis and increases mitochondrial size

and numbers (Rhoades & Bell, 2013). Lack of T3 binding to nuclear receptors represses basal transcription rates (Ishizuka & Lazar, 2005).

Thyroid hormones have both short-term and long-term effects on their target cells which can vary in latency from a few minutes to several days (Wrutniak-Cabello, Casas, & Cabello, 2001). Long term effects are mediated through nuclear receptor and increased gene transcription, while the short-term effect of thyroid hormones are mediated by non-genomic actions (Barreiro Arcos et al., 2011). Short term effects of thyroid hormone are due to inner mitochondrial membrane carrier adenine nucleotide translocase and can take as little as a few minutes (Sterling, 1986). T3 has been shown to increase oxygen consumption and mitochondrial oxidative phosphorylation in the liver of hypothyroid rats within 30 minutes of an intravenous injection (Sterling, 1986).

Thyroid hormone stimulation of mitochondrial gene transcription, basal metabolic rate, and protein expression take longer to manifest than changes in oxygen consumption, as these require nuclear thyroid hormone receptor activation of mRNA synthesis, phospholipid turnover and stimulation of uncoupling protein expression (Wrutniak-Cabello et al., 2001). T3 induced increased growth hormone gene transcription in GH_3 cells 1 hour after T3 incubation (Yaffe & Samuels, 1984). Injection of T3 into euthyroid men, led to an increase in oxygen consumption and plasma free fatty acids within 6 hours of an intravenous injection (Rich, Bierman, & Schwartz, 1959). A single subcutaneous injection of 20-30 μg T3 lead to an increase in oxygen consumption and basal metabolic rate in thyroidectomized rats after a latency period of 20-30 hours, maximal basal metabolic stimulation occurred around 70 hours after injection (Tata et al., 1963). Oxygen consumption by hepatocytes of hypothyroid rats increased 24 hours after administration of T3, with peak oxygen consumption reached between 72 and 96 hours (Ismail-Beigi, Bissell, & Edelman, 1979). The addition of 10 nM T3 to confluent fetal rat brown adipocytes increased levels of uncoupling protein mRNA synthesis after 24 hours, with mRNA levels peaking at 48 hours (Guerra, Roncero, Porras, Fernandez, & Benito, 1996). Treatment of hypothyroidism in female rats with T3 (12.5 μg/100g body weight), significantly increased mitochondrial mRNA in the liver within 24 hours (Wiesner, Kurowski, & Zak, 1992). Amino acid incorporation into mitochondrial protein of thyroidectomized rats increased 36 hours after

T3 administration, with peak amino acids uptake occurring 43 to 50 hours after T3 administration (Roodyn, Freeman, & Tata, 1965).

Additionally, the time lapse in thyroid hormone effect on oxygen consumption and metabolism depends on whether T4 or T3 is administered (Myant & Witney, 1967). While two injections of T4, given 12 hours apart to healthy rats, noticeably increased oxygen consumption after 24-27 hours and led to a significant increase in plasma free fatty acids after 5 days, T3 injections increased oxygen consumption after 18 hours and significantly increased plasma free fatty acid levels after 66 hours (Myant & Witney, 1967).

1.2 Thyroid dysfunction

Thyroid conditions cause an imbalance in the homeostasis of the body's systems which may affect an individual's ability to perform normal daily tasks or engage in physical activity (Ganapathy & Volpe, 1999; Hackney, Kallman, Hosick, Rubin, & Battaglini, 2012; Kahaly, Kampmann, & Mohr-Kahaly, 2002; Kaminsky et al., 2012). Among other things, thyroid problems disturb left ventricle function; thus affecting stroke volume and cardiac output, ultimately reducing exercise tolerance (Kahaly et al., 2002). Altered thyroid hormone levels have been associated with both insulin resistance (Maratou et al., 2009) and metabolic disorders (Chen et al., 2016). New research data shows that thyroid conditions are much more common than originally thought, with the incidence of thyroid disease increasing with age (Canaris, Manowitz, Mayor, & Ridgway, 2000). Most thyroid conditions are caused by an autoimmune condition that either over stimulates or destroys the thyroid gland (Rhoades & Bell, 2013; Vanderpump, 2011). Thyroid diseases are generally characterised into two major categories: hypothyroidism and hyperthyroidism.

1.2.1 Hypothyroidism

Hypothyroidism, a condition affecting nearly 4.6% of Americans aged 12 years and older (Golden, Robinson, Saldanha, Anton, & Ladenson, 2009), arises when tissues do not have access

to sufficient amounts of thyroid hormones. Hashimoto's disease is the most common cause of hypothyroidism in the United States (Golden et al., 2009). Additional causes of hypothyroidism include the destruction of the thyroid due to surgery or radiation, lack of dietary iodine, congenital thyroid malformation and consumption of certain medications (Coelho et al., 2007; Masood & Hakeem, 2011; Vanderpump, 2011; Yehuda-Shnaidman, Kalderon, & Bar-Tana, 2005). The lack of thyroid hormone binding with the thyroid hormone receptors has a repressive effect on most gene transcription and can cause the following symptoms: extreme fatigue, decreased muscle strength and endurance, decreased basal metabolic rate, cold intolerance, formation of a goitre, depression and reduced mental function, problems with reproductive systems, hypercholesterolemia and weight gain (Louwerens et al., 2012; Rhoades & Bell, 2013; Samuels, 2014). Daily supplementation of levothyroxine, a synthetic thyroxine hormone, is the standard treatment for hypothyroidism.

1.2.2 Hyperthyroidism

Hyperthyroidism is characterised by excessive amounts of thyroid hormones being produced and released into the bloodstream by the thyroid gland. Hyperthyroidism affects 1.3% of Americans over 12 years old (Golden et al., 2009). The majority of cases of hyperthyroidism are caused by an autoimmune disease called Grave's disease, where a T cell mediated immune response attacks the thyroid gland and causes the thyroid gland to become hyperactive, leading to a severe overproduction of thyroid hormones (Mihara et al., 1999). Other causes of hyperthyroidism include pituitary or thyroid adenoma and overconsumption of iodine or exogenous thyroid medication (Rhoades & Bell, 2013; Topliss & Eastman, 2004). Symptoms of hyperthyroidism include: high state of excitability, heat intolerance, profuse sweating, and extreme fatigue coupled with inability to sleep, muscle weakness, tachycardia, formation of a goitre, weight loss in spite of increased appetite, diarrhea and psychiatric disorders, including nervousness, restlessness, irritability, anxiety and sleeplessness (Hall & Guyton, 2011; Rhoades & Bell, 2013). Gauthier et al. found that cardiac symptoms caused by autoimmune hyperthyroidism could be significantly reduced with thyroidectomy (Gauthier et al., 2016). As thyroid hormones have a great impact on the overall regulation of body homeostasis, it is of great

importance to understand how thyroid hormones affect the regulation and function of the immune system.

1.3 The immune system and T cells

The immune system and the endocrine system interact together to preserve homeostasis. The immune system is responsible for protecting us from internal and external threats, such as viruses, bacteria, other invading microorganisms, and cancerous cells (Rhoades & Bell, 2013; Sompayrac, 2015). To function properly, the immune system must identify potentially dangerous invading pathogens while differentiating them from healthy innate tissue cells (Rhoades & Bell, 2013; Sompayrac, 2015). T cells are essential components of the immune system and cell-mediated immunity. T cells help regulate active immune responses by releasing cytokines, small signalling proteins, which direct the responses of other immune cells (Michalek & Rathmell, 2010). T cells are leukocytes, or white blood cells, which mature in the thymus and continuously circulate within the secondary lymphoid and peripheral tissues until they come in contact with an antigen presenting cell or pathogen (Michalek & Rathmell, 2010; Sompayrac, 2015). Upon detecting a foreign particle, T cells divide, exert their function, and die when they are no longer needed or able to perform their function appropriately. They may also remain as memory cells to help fight off secondary infections with the same pathogen (Green, 2011).

In order to mount a defence against a rapidly dividing pathogen, immune cells must also increase their numbers (Hughes & Mehmet, 2003). Activated T cells will release the T cell growth factor interleukin 2 which activates the proliferation pathways of the T cell cycle (Hughes & Mehmet, 2003). Proliferation is the action by which the cell population increases in number through mitosis, thus replacing cells that have died and increasing the number of live T cells (Hughes & Mehmet, 2003).

Hyperthyroidism has been linked to increased T cell proliferation (De Vito et al., 2011). Klecha et al. found an increase in proliferation responses in lymphocytes of hyperthyroid mice compared to euthyroid and hypothyroid mice (Klecha et al., 2006). Administration of T3 to hypothyroid mice increased T cell proliferation back toward levels seen in euthyroid mice

(Klecha et al., 2006).Thyroid hormone mediated-increases in T cell proliferation are especially remarkable in cells with mutated proliferation pathways. Both T3 and T4 cause increased proliferation in the human glioma (U-87 MG) cell line (Lin et al., 2009) and have been shown to have a growth factor-like effect on T cell lymphomas, leading to increased lymphoma proliferation (Cayrol et al., 2015). The proliferation of EL-7 T lymphoma cells was significantly increased in hypothyroid mice compared to euthyroid mice 10 days post tumor inoculation (H. Sterle et al., 2016). T cell proliferation can be measured using a carboxyfluorescein succinimidyl ester (CFSE) intercellular protein dye (Barreiro Arcos et al., 2011; Fulcher & Wong, 1999). The fluorescence intensity of the CFSE dye halves each time the cell divides, allowing for the quantification of cell proliferation using flow cytometry (Fulcher & Wong, 1999).

1.4 T cell metabolism

Naïve T cells and memory T cells do not require large quantities of energy to support their survival (Michalek & Rathmell, 2010). Non-activated cells rely mostly on glucose oxidation in the tricarboxylic acid cycle and mitochondrial fatty acid oxidation for their energy needs (Michalek & Rathmell, 2010). Once activated, T cells undergo a phase of increased growth and proliferation brought on by cytokine signaling (R. Wang & Green, 2012). To accumulate enough biomass and energy to undergo proliferation, T cells must have access to a substantial amount of adenosine triphosphate (ATP) (Lochner, Berod, & Sparwasser, 2015; Michalek & Rathmell, 2010). In order to support their increased need for substrates, activated T cells increase their consumption and catabolism of glucose, glutamine and oxygen, while simultaneously restricting their use of free fatty acids (R. Wang & Green, 2012).

Production of ATP through fatty acid oxidation occurs most often in the mitochondria of rapidly proliferating T cells (Byersdorfer et al., 2013; R. Wang & Green, 2012). Uptake of long-chain fatty acids into the mitochondria necessitates the conversion of FAs Acyl-CoA groups into acylcarnitines (Byersdorfer et al., 2013; Jansen, Cook, Song, & Park, 2000). This conversion is catalysed by the enzyme carnitine palmitoyltransferase I (CPT1) which can be pharmacologically blocked with the CPT1 irreversible inhibitor etomoxir (Ratheiser et al., 1991).

Etomoxir prevents the transformation of long-chain fatty acids into acylcarnitines, therefore preventing the uptake of these fatty acids into the inner mitochondrial membrane (Horn, Ji, & Friedman, 2004; Kruszynska, Stanley, & Sherratt, 1987). Etomoxir inhibits the oxidation of long-chain fatty acids without affecting the oxidation of short-chain or medium-chain fatty acids (Rupp, Schulze, & Vetter, 1995; Tutwiler & Ryzlak, 1980). Etomoxir is known to cause ATP depletion and decrease the efficiency of the electron transport chain (Hernlund et al., 2008; Pike, Smift, Croteau, Ferrick, & Wu, 2011). Etomoxir has also been shown to inhibit the oxidation of palmitoyl-CoA and palmitic acid (PA) in hepatic mitochondria of rats (Tutwiler & Ryzlak, 1980). Concentrations of etomoxir ranging from 100μM to 1mM have been used in cell culture to block CPT1 activity and fatty acid oxidation (Byersdorfer et al., 2013; Paumen et al., 1997; Pike et al., 2011).

Thyroid hormones play an important role in the regulation of mitochondrial fatty-acid oxidation. T3 increases oxidative metabolism and increase the uptake of fatty acids into hepatic mitochondria while increasing lipid autophagy (Liu & Brent, 2010; Sinha et al., 2012). Long-term T3 treatment has been shown to increase oxidation of fatty acids by increasing the expression and activity of CPT1α in rat liver mitochondria, as T3 is essential to the transcription of CPT1 mRNA (Jackson-Hayes et al., 2003). CPT1α mRNA levels were decreased by 80% in the livers of hypothyroid rats compared to hyperthyroid rats (Mynatt, Park, Thorngate, Das, & Cook, 1994). CPT1α enzyme activity was increased in hyperthyroid rats and decreased in hypothyroid rats compared to euthyroid rats (Mynatt et al., 1994).

Thyroid hormones also have a vital role in free fatty acid release from human adipose tissue (Debons & Schwartz, 1961). Experiments using labelled PA showed that thyroid hormones increased both the utilisation and mobilisation of fatty acids (Harlan, Laszlo, Bogdonoff, & Estes JR, 1963). Human subcutaneous fat deposits consist of 21 to 30% PA, with oleic, palmitoleic and steric acids making up the majority of the rest (Kingsbury, Paul, Crossley, & Morgan, 1961). PA is most abundant free fatty acid found in plants and animals (Takahashi et al., 2012). PA is often used in cell culture experiments, as it has been shown to be less toxic and better tolerated by Jurkat T cells and neutrophil cells than polyunsaturated fatty acids (Healy, Watson, & Newsholme, 2003; Lima, Kanunfre, Pompeia, Verlengia, & Curi,

2002). Cultured human fibroblast cells have the ability to change their fatty acid composition when PA is added to the culture media (Spector, Kiser, Denning, Koh, & DeBault, 1979). Although PA is abundantly found in T cell membranes as well as in the serum added to culture media, PA levels above 50μM may increase apoptosis in Jurkat T cells due to oxidative stress and increased pro-apoptotic Caspase 3 and Caspase 9 activity (Healy et al., 2003; Lima et al., 2002; Takahashi et al., 2012). Increasing T3 mediated CPT1 activity may decrease PA-induced apoptosis since increasing mitochondrial fatty acid oxidation in skeletal muscle cells help prevent apoptosis due to PA (Henique et al., 2010).

It is not yet knowing how T3 may affect substrate utilisation in T cell culture in vitro. Although thyroid hormones may increase T cells ability to utilise fatty acids to produce ATP, T3 also lead to a greater utilisation of ATP to maintain normal functions. T3 leads to greater use of ATP to maintain proper ion gradients in the sodium-potassium pumps (DeLuise & Flier, 1983). T3 also decreases the efficiency of the electron transport chain in the mitochondria due to uncoupling proteins, causing proton leakage (Gong, He, Karas, & Reitman, 1997).

1.5 Cell death

Regulation of cell death is essential for the homeostasis of the immune system and preventing disease states. Programmed cell death helps control T cell population numbers, preventing T cell population atrophy due to too much T cell death and preventing autoimmune disorders or formation of cancers due to T cell overcrowding and survival of mutated T cells (Michalek & Rathmell, 2010). Pathways for programmed cell death in T cells include apoptosis, necrosis and autophagy (Kroemer et al., 2009). T cell apoptosis is closely associated with the cell cycle. T cell cycle is essential for T cell proliferation and it allows the cell to undergo mitosis, leading to T cell replication (Vermeulen, Van Bockstaele, & Berneman, 2003). During the S-phase of the cell cycle, the cells genome is duplicated and during the M-phase, the mother cell divides into two daughter cells (Pucci, Kasten, & Giordano, 2000). Checkpoints at the G1 and G2 positions in cell cycle help prevent the mitosis of genetically damaged or abnormal cells (Green, 2011; Hughes & Mehmet, 2003; Pucci et al., 2000). Recognition of DNA damage by

protein kinases at the G1 checkpoint causes the release of caspases leading to the activation of the apoptotic pathways (Pucci et al., 2000).

Apoptotic T cells undergo a series of ATP-dependant morphological and biochemical changes which eventually lead to cell death including cell shrinkage, condensation, and fragmentation of the cell's nucleus (Ouyang et al., 2012). Early apoptosis causes phosphatidylserine to be externalized to the cell surface, while cellular membrane integrity is often conserved until the final stages of apoptosis where the cell membrane fragments are absorbed by the macrophages in the body (Green, 2011; Hughes & Mehmet, 2003; Kroemer et al., 2009). Apoptosis can also be triggered by addition of chemical drugs, high PA concentrations, Fas ligand, tumor necrosis factors, lack of growth factors, and viral or bacterial infection (Pucci et al., 2000; Takahashi et al., 2012). The apoptotic process is of short duration and T cell apoptosis is considered to be a transient state, therefore any measurement of apoptosis will only show the percentage of cells undergoing apoptosis at that very moment and not the total percent of cells that have undergone apoptosis (Darzynkiewicz & Traganos, 1998).

Necrosis is not dependent on caspases and is a much less regulated process (George et al., 2004; Ouyang et al., 2012). Necrosis is sometimes due to ATP depletion when cells no longer have enough energy to sustain survival (Eguchi, Shimizu, & Tsujimoto, 1997). Cells undergoing necrosis will increase in volume, undergo chromatin condensation, swelling of organelles and decreased cellular integrity, leading to the rupture of the cell and the release of the cell's contents into the local circulation (George et al., 2004; Green, 2011; Hughes & Mehmet, 2003). Necrosis is often caused by the permeabilization of the mitochondrial membrane by excessive reactive oxygen species production (Ouyang et al., 2012).

Flow cytometry and vital stains can be used to distinguish between T cells undergoing early-stage apoptosis and necrotic cells. Annexin V stains are used to detect cells with phosphatidylcholine lipids on their cell surface (Tatsuta et al., 2013). Normally, these lipids are found in the interior of the cell membrane, but phosphatidylserine externalizes to the cell surface at the onset of apoptosis (Green, 2011; Hughes & Mehmet, 2003). Necrosis can be measured with 7-amino-actinomycin D (7AAD) or propidium iodide (PI), as both these vital dyes have a

high affinity for DNA (Riccardi & Nicoletti, 2006; Rieger, Nelson, Konowalchuk, & Barreda, 2011). 7AAD and PI cannot cross the cell membrane and can only bind to the DNA of dead cells whose membrane has lost its cellular integrity (Chan, Wilkinson, Paradis, & Lai, 2012; Rieger et al., 2011). Early-stage apoptotic cells are positive for annexin V but negative for 7AAD (George et al., 2004). Necrotic cells are positive for both stains because the holes in the cell membrane allow both dyes to bind their targets. Flow cytometry and cell viability staining cannot distinguish between necrotic and late-stage apoptotic cells (George et al., 2004).

1.6 Research on thyroid hormones and T cells

Jurkat T cells have been used extensively in the literature and represent one of the gold standards for *in vitro* T cell research due to their reproducibility and compatibility with laboratory techniques (Koziel et al., 2010; Schoene & Kamara, 1999; C. J. Wang et al., 2014). Jurkat T cells are an immortal lymphoblastoid cell line originally taken from a patient with T cell leukemia. Jurkat T cells are human-derived and are clones of the original cell culture (Kon et al., 2013) and have a population doubling time of 20.7 hours (Schoene & Kamara, 1999). These cells have a gene mutation in the phosphatase and tensin homolog gene causing their proliferation pathways to always be turned on (Shan et al., 2000).

Jurkat T cells cultured in optimal conditions with 500nM to 1000nM of T3 had an increase in apoptosis promoting proteins in their mitochondria (Yehuda-Shnaidman et al., 2005; Yehuda-Shnaidman, Kalderon, Azazmeh, & Bar-Tana, 2010). Apoptosis rates also increased in human lymphocytes cultured with T3 and T4 compared to lymphocytes cultured without added thyroid hormones (Mihara et al., 1999). However, apoptosis rates were decreased and there was a trend towards increased monocyte counts when human subjects had serum T3 concentrations above 1.95 nmol/L, these values being in the higher section of the normal physiological range of 1.49 to 2.60 nmol/L (Hodkinson et al., 2009). Low T3 concentrations were associated with higher levels of apoptosis causing tumor necrosis factors in patients with non-thyroidal disease (Mooradian, Reed, Osterweil, Schiffman, & Scuderi, 1990). T cells from patients' with Graves' disease have greater apoptosis rates than T cells from control subjects (Sera et al., 2001). Neutrophil cells of hypothyroid patients have a decreased ability to undergo phagocytosis

(Palmblad, Adamson, Rosenqvist, Udén, & Venizelos, 1981). Additionally, immune cells numbers significantly decreased in animals treated with an antithyroid drug for a week (Csaba, Kovács, & Pállinger, 2005).

Research on healthy adult subjects found that thyroid hormone variations within normal physiological ranges lead to changes in levels of certain T cell and immune markers (Hodkinson et al., 2009). In this study, thyroid hormones increased natural killer cell counts, which are known to increase the risk of autoimmune disorders (Miyake & Yamamura, 2007). Ingestion of oral T3 was found to increase in the immune marker IL-6 (Mariotti et al., 1992). Alteration in IL-6 concentrations was also found in subjects with thyrotoxicosis (Bartalena et al., 1994). A significant increase in soluble IL-2 receptor levels was found in peripheral blood cells of subjects with untreated Graves' disease and hyperthyroid patients with Hashimoto's thyroiditis (Nakanishi, Taniguchi, & Ohta, 1991). T3 administration lead to significantly higher soluble IL-2 receptor levels in both subjects in remission from Graves' disease and in normal controls (Nakanishi et al., 1991).

In summary, fundamental knowledge of how thyroid hormones affect the cells of the immune system and how these two systems work together will help doctors to better treat their patients and allow for the creation of better pharmacological guidelines. This knowledge will help healthcare providers determine if a patient with a thyroid condition is more or less susceptible to certain common pathogens.

2.0 Rationale and Objectives

The primary purpose of this study is to quantify how different concentrations of T3 affect T cell survival and proliferation. The second aim of this study is to differentiate the types of cell death T cells undergo in the presence different T3 concentrations. The third aim of this study is to assess the effect of T3 on T cell metabolism, by measuring T cell survival and proliferation when fatty acids are added to the culture medium. Finally, the fourth aim of this study is to measure the effect of blocking mitochondrial fatty acid oxidation on T3 mediated T cell survival.

To our knowledge, no previous study has looked at the thyroid hormone mediated effects on Jurkat T cell survival, proliferation and metabolism in such a complete way. T cells were measured every day until cell viability in the conditions had decreased to a point where T cells in the control condition were considered to be over 90% dead. Measurements were conducted every 24 hours for the entire life cycle of the cells to ensure the data collected was as complete and accurate as possible and that the exact time point of changes in T cell survival, proliferation or metabolism could be determined. No other study has looked at the cumulative effect of T3 and PA supplementation on Jurkat T cell survival and proliferation in a glucose-restricted environment.

2.1 Objectives

- Aim 1: Measure survival and proliferation of T cells cultured with a hyperthyroid, euthyroid and hypothyroid T3 levels compared to control condition using both microscopy and flow cytometry staining.

- Aim 2: Determine the levels of early-stage apoptosis and necrosis rates of T cells cultured with different levels of T3.

- Aim 3: Assess the effects of PA supplementation on survival and proliferation of Jurkat T cells cultured in media containing T3 10 µg/mL compared to a control condition using both cell parameter and PI cell staining techniques

- Aim 4: Demonstrate the involvement of T3 on mitochondrial fatty acid oxidation in Jurkat T cells cultured in media containing T3 10 µg/mL by adding the CPT1 inhibitor etomoxir.

2.2 Hypothesis

- Hypothesis 1: T3 10 µg/mL will increase T cell survival and increase T cell proliferation compared to T cells in the other conditions.

- Hypothesis 2: T3 10 µg/mL will increase apoptosis and decrease necrosis compared to the other conditions.

- Hypothesis 3: Supplementing T cells in the T3 10 µg/mL condition with palmitic acid will have an increase in survival and increase in proliferation compared to the T cells in the control condition.

- Hypothesis 4: Adding etomoxir to T cells in the T3 10 µg/mL condition will negate the survival effects of T3.

2.3 Rational

The overall goal of this project is to determine the effect of T3 levels on survival, proliferation, and metabolism of Jurkat T cells. In aim 1, the effects of T3 on T cell survival, proliferation, and cell characteristics were measured. Initial T cell counts were done with microscopy and trypan blue staining. Trypan blue is a technique where a mixture of cells and trypan blue dye is placed into a glass hemocytometer and cells are analysed by microscopy. The dye stains the outer cell membrane of live cell blue while it enters the cytoplasm of apoptotic or necrotic cells and dyes the whole cell a deep blue (Stoddart, 2011). A miniature grid on the glass hemocytometer allows the researcher to count the T cells in each grid section under the microscope. If T3 did have an effect on T cell survival and proliferation, it would be captured with flow cytometry techniques. T cell proliferation was measured by using CFSE staining and measuring mean CFSE emission of live cells using flow cytometry. The fluorescence of the CFSE dye allowed for accurate measurement of how many times the cell population had undergone proliferation. As each mother cell underwent mitosis, the CFSE dye concentration and its emission in the subsequent daughter cells were halved when measured on the flow cytometer (Fulcher & Wong, 1999). The T3 levels used in all the experiments were based on the euthyroid physiological range of 0.45 µg/mL to 1.32 µg/mL for total serum T4 (Hollowell et al., 2002). Since T4 must be converted into T3 to become biologically active (Olivares et al., 2012) and Jurkat T cells do not respond as well to T4 (Appendix A, Figure 12), T3 was used instead of T4. Additionally, T3 has a ten to a hundred-fold greater affinity for the thyroid hormones receptor than T4 (Visser, 1988).

In aim 2, the early-stage apoptosis and necrosis rates of T cells were measured. Cell death was expected to occur as a result of apoptosis, but with the suboptimal growth conditions, necrosis was also possible. Flow cytometry was used to determine the rates of apoptosis and necrosis in each of the different experimental conditions. Apoptosis rates were measured using an annexin V stain which attached to the membrane of cells undergoing early-stage apoptosis (Tatsuta et al., 2013). Since apoptosis is a transient state, measurement of apoptosis with Annexin V will only show the percentage of T cells undergoing early-stage apoptosis at that exact time and cannot measure to total percentage of cells that have undergone the apoptotic

process (Darzynkiewicz & Traganos, 1998). A 7AAD dye which stained the interior of necrotic cells was used to determine necrosis rates in each condition (George et al., 2004).

In aim 3, the effects of T3 on the survival, proliferation and cell characteristics of T cells cultured in media with T3 and the free fatty acid PA was measured. A dose response was conducted in order to find a moderate PA concentration to supplement the T cells with. 50 μM was the maximum dose added to T cells since levels above 50 μM have been seen to cause T cell death (Takahashi et al., 2012). It was predicted that T3 would allow T cells to increase their uptake of fatty acids into the mitochondria and help T cells use PA as an additional source of nutrients, leading to increased T cell survival and proliferation rates. T cell survival will be measured using live cell gating and propidium iodide (PI) staining. T cell proliferation was measured by staining T cells with CFSE.

In aim 4, the effect of adding the CPT1 inhibitor etomoxir on the survival of T cells cultured with or without T3 was measured. It was predicted that adding etomoxir would inhibit T cell uptake and metabolism of fatty acids. Therefore, negating the T3 mediated increase in T cell survival, leading to a sudden decrease in the T3 mediated T cell survival rates. T cell survival was measured using cell parameter gating on the flow cytometer.

3.0 Methodology

3.1 Jurkat T cell and T3 preparation

Jurkat T cells were thawed from a cryogenic vial and cultured in vented flasks with Roswell Parks Memorial Institute (RPMI) culture media (Thermo Fisher Scientific, Ottawa, ON) with 5% fetal bovine serum (FBS) (Wisent Inc., Saint-Jean-Baptiste, QC) for two weeks, giving the cells enough time to recover from the cryogenic storage. The RPMI culture media contains 2 mg/mL of glucose supplemented with 1mM L-glutamine and 1 mM pen-strep.

The vented flasks were left in a CO_2 incubator at 37^0 C and at a 5% CO_2 concentration and atmospheric oxygen levels. No additional oxygen was added to any of the conditions and the pH of the culture media was not controlled or measured. The cells were passaged every 2 to 3 days to ensure that the cells did not become over crowded. For the experiment, the Jurkat T cells were grown in RPMI culture media containing 2 mg/mL of glucose supplemented with 1mM L-glutamine, 1 mM pen-strep. Glucose and fatty acids concentrations in the culture media were restricted by only adding 1% FBS to RPMI media, instead of the usual 5 to 10% used to culture Jurkat T cells. It is unlikely that there would be enough glucose in the 1% FBS media to prevent T cell from starving after a week.

Thyroid hormone media was made by dissolving powdered T3 (Sigma-Aldrich, Oakville, ON) in 0.02N NaOH and sterile phosphate buffered saline (PBS) (Wisent Inc., Saint-Jean-Baptiste, QC) to make a 2000 µg/mL stock which was then diluted into culture media. Four T3 conditions were analysed: T3 at approximated hypothyroid levels (0.1 µg/mL), T3 at normal physiological (euthyroid) levels (1.0 µg/mL), T3 at hyperthyroid levels (10 µg/mL), and a control group with no added thyroid hormones. T cells were cultured at an initial concentration of 50 000 cells/mL, to help ensure consistency between experiments and decrease overcrowding of cells, in flasks containing one of the four previously described conditions. Cells were analysed on a daily basis for the entirety of the experiments.

For the preparation of thyroid hormone media protocol, please see Appendix D.

3.2 T cell survival and proliferation experiments

Initial cell proliferation and viability were measured using a trypan blue counting technique. T cell survival was measured using the BD Accuri ™ C6 flow cytometer (BD Biosciences, Mississauga, ON) based on cell size and granularity parameters in addition to measuring the fluorescence of every individual cell. T cell proliferation was measured by incubating T cells with CFSE (Sigma-Aldrich, Oakville, ON) and measuring mean CFSE emission of live cells using flow cytometry. Software on the flow cytometer allowed for gating around specific population subtypes and measurement of the percentages of cells contained in a specific subpopulation.

For additional information on flow cytometry analysis, please see Appendix B
For CFSE staining protocol, please see Appendix E.

3.3 T cell apoptosis and necrosis experiment

In order to quantify whether T3 affected T cell death pathways, flow cytometry and cell staining were used to differentiate between apoptotic and necrotic cells. To measure T cell necrosis and apoptosis rates, T cells from each experimental condition were analysed by flow cytometry after being stained with annexin V PE (eBioscience, Inc. San Diego, CA) to measure T cell apoptosis and 7AAD (eBioscience, Inc. San Diego, CA) to measure necrosis.

For 7AAD and annexin V staining protocol, please see Appendix F.

3.4 PA supplementation experiments

PA was added to T cells with and without T3. A dose response to PA (Sigma-Aldrich, Oakville, ON) was set up with T cells incubated in 1% FBS with 50 µM, 10 µM, 2 µM, 0.4 µM or 0 µM of PA. Cell viability was measured over 10 days. Following the PA dose response experiment (Figure 6), a concentration of 2 µM of PA was chosen for PA supplementation

experiments. For all the PA supplementation experiments, T cells were cultured over 12 days in 1% FBS media containing one of the following conditions: 10 µg/mL of T3 with 2 µM of PA (T310-PA2), 10 µg/mL of T3 with no added PA (T310-PA0), 2 µM of PA with no added T3 (T30-PA2) and a control condition (T30-PA0).

For PA preparation protocol, please see Appendix G.

The T3 PA dose response was measured using flow cytometry and cell size and granularity parameters. CFSE staining was used to measure T cell proliferation. T cell survival was measured by staining T cells with propidium iodide (PI) (Sigma-Aldrich, Oakville, ON) before analysing the cells by flow cytometry

For PI staining protocol, please see Appendix H.

3.5 Etomoxir experiments

Additionally, blocking the uptake of fatty acids into the cells mitochondria was used to measure whether fatty acids used as an energy source helped T cells survive in the presence of T3. T cells were cultured for 6 days in 1% FBS media containing 10 µg/mL of T3 or no added T3. On the 6th day, etomoxir (Sigma-Aldrich, Oakville, ON) at a concentration of 100 µM (Byersdorfer et al., 2013) was added to half of the flasks of each condition. The other flasks received an equal volume of PBS. Cells were analysed by flow cytometry for an additional 5 days.

For etomoxir experiment protocol, please see Appendix I.

3.6 Statistics

All data collected by flow cytometry was analysed using Flow Jo Single Cell Analysis Software (FlowJo LLC, Ashland, OR). Flow Jo allowed for the gating of T cells and live cells, in addition to setting quadrants and bisector limits to distinguish between positively and negatively stained cells (Figure 13, Appendix B). Raw values were then exported to Excel, where each value was corrected using subtraction of within-subject variation from the group mean according to Loftus *et al.,* (Loftus & Masson, 1994). The area under the curve (AUC) was then calculated for the duration of the experiment according to Pruessner *et al*. (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). AUC, a mathematical calculation based on the calculation of the definite integral, is often used in pharmacokinetics to measure total dosage of a drug. AUC was used to simplify the analysis of the multiple time points collected and comparing the total effect of each condition without forfeiting the accuracy of the values collected (Pruessner et al., 2003). AUC with respect to ground was calculating by adding half the difference between the y values of two days to the y value of the previous day. The sum of daily AUC values was calculated for the entire length of the experiment.

Single factor analysis of variance (ANOVA) was performed using a data analysis extension on Excel. If the p-value of the ANOVA was below 0.05, the ANOVA was considered significant and a subsequent 2-tailed paired Student's T-test (T-test) was performed. T-tests were considered significant if the p-values were < 0.05. P-values above 0.05 were considered not significant (NS). P-values between 0.1 and 0.05 were described as a trend (TR). No power calculations were performed on the data sets.

4.0 Results

4.1 Effect of T3 on T cell growth and proliferation

In preliminary experiments, T cells were cultured over 10 days in 1% FBS media containing 10 µg/mL T3, 0.1 µg/mL T3 or a control condition with no added T3 (Figure 1). Total live T cell counts were measured every 24 hours using the trypan blue counting technique to ensure results were as complete and detailed as possible. After day 7, there was a noticeable decrease in live T cell numbers in the control and 0.1 µg/mL T3 conditions, while live T cell counts in the T3 10 µg/mL condition continued to increase. These experiments suggest that T3 10 µg/mL helps increase T cell numbers *in vitro*. The amount of serum used and the choice of using T3 over T4 was based on other preliminary experiments presented in appendix A (Figure 12). The 5-day delay before any thyroid hormone effect on T cell numbers can be seen, is most likely due to the latency of T3 activation of the thyroid hormones receptor and the genomic changes that follow. In the first few days of the experiment, there is most likely still enough glucose in the media to sustain the cell in the control and T3 0.1 µg/ml conditions.

Figure 1. T3 increases Jurkat T cell counts

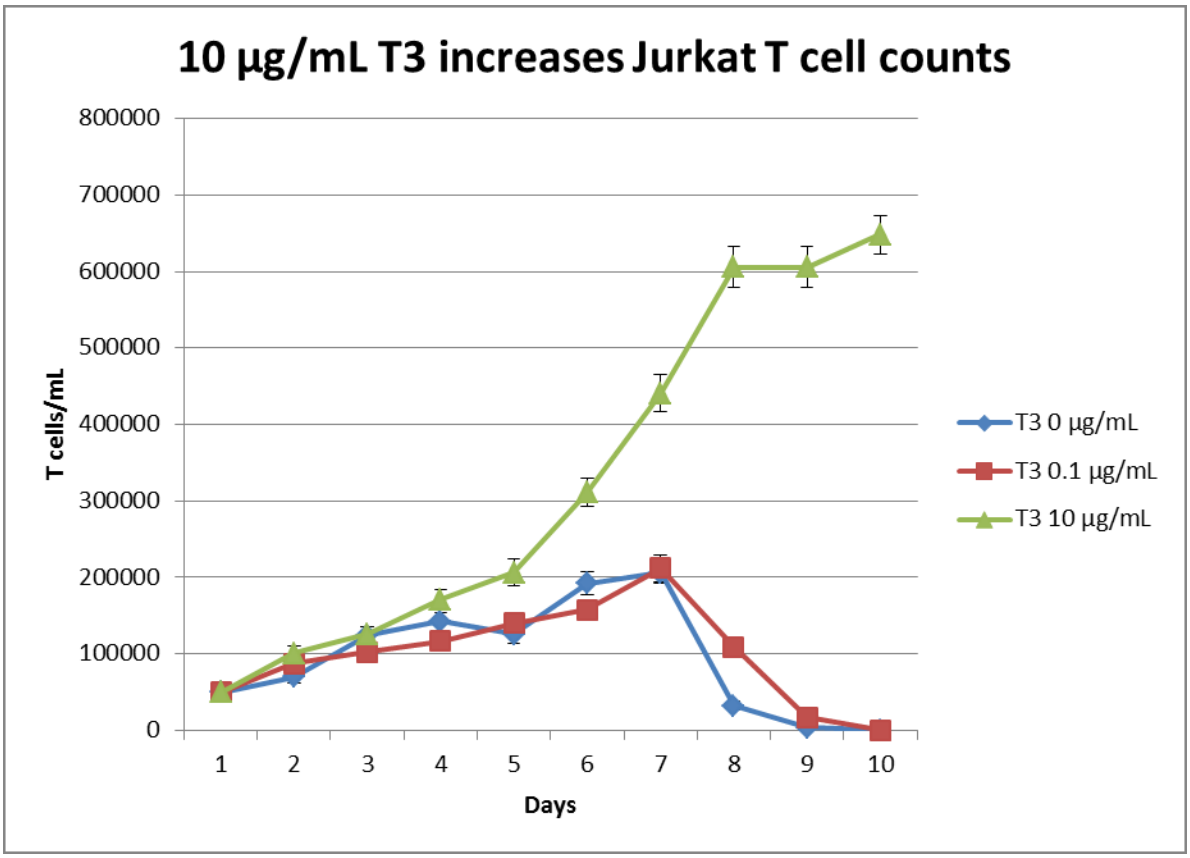


Figure 1. T cells were cultured in 1% FBS containing media with or without T3. Live cells counts were determined by trypan counting. Data from an experiment with duplicate flasks. Error bars based on SE.

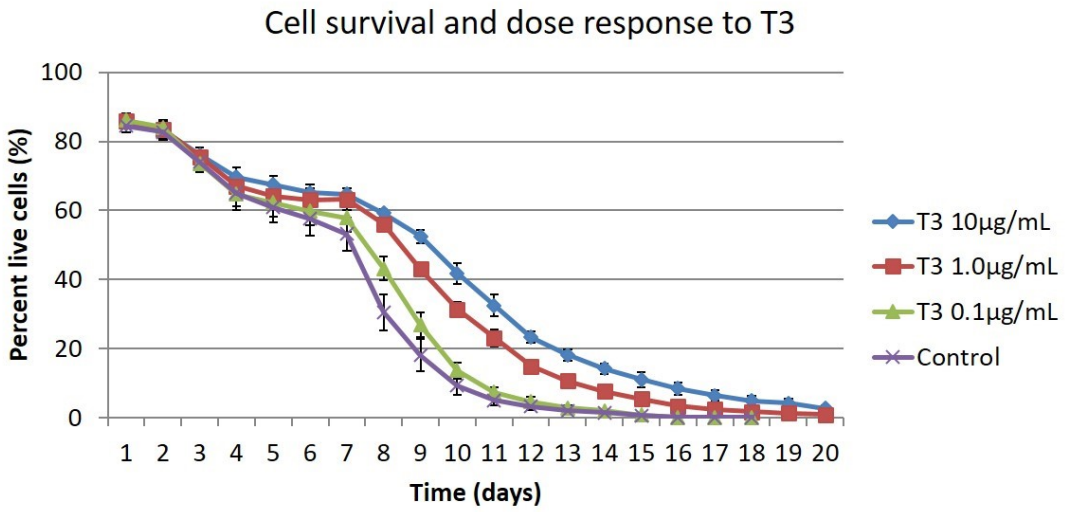
4.1.1 T3 and survival

In order to investigate the mechanism leading to increased T cell population numbers, experiments were done to measure whether adding T3 10 µg/mL to culture media would help increase T cell survival or increase T cell proliferation.

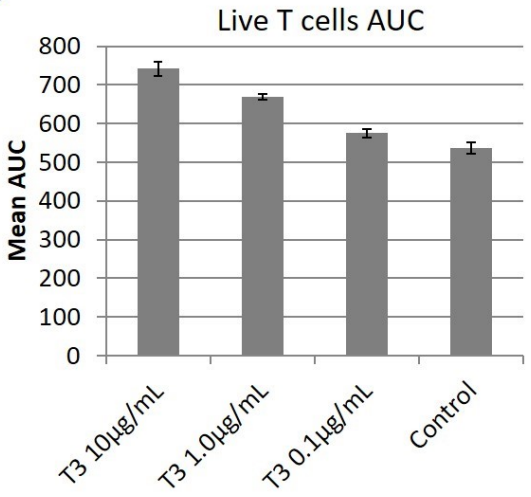
A dose response was conducted over 20 days in media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL T3 or a control condition, in order to measure the T3 mediated effect on T cell survival until cells in all the conditions had crashed. T cell survival was based on flow cytometry gating (see appendix B for gating example). Although the percentage of live cells slowly diminished in all the conditions, T cells in the T3 10 µg/mL condition survived longer than the cells in the other conditions. The dose response curve showed two distinct phases in cell survival over the course of the experiment. There was an initial decline in cell survival which leveled off around day 6, followed by a sharp decline in cell survival which lead to the complete death of T cells in the T3 0.1 µg/mL and control conditions (Figure 2A). The sharp decline in T cell survival is most likely due to the depletion of glucose. Thyroid hormone-mediated increase in gene transcription take several days to become apparent, this is the most likely reason there does not seem to any great differences in T cell survival for the first 7 days of the experiments. To make statistical comparisons, the AUC was calculated for 6 experiments over 20 days (Figure 2B). The single factor ANOVA performed on the AUC values was considered significant, indicating that T3 10 µg/mL promoted an increase in T cell survival compared to the other conditions. Subsequent paired two-tailed T-tests were performed and showed a significant difference ($p<0.02$) between all the conditions (Figure 2C). These results suggest that increased levels of T3, lead to increased T cell survival over 20 days.

Figure 2. T cell survival and dose response to T3

A



B



C

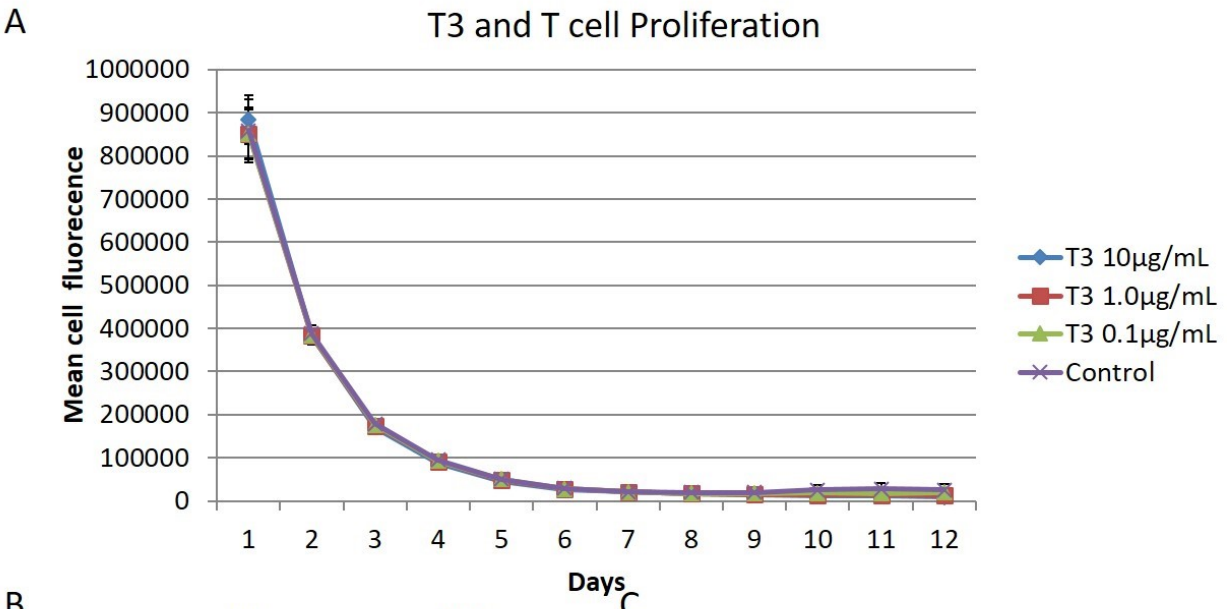
	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	** 0.003	** 0.002	** 0.001
T3 1.0 µg/mL		** 0.003	** 0.001
T3 0.1 µg/mL			* 0.013

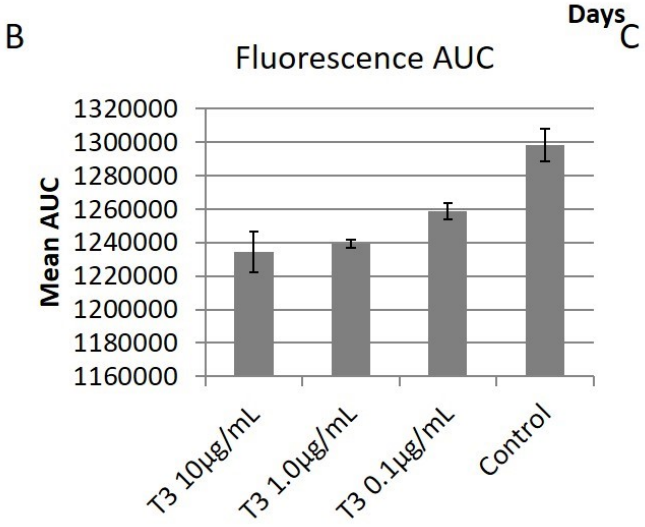
Figure 2. T cells were cultured in 1% FBS containing media with or without T3. Live cells were determined by flow cytometry using size parameters. A) Data pooled from 7 experiments. B) AUC was calculated for the 6 experiments that lasted at least 20 days. C) *$p < 0.05$, ** $p < 0.01$, p-value for paired two-tailed T-tests performed on the mean AUC values.

4.1.2 T3 and proliferation

To determine if the T3 mediated increase in T cell survival was due to enhanced proliferation rates, T cells were labelled with a proliferation dye and cultured over 12 days in media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL or a control condition. The concentration and intensity of the CFSE dye are reduced every time the cell divides, leading to an inverse relation between proliferation rates and CFSE emission intensity. Therefore, the lower the mean cell fluorescence and cell fluorescence AUC, the more the cells have undergone proliferation. The graph depicting T cell proliferation did not show an obvious change in the mean CFSE levels (Figure 3A). The AUC was calculated for 4 experiments over 12 days (Figure 3B). T cells in the control condition had a slightly higher CFSE emission, meaning that T cells in this condition had proliferated the least. The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a small but significant difference in T cell proliferation between some of the conditions (Figure 3C). As a result of these significance tests, it was apparent that T cells cultured with T3 10 µg/mL had a trend towards increased proliferation when compared to the control condition. T cells cultured with T3 1.0 µg/mL had a significant increase in T cell proliferation compared to cell cultured with T3 0.1 µg/mL ($p<0.03$) and the control condition ($p<0.02$). T cells cultured with T3 0.1 µg/mL had a significant increase in T cell proliferation compared to T cells in the control condition ($p<0.03$).

Figure 3. T3 and T cell proliferation





C

	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	NS	NS	TR 0.056
T3 1.0 µg/mL		* 0.027	* 0.014
T3 0.1µg/mL			* 0.02

Figure 3. T cells were cultured in media with or without T3. Proliferation was measured each day with CFSE staining and flow cytometry. A) Data pooled from 4 experiments. B) AUC was calculated for 4 experiments over 12 days. C) *$p < 0.05$, p-value for paired two-tailed T-tests performed on the mean AUC values.

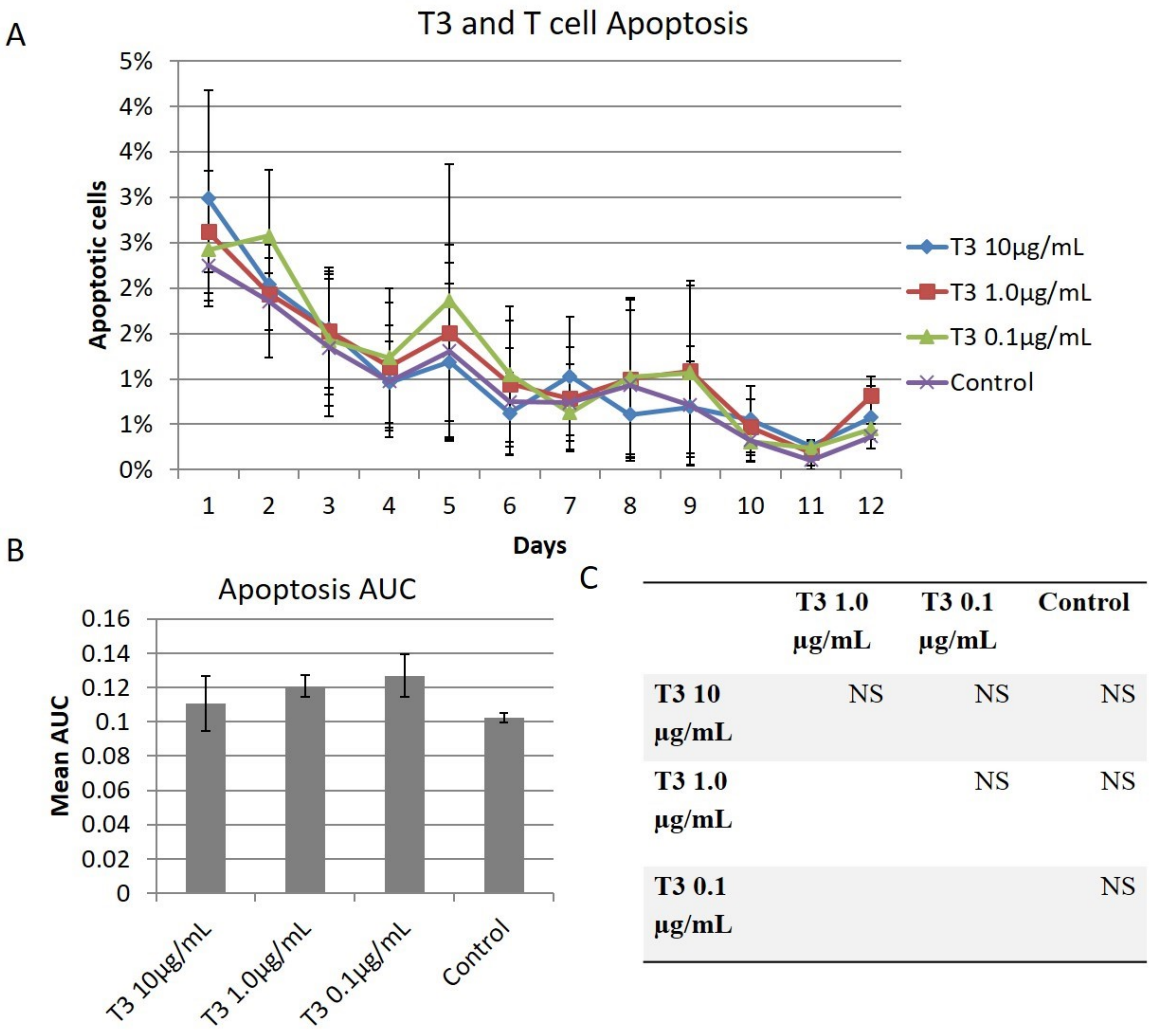
4.2 T3 and cell death

4.2.1 T cell apoptosis

In order to measure the effect of T3 on T cell death, T cells were analysed after being stained with annexin V and 7AAD. T cells undergoing early-stage apoptosis are positively stained with annexin V, but negative for 7AAD. Necrotic T cells are positively stained by both annexin V and 7AAD while live cells are negative for both dyes.

To see if apoptosis was affected by adding T3 to culture media, T cells were cultured over 12 days in media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL or a control condition. There was no visible difference between the mean apoptosis levels of the measured condition (Figure 4A). This curve depicts the percent of cell undergoing apoptosis in the culture and cannot be compared with the T cell survival curve seen in Figure 2A, as apoptosis is only one of the programmed T cell death pathways measured in this study and apoptosis rates stayed relatively low during the entire experiment. AUC was calculated for 3 experiments over 12 days (Figure 4B). Although the mean apoptosis AUC seemed lower for the control condition compared to the cells with added T3, the single factor ANOVA performed on the corrected AUC values was not considered significant (Figure 4C). No further statistical tests were performed. Although T cells in the control condition seem to have slightly less apoptosis than T cells in the other conditions, T3 did not have any significant effect on T cell apoptosis rates and apoptosis rates seemed to decrease in all the conditions as the experiments progressed. In conclusion, T3 did not affect T cell early-stage apoptosis rates in these experiments.

Figure 4. T3 and T cell apoptosis



C

	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	NS	NS	NS
T3 1.0 µg/mL		NS	NS
T3 0.1 µg/mL			NS

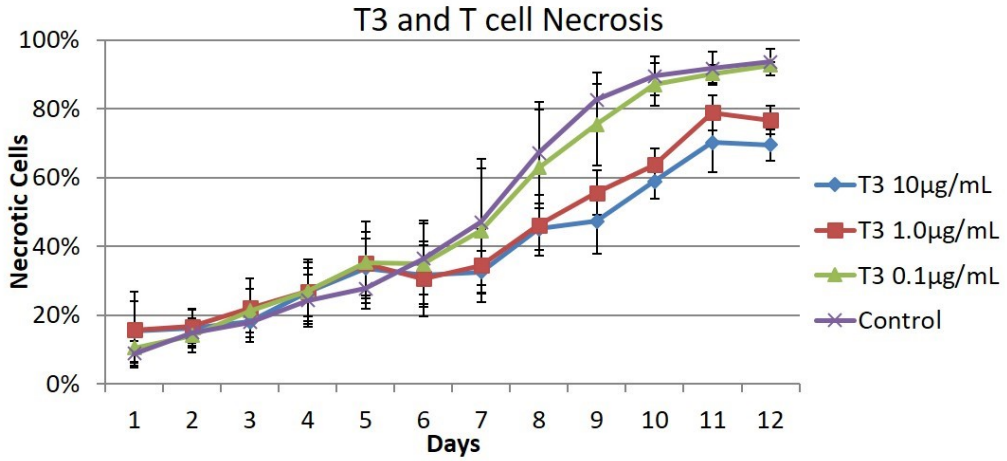
Figure 4. T cells were cultured in media with or without T3. Apoptosis was measured each day with flow cytometry and cell staining. A) Data pooled from 3 experiments. B) AUC was calculated for 3 experiments over 12 days. C) T-tests were not performed.

4.2.2 T cell necrosis

While T3 did not have a significant effect on the apoptosis rates of T cells, T cell necrosis rates could still be affected by the presence of T3. Necrotic cells are positive for annexin V and positive for 7AAD. T cells were cultured over 12 days in media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL T3 or a control condition. In the line graph showing T cell necrosis rates, T3 0.1 µg/mL and the control conditions seemed to have a greater percentage of necrotic cells compared to the T3 10 µg/mL and T3 1.0 µg/mL conditions (Figure 5A). This curve depicts the percent of necrotic cells in the culture and is the inverse of the curve seen in Figure 2A, where the percentage of T cells still alive were depicted in the graph. The AUC was calculated for 3 experiments over 12 days (Figure 5B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a trend towards decreased T cell necrosis between some of the conditions (Figure 5C). T cells cultured with T3 10 µg/mL had a trend towards decreased necrosis when compared to the control condition. While there was a trend towards less T cell necrosis, T3 10 µg/mL did not significantly decrease T cell necrosis rates. In conclusion, there is a weak trend toward decreased rates of T cell necrosis in the T3 10 µg/mL condition compared to the control condition.

Figure 5. T3 and T cell necrosis

A



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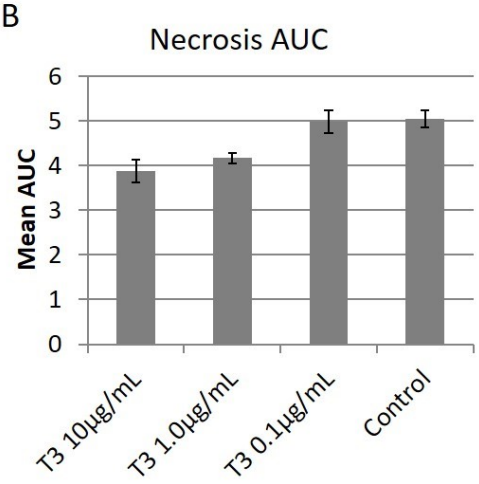


C

	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	NS	NS	TR 0.095
T3 1.0 µg/mL		NS	NS
T3 0.1 µg/mL			NS

Figure 5. T cells were cultured in media with or without T3. Necrosis was measured each day with flow cytometry and cell staining. A) Data pooled from 3 experiments. B) AUC was calculated for 3 experiments over 12 days. C) Paired two-tailed T-tests performed on the mean AUC values.

4.3 T3 and PA supplementation

Given that T3 increased survival in T cells, but had no effect on apoptosis and only a trend towards decreasing necrosis, exogenous PA supplementation was used to study whether the presence of added T3 allowed the cells to use fatty acid as another energy choice when glucose levels in the media were depleted. The appropriate level of PA was chosen after performing a dose-response experiment over 10 days (Figure 6). T cell survival was measured instead of total T cell numbers (Figure 1), because the goal of this experiment was to determine which concentration of PA were tolerated by or toxic to Jurkat T cells cultures with 1% FBS media. T cells were cultured for 10 days with 50 µM, 10 µM, 2 µM, 0.4 µM or no added PA. The T cells in the 50 µM PA condition had greatly reduced viability from the first day, this was expected as PA 50 µM is the highest concentration tolerated by Jurkat T cells cultured in optimal conditions (Lima et al., 2002; Takahashi et al., 2012). The sudden 30 % decrease in T cell survival between day 6 and day 7 is most likely due to cell starving from lack from glucose depletion. After reviewing the dose response results, 2 µM PA was chosen as the desired PA dosage for the following experiments. T cells in the 2 µM PA condition seemed to have slightly greater survival rates at almost all the time point during the 10 day experiment. The concentration of PA added to the culture media would be considered to be quite low physiologically, as PA concentration in the blood in euthyroid subjects are 0.19±0.03mM (190±30µM) and have been reported to be as high as 0.49 ±0.10mM (490±100µM) in subjects with non-thyroidal illnesses with thyroid hormone binding inhibitor (Chopra et al., 1985).

Figure 6. PA dose response

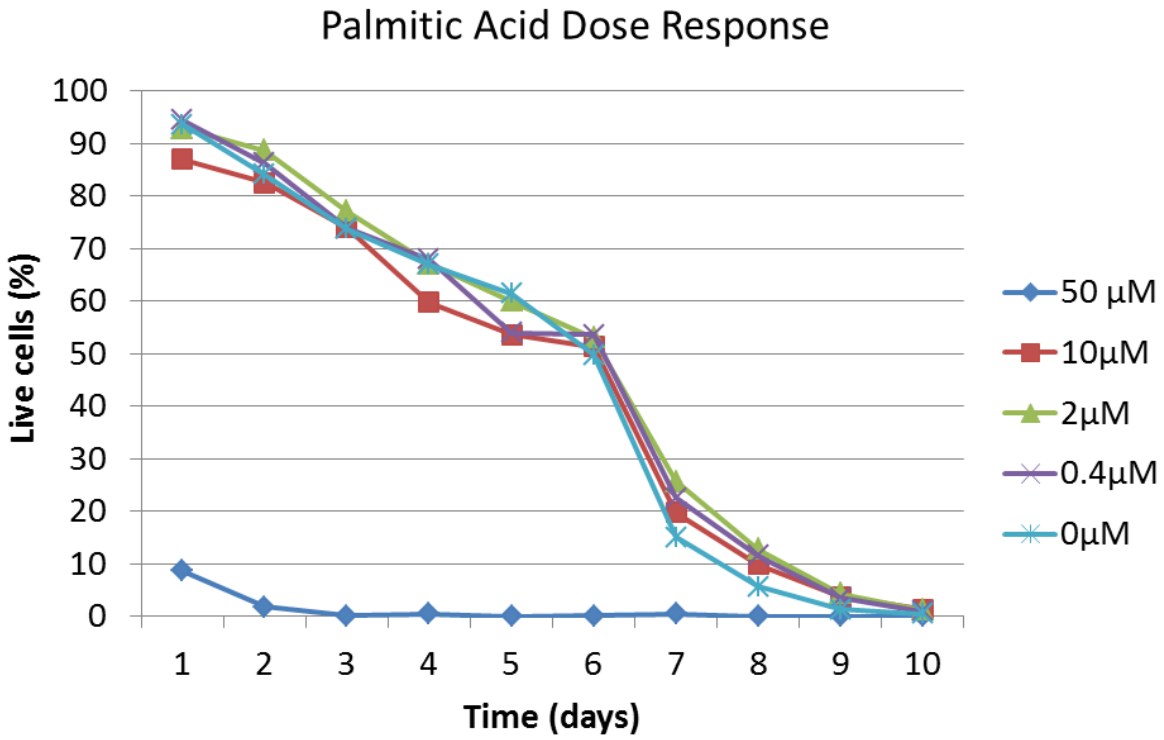
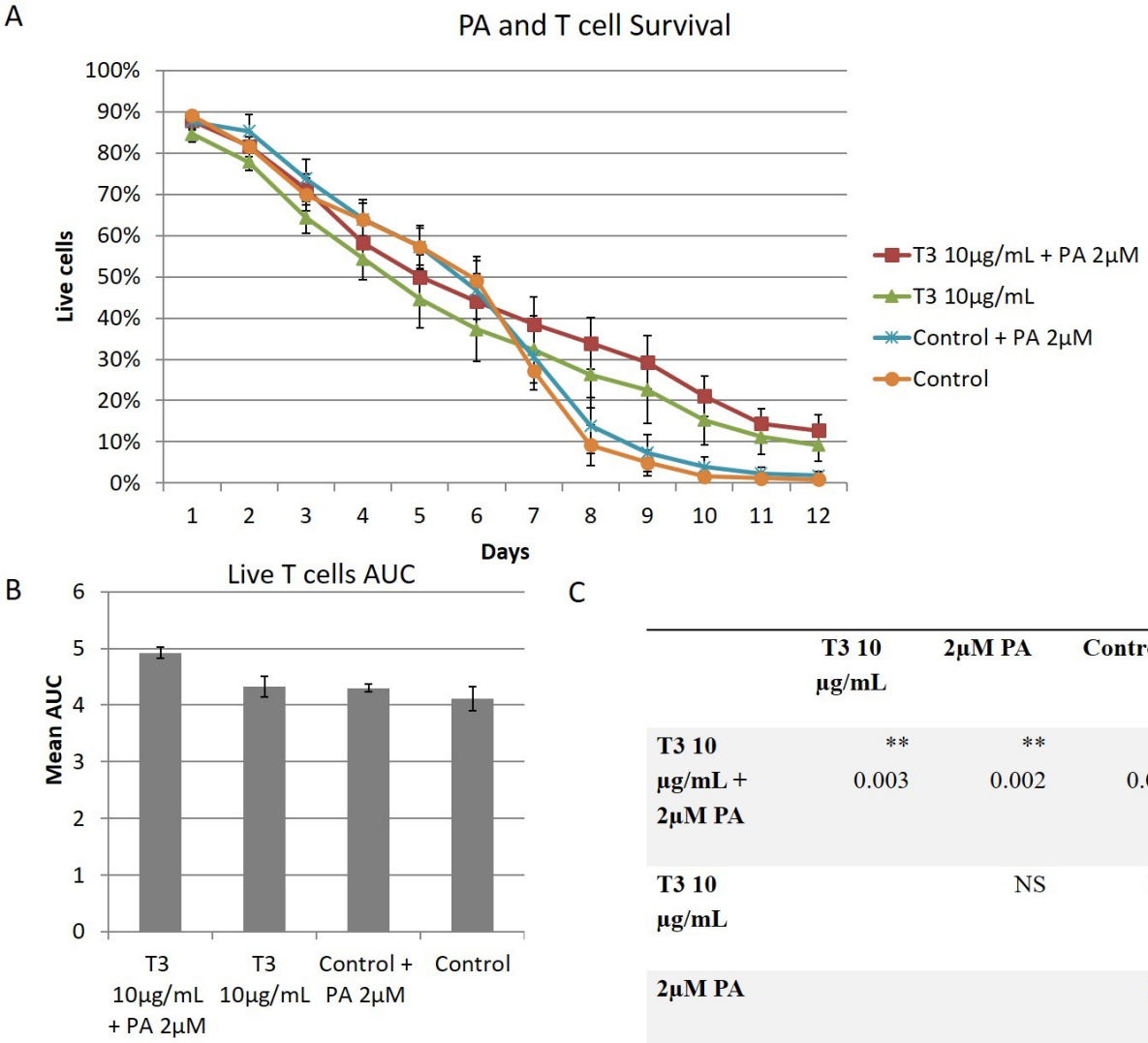


Figure 6. T cells were cultured for 10 days in 1% FBS containing media, with 50 µM, 10 µM, 2 µM, 0.4 µM or no added PA.

4.3.1 PA and T cell survival

A detailed dose response was conducted over 12 days in 1% FBS media containing T3 10 µg/mL + 2 µM PA (T3 10-PA 2), 10 µg/mL T3 (T3 10-PA 0), control + 2 µM PA (T3 0-PA 2) and the control condition (T3 0-PA 0). The percentage of live cells slowly diminished in all the conditions. T cells in the T3 10-PA 2 and T3 10-PA 0 initially had a slighter percentage of live cells, then around day 7, T cell survival stabilised (Figure 7A). The AUC was calculated for 8 experiments over 12 days. The mean AUC for the T3 10-PA 2 condition was visually greater than the AUC for the other conditions (Figure 7B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent paired two-tailed T-tests were performed and showed a significant increase in T cell survival between some of the conditions (Figure 7C). T cells cultured in the T3 10-PA 2 condition had a significant increase in survival when compared to the other conditions ($p<0.04$), suggesting that the addition of PA in the presence of T3 helps the cell survive longer. There was no significant difference in survival between the T3 10-PA 0 and the T3 0-PA 2 conditions.

Figure 7. PA and T cell survival



	T3 10 µg/mL	2µM PA	Control
T3 10 µg/mL + 2µM PA	** 0.003	** 0.002	* 0.032
T3 10 µg/mL		NS	NS
2µM PA			NS

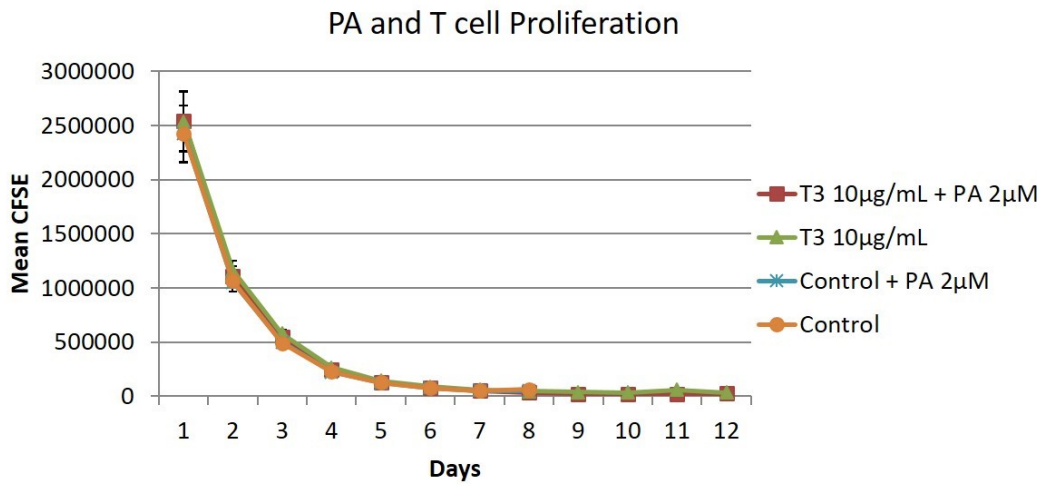
Figure 7. T cells were cultured in media with or without T3 and with or without 2 µM of PA. Live cells were measured with flow cytometry using size parameters. A) Data pooled from 8 experiments. B) AUC was calculated for all time point for 8 experiments over 12 days. C) *$p < 0.05$, ** $p < 0.01$, p-value for paired two-tailed T-tests performed on the mean AUC values.

4.3.2 PA and T cell proliferation

To determine if the T3 and PA-mediated increase in T cell survival was due to enhanced proliferation rates, T cells were labelled with a proliferation dye and cultured over 12 days in 1% FBS media containing one of the T3- PA conditions. The line graph depicting T cell proliferation did not show any visible change in the mean CFSE levels (Figure 8A). AUC was calculated for 6 experiments over 12 days (Figure 8B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a significant increase in T cell survival between some of the conditions (Figure 8C). T cells cultured with T3 10-PA 2 condition had a significant increase in proliferation compared to the cells in the T3 10-PA 0 ($p<0.02$) and T3 0-PA 0 ($p<0.05$) conditions. T cells cultured in the T3 10-PA 2 condition showed a trend towards increased proliferation compared to cells in the T3 0-PA 0 condition. T cells cultured in the T3 0-PA 2 condition indicated a trend towards increased proliferation compared to cells in the T3 0-PA 0 condition. Supplementation with 2 μM PA had a trend toward increasing T cell proliferation, suggesting that the T cells were starved of nutrients and any additional energy source would allow the cells to improve their function.

Figure 8. PA and T cell proliferation

A



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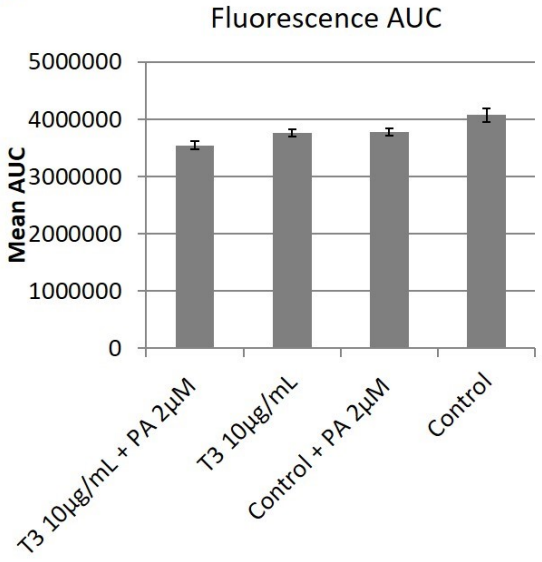


C

	T3 10 µg/mL	2µM PA	Control
T3 10 µg/mL + 2µM PA	* 0.012	TR 0.074	* 0.042
T3 10 µg/mL		NS	NS
Control + 2µM PA			TR 0.087

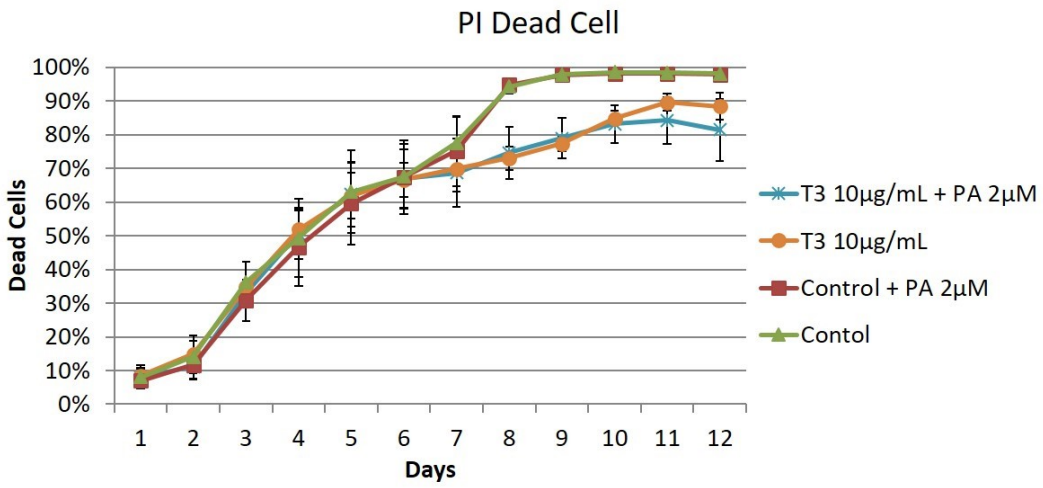
Figure 8. T cells were cultured in media with or without T3 and with or without 2 µM of PA. Live cells were measured by flow cytometry and cell staining. A) Data pooled from 6 experiments. B) AUC was calculated for all time points for 6 experiments over 12 days. C) *$p < 0.05$, p-value for paired two-tailed T-tests performed on the mean AUC values.

4.3.3 PI positive cells

Given the results seen with the PA supplementation experiments, a more stringent cell survival experiments were performed using PI. T cell death was measured by growing T cells over 12 days in one of the T3 - PA conditions and staining them with PI. T cells positively stained with PI were considered dead. T cells in the T3 10-PA 2 and the T3 10-PA 0 conditions seemed to have fewer dead T cells compared to the T3 0-PA 2 and T3 0-PA 0 conditions (Figure 9A). The AUC was calculated for 4 experiments over 12 days. The mean AUC for the T3 0-PA 2 and T3 0-PA 0 conditions was visually greater than in the other conditions, suggesting that T3 helped T cells survive better (Figure 9B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a significant increase in T cell survival between some of the conditions (Figure 9C). T cells cultured with T3 10-PA 2 condition had significantly less death compared to the cells in the T3 0-PA 2 ($p<0.05$) and T3 0-PA 0 ($p<0.04$) conditions. T cells cultured with T3 10-PA 0 condition had significantly less death compared to the cells in the T3 0-PA 2 ($p<0.04$) and T3 0-PA 0 ($p<0.03$) conditions. T cells cultured in the T3 0-PA 2 condition had significantly less death compared to the cells in the T3 0-PA 0 condition. There was no significant difference in dead cells between the T3 0 -PA 2 and the T3 10-PA 0 conditions, suggesting that T3 has a greater impact on T cell survival than PA supplementation.

Figure 9. PI positive cells

A



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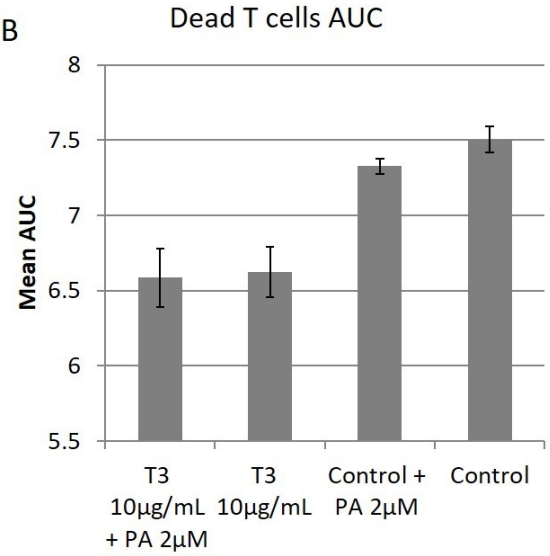


C

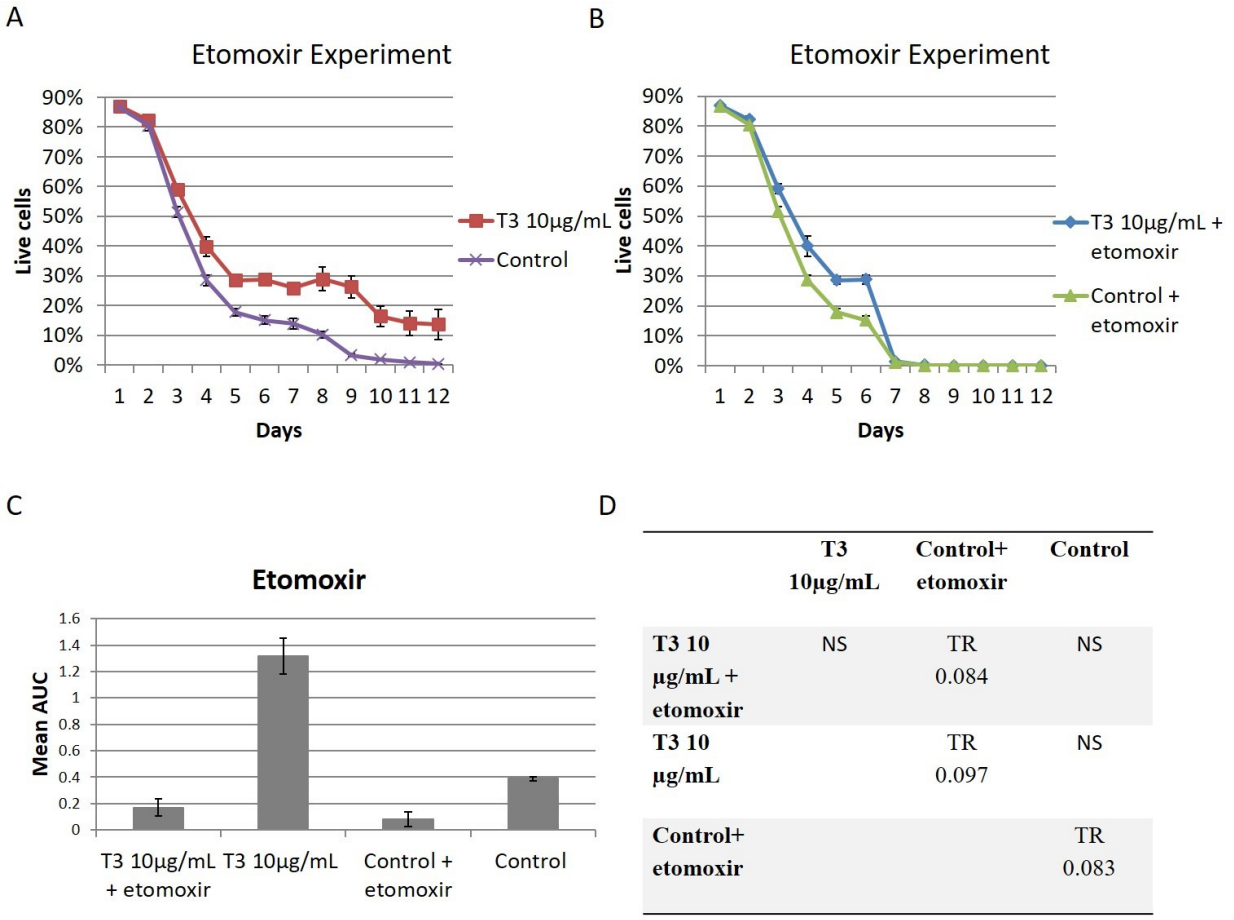
	T3 10µg/mL	Control + 2µM PA	Control
T3 10 µg/mL + 2µM PA	NS	* 0.043	* 0.037
T3 10 µg/mL		* 0.033	* 0.02
Control + 2µM PA			* 0.049

Figure 9. T cells were cultured in media with or without T3 and with or without 2 µM of PA. Dead cells were measured by flow cytometry and PI staining. A) Data pooled from 4 experiments. B) AUC was calculated for 4 experiments. C) *$p < 0.05$, p-value for paired two-tailed T-tests performed on the mean AUC values.

4.4 T3 and etomoxir

In the previous experiments, supplementing T cells with T3 and PA resulted in a small but significantly increased T cell survival and proliferation, this was most noticeable as of the 6th day of the experiment. This lead to the conclusion that T3 might allow T cells to utilise fatty acids as an energy source by increasing fatty acid uptake into the mitochondria and mitochondrial fatty-acid oxidation. Utilisation of mitochondrial fatty acid oxidation as an energy source was measured by adding etomoxir, a beta-oxidation blocker. T cells were cultured for 6 days in media containing 10 µg/mL T3 or no added T3 (Figure 10 A). On the 6th day after all the conditions were analysed, half of the samples were supplemented with 100 µM of etomoxir, the rest of the samples were supplemented with the same volume of PBS (Figure 10 B). Etomoxir lead to a sharp decline in live T cells in both the T3 10 µg/mL and the control conditions within 24 hours. The AUC was calculated for 2 experiments over 12 days (Figure 10C). The AUC values for the T3 10 µg/mL appeared greater than the AUC for the other conditions. The single factor ANOVA performed on the AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a trend in T cell survival between some of the conditions (Figure 10 D). T cells cultured in the control condition showed a trend towards an increased in live cells compared to the cells cultured in the control+ etomoxir condition. These results suggest that T cells already use beta-oxidation to increase their access to nutrients. T cells cultured within the control + etomoxir had a trend towards decreased survival when compared to the T3 10 µg/mL and the T3 10 µg/mL+ etomoxir condition. Even though there was a sharp decline in live T cells in the T3 10 µg/mL condition after etomoxir was added, the difference in mean AUC values over 12 days was not considered significant.

Figure 10. T3 and etomoxir



	T3 10µg/mL	Control+ etomoxir	Control
T3 10 µg/mL + etomoxir	NS	TR 0.084	NS
T3 10 µg/mL		TR 0.097	NS
Control+ etomoxir			TR 0.083

Figure 10. T cells were cultured in media with or without T3 and half of the sampled were supplemented with etomoxir on the 6th day. Live cells were measured by flow cytometry and cell size parameters. A) Data pooled from 2 experiments. B) Data pooled from 2 experiments. C) AUC was calculated for 2 experiments. D) *$p < 0.05$, p-value for paired two-tailed T-tests performed on the mean AUC values.

5.0 Discussion

The primary purpose of this project was to measure the effects of T3 on T cell survival and proliferation. Additionally, the effect of T3 on the T cell's ability to utilise fatty acids as an energy source in a glucose-restricted environment was studied. This research project is the first to characterise the T3 mediated effects on T cell survival and proliferation on a daily basis over the course of 12 days. This study is also the first to measure these parameters when T cells are supplemented with both T3 and PA.

5.1 T3 increases T cell proliferation and survival

In the first aim of the study, the role of T3 in T cell survival and proliferation was studied. Adding T3 significantly increased T cell survival in the glucose-deprived environment. Glucose and fatty acids concentrations in the culture media were restricted by only adding 1% FBS to RPMI media, instead of the usual 5 to 10% used to culture Jurkat T cells. It is unlikely that there was enough glucose in the 1% FBS media to prevent T cell from starving after a week.

The results of the T3 dose response on T cell survival showed two separate phases to the decline in T cell survival. This biphasic process of death may initially be due to the decrease in available glucose, and that the second part may be due to the decreasing availability of fatty acid as they get used up by the T cells. According to these results, the presence of T3 may allow T cells to increase their use of fatty acid as an energy source, therefore allowing T cells to postpone death from starvation. As mentioned in the literature review, activated T cells preferentially use glucose as an energy substrate, but T3 has been shown to increase fatty acid uptake into mitochondria of rats (Jackson-Hayes et al., 2003; R. Wang & Green, 2012).

Although the half-life of T3 in Jurkat T cell culture is not known, T3 has a half-life of 12 to 15 hours when added to cultured rat neuroglial cells (Courtin et al., 1986). Even if T3 concentration in the conditions had decreased during the course of the experiment, T3 uptake

into the cell would have induced genomic changes in the T cell's proliferation and metabolic rates which would have taken several days to become apparent. This is most likely why there was a 5 to 7 day delay before any thyroid hormone effect on T cell numbers, survival or proliferation was seen, as the latency of T3 activation of the thyroid hormones receptor and the changes in gene transcription of proliferative and metabolic pathways took several days to become complete.

The proliferation of T cells was significantly increased in cell cultured in the T3 1.0 µg/mL condition when compared to the 0.1 µg/mL T3 and the control condition. Proliferation was also increased in cells cultured with T3 0.1 µg/mL when compared to cells cultured in the control condition. These results suggest that T3 increases T cell numbers by both allowing T cells to divide more rapidly and by helping T cells to survive longer, as a T cell which survives longer with be able to divide more times than a cell which dies off sooner. The proliferation effects of T3 10 µg/mL were relatively small, as there was only about a 5% difference between the mean AUC of T cell in the T3 10 µg/mL and the control condition. The progressive increase in T cell proliferation combined with the increased in T cell survival rates seen in the T3 10 µg/mL conditions could significantly increase live T cell counts over time and corroborate the exponential increase live T cell population seen in Figure 1. The T3 mediated increase in Jurkat T cell proliferation observed in the previously described experiments are compatible with the current published literature as T3 has been shown to increase proliferation of mice lymphocytes, human glioma cells and T cell lymphomas (Cayrol et al., 2015; Klecha et al., 2006; Lin et al., 2009; H. A. Sterle et al., 2014).

These results are consistent with the result of the study by Hodkinson et al., where T3 concentration in the higher levels of the normal physiological range lead to an increase in monocyte counts (Hodkinson et al., 2009). This study by Hodkinson *et al.* also found an increase in neutrophil cell counts and an increase in total memory T cells expression when T4 levels were increased. Together with the results of this study, this could mean that subjects with hypothyroidism may have altered levels of T cells and other immune cells leading to a

potentially compromised immune system, while subjects with hyperthyroidism may have an overactive immune system, leading to an increased risk of autoimmunity.

Additionally, the increased T cell proliferation with T3 is consistent with the study by Klecha *et al.,* that found an increase in proliferation responses in lymphocytes of hyperthyroid mice compared to euthyroid and hypothyroid mice (Klecha et al., 2006). If these results were extrapolated to the effect of thyroid hormones in human, a decrease in T cell proliferation and T cell survival in hypothyroid subjects could potentially lead to a decreased ability to mount an immune defense against pathogens. This could hypothetically increase the risk that the immune system of a person with severe hypothyroidism would not be able to mount a proper defense against an invading pathogen.

5.2 T3 does not significantly alter T cell early-stage apoptosis and necrosis rates

The second aim looked at whether the T3-mediated increase in T cell survival altered T cell apoptosis or necrosis rates. This was important because apoptosis is a self-defense mechanism whereby cells auto-destruct when infected or become otherwise compromised. The experiments showed no significant changes in levels of apoptosis regardless of T3 concentration. In these experiments, T3 might not have an effect on apoptosis rates because early-stage apoptosis is a transient state and cells that underwent the apoptotic process would later be measured as being necrotic or debris once the cell has completely disintegrated (Darzynkiewicz & Traganos, 1998). Additionally, since necrosis occurs due to ATP depletion, while apoptosis is a highly ATP-dependent process, it is logical that T cells in a nutrient restricted environment would preferentially undergo necrosis instead of apoptosis (Darzynkiewicz & Traganos, 1998; Eguchi et al., 1997). The findings in this study suggested that apoptosis rates stayed below 5% for the entirety of the study while more T cells became necrotic due to ATP depletion as the experiment progressed. There was a trend towards decreased necrosis in the T3 10 µg/mL condition compared to the control condition seen as of day 7. This could be due to T3 increasing

CPT1 transcription and increasing mitochondrial fatty acid oxidation in the T cells, therefore increasing the amount of ATP available to the cells.

It is difficult to compare our results on T cells apoptosis levels with other published work, as the other studies used different types of cells and used different criteria to define apoptosis. There has been much controversy concerning the definition and classification of cell death (Kroemer et al., 2009). In this study, T cells with phosphatidylcholine lipids on their cell surface (stained with Annexin V) and intact membranes were considered to be apoptotic (not stained with 7AAD). Cells that had lost their cellular integrity and were positively stained with 7AAD were considered to be necrotic.

The previously mentioned studies reporting an increase in apoptosis promoting protein in the mitochondria of Jurkat T cells cultures with 500nM to 1000nM of T3 did not measure apoptosis using phosphatidylcholine lipids staining (Yehuda-Shnaidman et al., 2005; Yehuda-Shnaidman et al., 2010). Instead, these studies relied on measurement of changes in mitochondrial membrane potential and permeabilization to define apoptosis and used western blotting of lysed T cell to measure pro-apoptosis protein concentrations. Another study which mentioned an increase in apoptosis rates in human lymphocytes cultured with thyroid hormones used the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling assay, which measures DNA degradation, to define apoptosis (Mihara et al., 1999). Both the mitochondrial membrane potential and the DNA degradation methods do not measure cell in the same stage of apoptosis as the Annexin V dye and are better suited to measuring later stages of apoptosis. The difference in definition and methodology in measuring cell apoptosis makes it difficult to compare the current T cell apoptosis rates to those reported in the published studies (Kroemer et al., 2009).

5.3 T3 PA supplementation in the presence of T3 increases T cell survival and proliferation

In the first experiments, T3 extended the T cell cultures for up to 20 days. It was unlikely that there would be enough glucose to keep the cells alive that long and there was no media

change or additional glucose supplementation. This suggested that the T cells were using an alternate fuel source to get energy. PA was the most likely factor because it is very abundant in the serum, and could be derived from the cell membranes of dying cells. The third aim looked at whether adding exogenous PA in the presence of T3 would increase T cell survival and proliferation compared to PA supplementation alone. The concentration of PA added to the culture media was quite low (2μM), as PA concentration in the blood in euthyroid subjects are 0.19±0.03mM (190±30μM) and have been reported to be as high as 0.49 ±0.10mM (490±100μM) in subjects with non-thyroidal illnesses with thyroid hormone binding inhibitor (Chopra et al., 1985).

T cell survival rates were very similar when measured with cell size parameter gating and PI staining. Culturing T cells with both T3 and moderate levels of PA increased T cell survival significantly compared to T cells cultured without T3. This may be due to T3 increasing mitochondrial fatty acid oxidation in T cells and allowing them to use the additional fatty acid as an energy source when glucose is scarce. These results are compatible with the current literature as T3 has been shown to regulate the metabolism of fatty acids by increasing the transcription and activity of the CPT1, the enzyme needed for the uptake of fatty acids into the mitochondrial oxidative cycle (Jackson-Hayes et al., 2003; Sinha et al., 2012).

This could mean that subjects with hypothyroidism may be more reliant on circulating glucose and may not be able to use fatty acids as effectively as euthyroid subjects. Additionally, T cells cultured with T3 10 μg/mL proliferated quicker than T cells without T3 regardless of PA supplementation. Therefore, T3 plays a more important role in T cell proliferation than PA supplementation.

Mean CFSE fluorescence and AUC of cell fluorescence were higher in the Figure 8 PA and T cell proliferation than in the Figure 3 T3 and T cell proliferation experiment because higher initial concentrations of CFSE were used to stain Jurkat T cells at the begging of the PA supplementation experiments than in the previous T3 and T cell proliferation experiments. The

change in CFSE concentrations used to initially stain T cells and differences in standard error may account for the significant difference between the T3 10 µg/mL and control conditions in Figure 3C, while the difference in proliferation between the T3 10-PA 0 and control conditions in Figure 8C was not considered significant.

5.4 Etomoxir does not significantly affect T cell survival

In the fourth aim, etomoxir was added to T cells as a way to inhibit the T3 mediated increase in cell beta-oxidation. Although the addition of etomoxir led to a visible decline in live T cells in the T3 10 µg/mL condition, it did not have a statistically significant effect on T cell survival. Adding etomoxir did lead to a statistically significant decline in live T cells in the control condition. The lack of significance is most likely due to the lack of statistical power. Even though the results did not significantly show the importance of mitochondrial fatty acid uptake and oxidation on T cell survival, the drastic decrease in live T cells rates does corroborate the results of the PA supplementation experiment. This could imply that subjects with decreased thyroid hormones levels may not have the ability to use mitochondrial fatty acid oxidation compared to euthyroid subjects leading to potentially higher circulating free fatty acid level in the blood and a need to carefully monitor blood glucose levels (Chopra et al., 1985). Subjects with untreated hypothyroidism may need to alter their diet or regulate their blood glucose levels in order to help protect the integrity of their immune system.

5.5 Conclusion

Thyroid hormones are essential to the proper functioning of the immune system. These experiments show the impact that T3 has on T cell survival and proliferation, regardless of supplementation with PA. Adequate levels of thyroid hormones are essential for the body to respond properly to an infection that requires T cells to detect the pathogen.

T3 has a role in both increasing T cell proliferation and decreasing cell death. T3 did not have any significant effect on T cell early-stage apoptosis rates measured using Annexin V, while showing a trend towards decreasing T cell necrosis rates measured with 7AAD in this study. Culturing T cells with both T3 and moderate levels of PA increased T cell survival significantly compared to T cells cultured without T3. Blocking the T cell's uptake of fatty acids by inhibiting CPT1 activity with etomoxir could potentially negate the T3 mediated effects on T cell survival, but those effects were not statistically significant in this study.

5.6 Limitations and strengths

Jurkat T cells were used for these experiments since they are lymphoblastoid cells and have less genetic variability than primary cells. This was a major advantage compared to research using primary human blood cells which have substantial person-to-person variability, or using rodent cells which do not always reflect an actual human scenario. The Jurkat T cells robustness allowed for longer term experiments than would be possible with primary human blood cells, allowing experiments to last 10 to 20 days.

One of the major limitations of using Jurkat T cells is that they constantly proliferate at an exponential rate, making it difficult to measure effects on their proliferation. The concentration of FBS in the culture media was reduced from the standard 10% to 1% in order to decrease the rate of T cell proliferation in this study. The mutation which allows Jurkat T cells to proliferate constantly makes them a good model to study the immune system and T cells, as their metabolic use of energy substrates is very similar to that of activated T cells. The use of Jurkat T cells has allowed for the study the effect of T3 on T cells over a much longer period of time than would be possible with primary human blood cells. But the mutation that makes Jurkat T cells so useful in cell culture also makes the results of these experiments less physiologically relevant. The results collected during these experiments are not directly transferable to the effects of thyroid hormones *in vivo* and should be seen as an extrapolation of the possible effects of extreme thyroid hormone disease.

Glucose levels in the media were not measured throughout the experiments, therefore it is not known if glucose levels were completely depleted and T cell starvation due to glucose depletion can only be hypothesised. PA concentrations in the media were not measured and the amount of PA metabolised by T cells in the different conditions is not known. Oxygen consumption by T cells was not measured or controlled and formation of reactive oxygen species in the experimental conditions is unknown. Additionally, the pH of the culture media was not measured or controlled. Therefore, it is unknown if acidity, oxygen consumption or reactive oxygen species played a part in the results collected.

5.7 Future studies

In the future, PA levels in the culture media could be measured using High-Performance Liquid Chromatography and compared with initial levels used to supplement the culture media. Testing daily PA levels could help determine whether changing levels of PA in the media could be due to T cells releasing PA during necrosis or using PA as an energy source. Adding PA to the etomoxir experiments could help strengthen the hypothesis that T3 does increase fatty acid metabolism in Jurkat T cells.

Further studies could also be conducted on primary human blood cells from healthy people, which would more closely mimic *in vivo* immune system conditions. Conducting the same experiments on primary blood cells collected from individuals with untreated hypothyroidism and hyperthyroidism, could help measure the long-term effect of T3 of T cell survival, proliferation and metabolism in a more physiologically relevant way.

Measuring daily glucose levels in the culture media would help solidify the hypothesis that T3 help T cell survive even when glucose levels are depleted. Finally, inhibiting T cell uptake of glucose with fasentin (a glucose transport inhibitor) would help measure if T3 increases fatty acid metabolism in Jurkat T cells when glucose is unavailable (Wood et al., 2008).

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Appendix

Appendix A: Preliminary Data

These preliminary experiments were done to optimize the correct serum level at which Jurkat T cells would proliferate sub-optimally while not dying off too quickly (Figure 11). Preliminary experiments monitoring the effect of T3 on T cell viability (Figure 12) was conducted using a trypan-based counting technique.

In preliminary experiments, Jurkat T cells were cultured in culture media containing different concentrations of FBS over a period of 8 days to measure a dose response in proliferation (Figure 11). At 10% FBS T cell counts were the highest, while cell proliferation at 1% FBS was roughly intermediate. At 0.1% FBS cells did not proliferate and died. After 8 days, there was a noticeable dose response of cell proliferation to the different concentration of FBS added to the culture media. The T cells cultured in 10% FBS media were proliferating exponentially, while the cell cultures in 0.1% and 0.5% FBS containing media demonstrated greatly decreased proliferation. 1% FBS was chosen to be used in all the following experiments because Jurkat T cells cultured in 1% FBS culture media still survived well without becoming overcrowded within a few days.

Figure 11. FBS Serum response

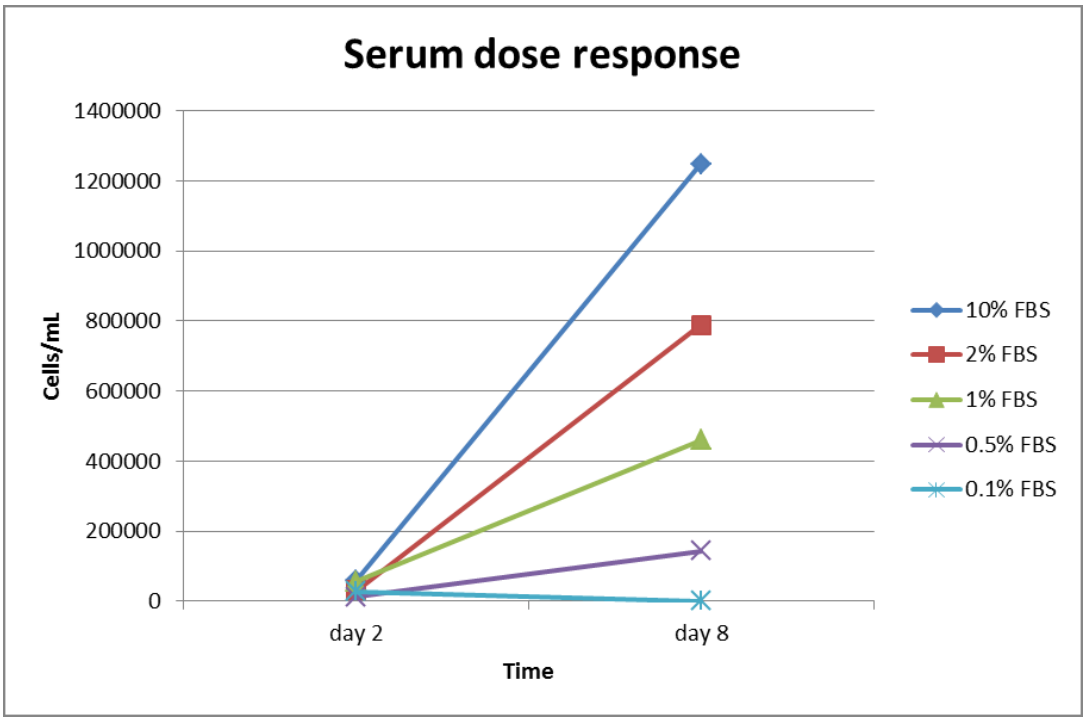


Figure 11. T cells were cultured in media containing various levels of FBS. Live cells counts were determined by trypan counting on day 2 and day 8.

In preliminary experiments, Jurkat T cells were cultured in different concentrations of thyroid hormones for a period of 10 days and their viability was measured using the trypan blue counting technique. After day 7, there was a noticeable decrease in the percentage of live cells in the control and 0.1 µg/mL of T3 and T4 conditions, while the Jurkat cells in the flasks with 10 µg/mL T3 continued to survive and the cells in the T4 10 µg/mL condition were slowly dying. T3 10 µg/mL seems to have had an effect on Jurkat cell survival rates as compared to the other conditions tested. The greater survival rates in the T3 10 µg/mL condition compared to the T4 10 µg/mL condition are most likely due to that fact that T3 has a ten to hundred-fold higher affinity for the thyroid hormones receptor than T4, and T4 must be converted into T3 to become biologically active (Visser, 1988).

Figure 12. T3 maintains Jurkat T cell viability

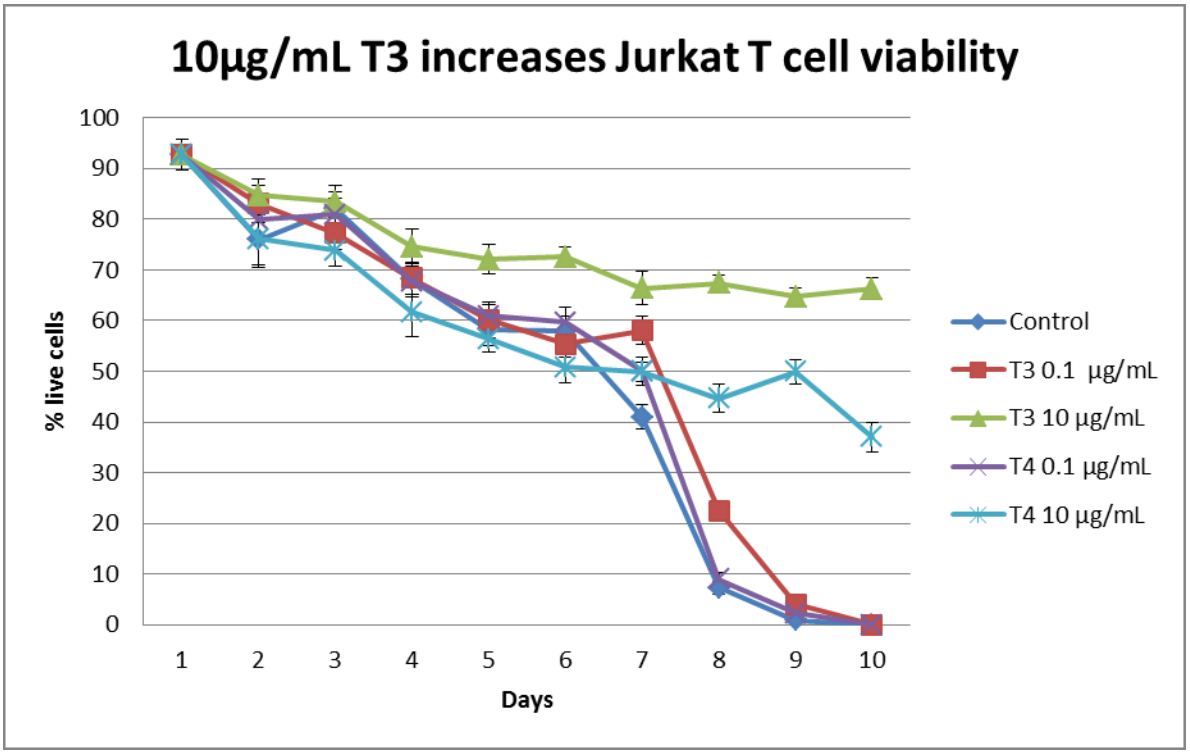


Figure 12. T cells were cultured in 1% FBS containing media with or without T3. Live and dead cell counts were determined by trypan counting. Data from an experiment with duplicate flasks. Error bars based on SE.

Appendix B: Flow cytometry analysis

FlowJo Single Cell Analysis Software was used to analyse the data collected by flow cytometry. The flow cytometer collects many parameters on each event it detects using two lasers for excitation. These parameters include cell size (FSC), cell granularity (SSC) and the emission levels of fluorescent dyes that cells were stained with. To differentiate between cellular debris and T cells and determine the proportion of live T cells in each condition, gates were drawn around events visualised according to their FCS and SSC properties. Live cells are large with intermediate granularity; apoptotic cells are smaller in size and have a high granularity, necrotic cells are larger with high granularity, debris is small with variable granularity (Darzynkiewicz & Traganos, 1998). A rectangular gate was used to distinguish lymphocytes from cellular debris. A polygon gate was drawn around live T cells to separate them from dead T cells. (Figure 13A)

Proliferation was measured by recording the mean CFSE emission of the events in the live cell gate using a histogram (Figure 13B). The flow cytometer measured the fluorescence intensity of each cell and displays both the peak and mean emission of each event.

In order to measure apoptosis and necrosis, T cells stained with annexin V and 7AAD were represented on a dot plot according to their emission levels for each dye (Figure 13C). Quadrants were set according to single stain control, differentiating between positively and negatively stained cells. Cellular debris is found in Q1, these events are negative for annexin V and positive for 7AAD. Necrotic T cells are found in Q2, they are positive for annexin V and positive for 7AAD. T cells undergoing apoptosis are found in Q3, they are positive for annexin V and negative for 7AAD. Live T cells are found in Q4, they are negative for annexin V and negative for 7AAD.

In the PA supplementation experiments, PI staining was used to distinguish between live and dead T cells (Figure 13D). T cells were represented on a histogram according to their PI emission levels and a bisector tool was used to split the histogram into two sections: dead cells which were positively stained by PI and live cells which were negatively stained.

Figure 13. Flow cytometry analysis

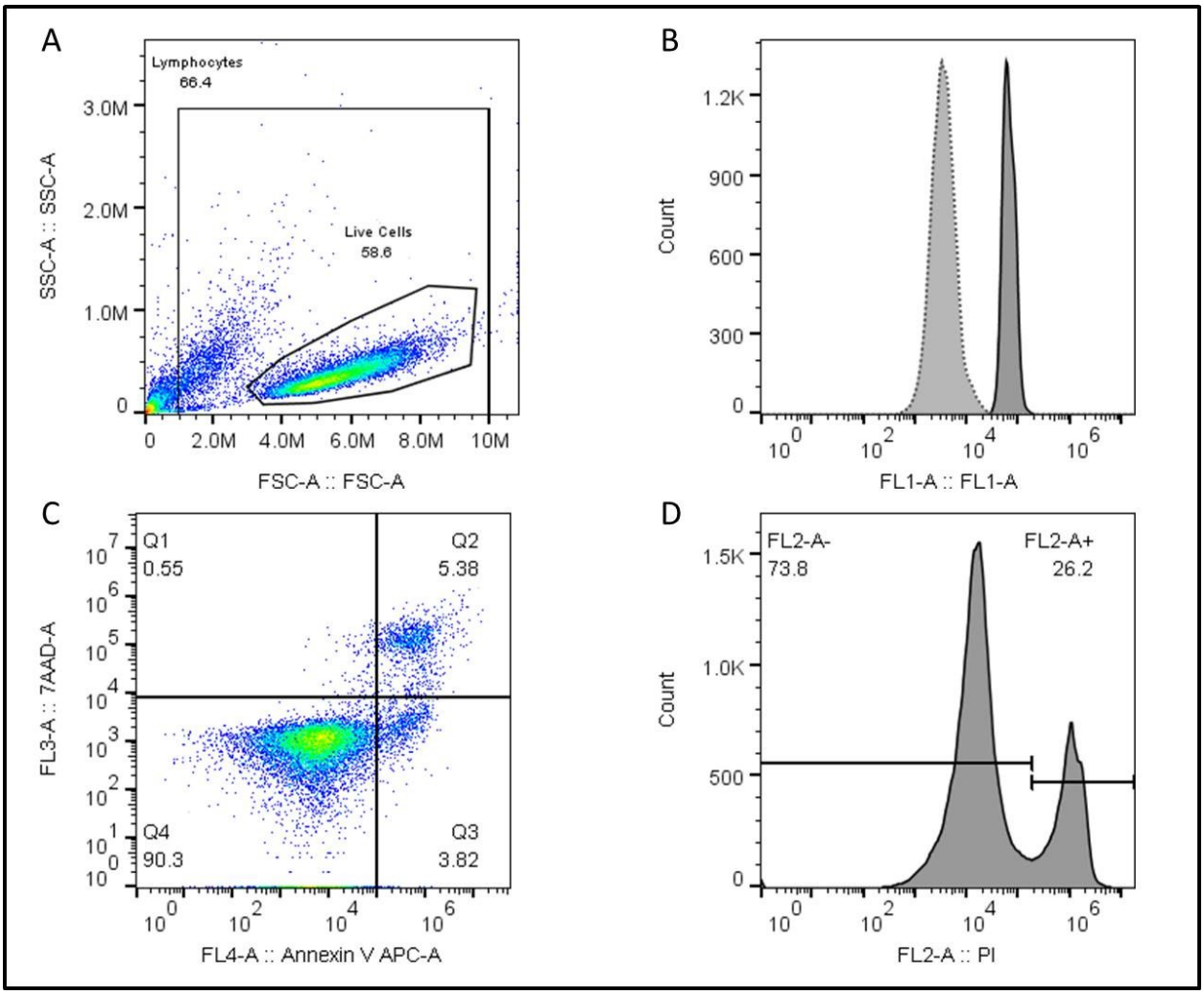


Figure 13. A) Live cell gating according to SSC and FCS on a 2 dimensional dot plot B) CFSE overlay comparing mean CFSE emission of live cells on day 1 (solid line) and day 4 (dashed line) using histograms C) Quadrants used to determine the proportion apoptosis and necrosis based on the emission levels of Annexin V and 7AAD. D) A bisector gate tool was used to distinguish between live cells and dead cells after PI staining.

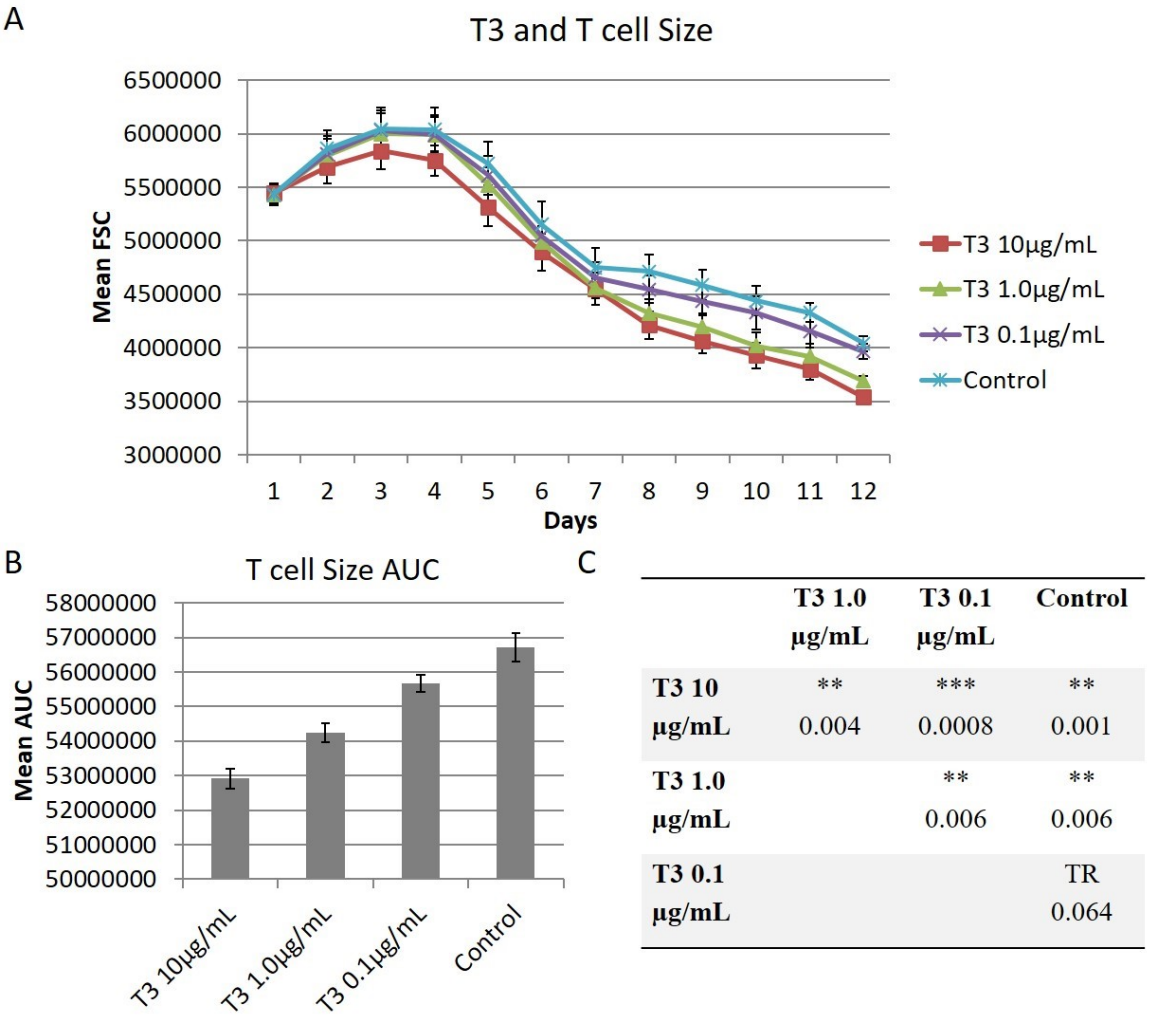
Appendix C: Secondary data analysis

The flow cytometer quantified the size and granularity of each cell based on the FSC and SSC properties of the laser. This flow cytometer measured FSC and SSC of all the cells in addition to measuring the fluorescence of every individual cell. FSC refers to the cells size while SSC refers to the cells granularity and density. An increase in SSC could indicate that the cell's nucleus is fragmenting and that the cell is dying.

T3 and T cell size

While T cells proliferated slightly faster in the presence of T3, this increase in proliferation did not seem to account for the increased T cell survival with T3. T cells were cultured over 12 days in 1% FBS media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL or in a control condition (Figure 14A). AUC was calculated for 7 experiments over 12 days (Figure 14B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a significant difference in T cell size between some of the conditions (Figure 14C). T cells cultured with T3 10 µg/mL had a significantly decreased cell size when compared to the cells in the T3 1.0 µg/mL T3 ($p<0.004$), T3 0.1 µg/mL ($p<0.0001$) and the control ($p<0.002$) conditions. T cells cultured with T3 1.0 µg/mL had a significantly decreased cell size when compared to the cells in the T3 0.1 µg/mL ($p<0.007$) and the control ($p<0.007$) conditions. T cells cultured with T3 0.1 µg/mL had a trend towards decreased cell size when compared to the cells in the control condition. The overall decreased Jurkat T cell size is most likely due to the insufficiency of extrinsic growth factors in the culture media (Rathmell, Vander Heiden, Harris, Frauwirth, & Thompson, 2000). Since keeping an increased biomass necessitates a lot of energy, decreasing cell size may help increase T cell survival (Rathmell et al., 2000).

Figure 14. T3 and T cell size



C

	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	** 0.004	*** 0.0008	** 0.001
T3 1.0 µg/mL		** 0.006	** 0.006
T3 0.1 µg/mL			TR 0.064

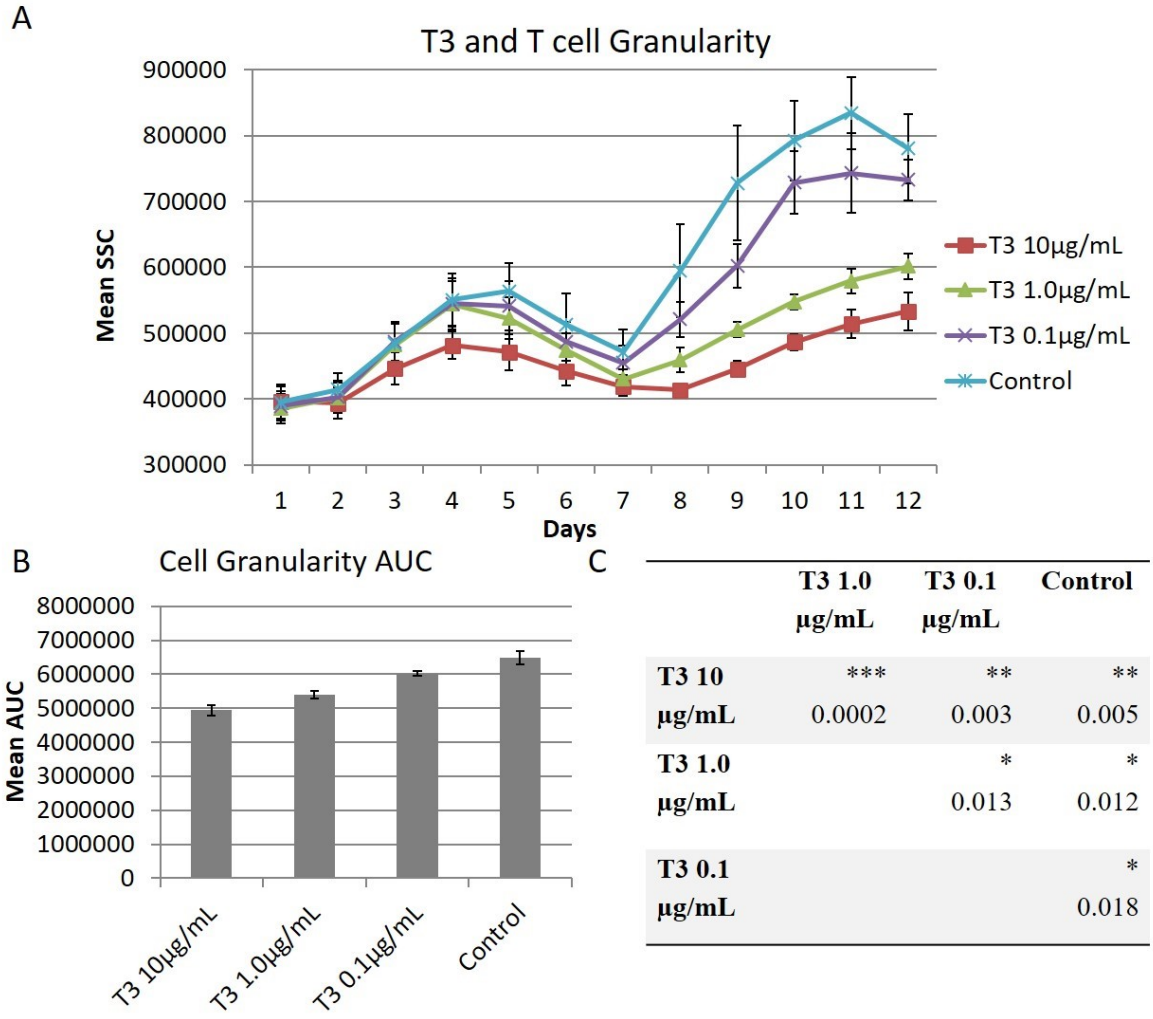
Figure 14. T cells were cultured in media with or without T3. FSC was measured each day with flow cytometry using size parameters. A) Data pooled from 7 experiments. B) AUC was calculated for 7 experiments. C) *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, p-value for paired two-tailed T-tests performed on the mean AUC values.

T3 and T cell granularity

As T cells die they become more granular as the cellular membrane and nucleus fragment. The SSC parameter was used to analyse changes in T cell granularity. T cells were cultured over 12 days in 1% FBS media containing 10 µg/mL T3, 1.0 µg/mL T3, 0.1 µg/mL or a control condition (Figure 15A). AUC was calculated for 7 experiments over 12 days (Figure 15B). The single factor ANOVA performed on the corrected AUC values was considered significant. Subsequent 2 tailed T-tests were performed and showed a significant difference in T cell granularity between all of the conditions (Figure 15C). T cells cultured with T3 10 µg/mL had a significantly decreased cell granularity when compared to the cells in the T3 1.0 µg/mL ($p<0.0003$), T3 0.1 µg/mL ($p<0.004$) and the control ($p<0.006$) conditions. T cells cultured with T3 1.0 µg/mL had a significantly decreased cell granularity when compared to the cells in the T3 0.1 µg/mL ($p<0.02$) and the control ($p<0.02$) conditions. T cells cultured with T3 0.1 µg/mL had a significantly decreased cell granularity when compared to the cells in the control condition. Thus, T3 10 µg/mL caused the lowest levels of cell granularity; while the T cells in the control condition had the highest granularity was seen without any T3 supplement.

This increase in cell granularity in the control condition suggests that the cells were dying, confirming the results of the T3 dose-response experiment (Figure 2). Taken together with the size parameter data, it is not clear how T3 10 µg/mL affects cell death. To address the effects of T3 on T cell death, specific dyes for apoptosis and necrosis were used in the following experiment.

Figure 15. T3 and T cell granularity



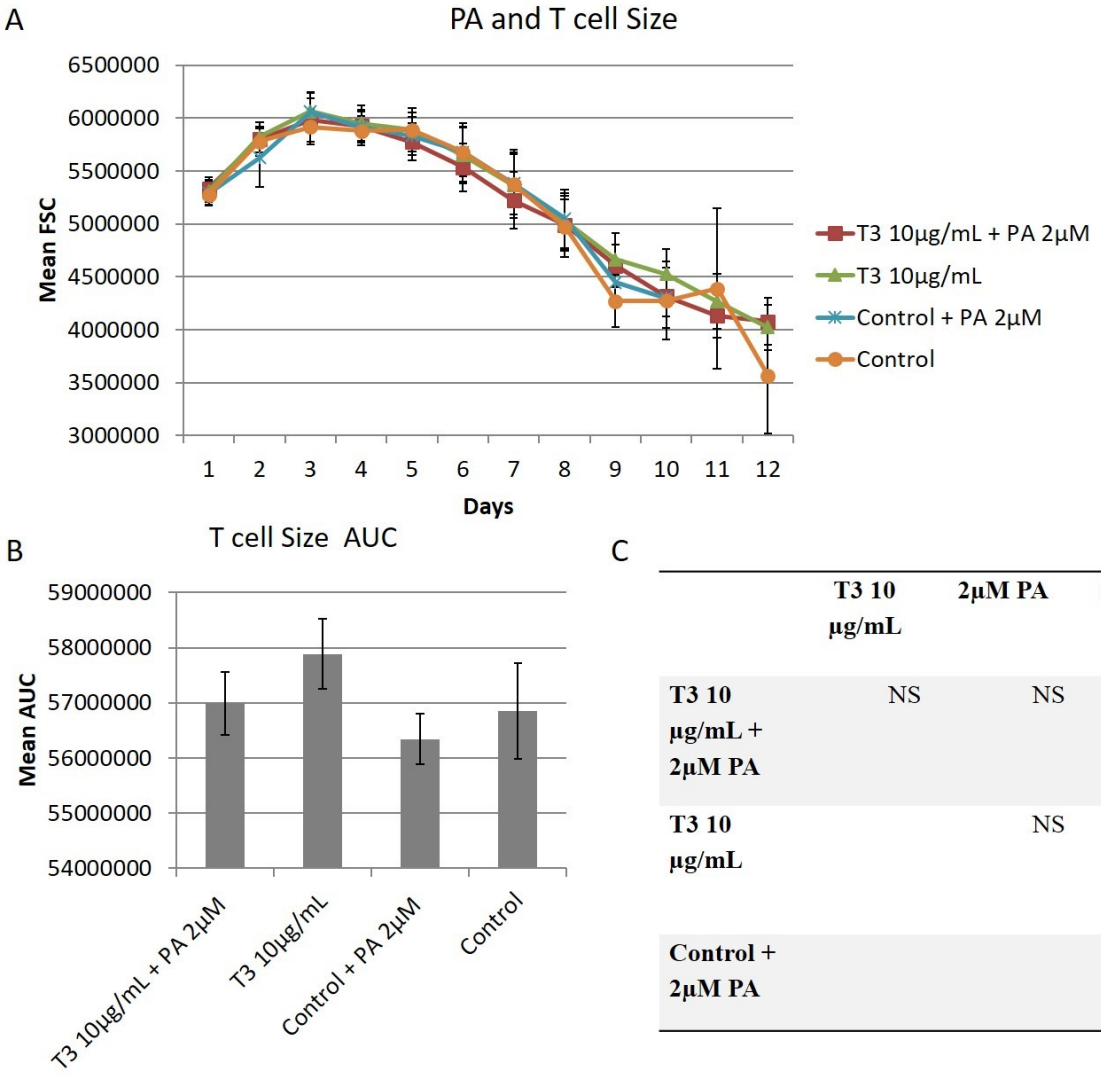
	T3 1.0 µg/mL	T3 0.1 µg/mL	Control
T3 10 µg/mL	*** 0.0002	** 0.003	** 0.005
T3 1.0 µg/mL		* 0.013	* 0.012
T3 0.1 µg/mL			* 0.018

Figure 15. T cells were cultured in media with or without T3. SSC was measured each day with flow cytometry using size parameters. A) Data pooled from 7 experiments. B) AUC was calculated for 7 experiments. C) *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, p-value for paired two-tailed T-tests performed on the mean AUC values.

PA and T cell size

Since adding PA to cells allowed them to survive and proliferate more efficiently, it was unknown whether adding PA might have any effect on T cell size due to the increased availability of fatty acids in the media. FSC was measured by growing T cells over 12 days in one of the T3 and PA conditions. Through the experiment, T cell size diminished for all the conditions, but there was no visible size difference between the T3 and PA conditions (Figure 16A). AUC was calculated for 8 experiments over 12 days. The mean AUC seemed higher in the T310-PA0 condition even if the error bars were quite large (Figure 16B). The single factor ANOVA performed on the corrected AUC values was not considered significant. No further statistical tests were performed (Figure 16C).

Figure 16. PA and cell size



C

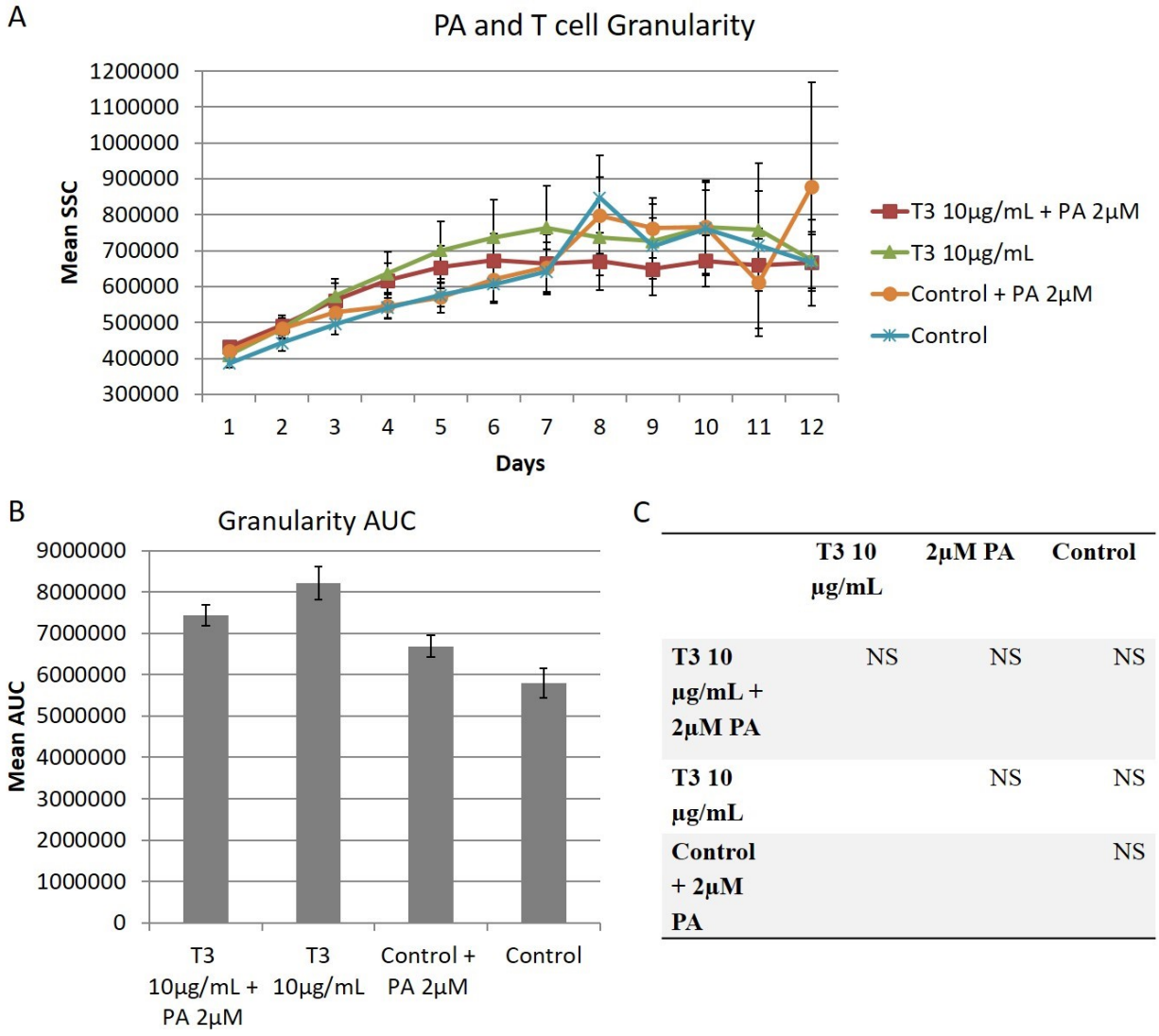
	T3 10 µg/mL	2µM PA	Control
T3 10 µg/mL + 2µM PA	NS	NS	NS
T3 10 µg/mL		NS	NS
Control + 2µM PA			NS

Figure 16. T cells were cultured in media with or without T3 and with or without 2 µM of PA. FSC was measured with flow cytometry using size parameters. A) Data pooled from 8 experiments. B) AUC was calculated for 8 experiments. C) T-tests were not performed.

PA and T cell granularity

SSC was measured by growing T cells over 12 days in one of the T3 and PA conditions AUC was calculated for 8 experiments over 12 days. The was no visual difference in granularity between the condition even though the mean SSC levels varied a lot from day to day in the T30-PA2 and T30-PA0 conditions. The variability in mean SSC levels is probably due to the decreased number of live cells in these conditions compared to the T310-PA2 and T310-PA0 conditions (Figure 17A). AUC was calculated for 8 experiments over 12 days. The mean SSC AUC appeared higher in the T310-PA0 condition compared to the other conditions, suggesting that cells most likely to die soon (Figure 17B). The single factor ANOVA performed on the corrected AUC values was not considered significant. No further statistical tests were performed (Figure 17C).

Figure 17. PA and cell granularity



C

	T3 10 µg/mL	2µM PA	Control
T3 10 µg/mL + 2µM PA	NS	NS	NS
T3 10 µg/mL		NS	NS
Control + 2µM PA			NS

Figure 17. T cells were cultured in media with or without T3 and with or without 2 µM of PA. SSC was measured with flow cytometry using size parameters. A) Data pooled from 8 experiments. B) AUC was calculated for 8 experiments. C) T-tests were not performed.

Appendix D: Preparing T3 Solutions

How to dissolve T3.

T3 (3,3',5-triiodo-L-thyronine), SIGMA, cat # T6397

The final concentration is going to be 10 µg/mL, 1 µg/mL or 0.1 µg/mL

1. Measure 5 mg of T3 (in a 50 mL tube) on an analytical balance.

2. Add 5.51 mL of 0.02N NaOH per 5 mg (recalculate based on how much you measured).

Vortex solution to completely dissolve T3.

Note-To make 0.02M NaOH, dilute the 1N NaOH by a factor of 50 (add 1 mL of 1N NaOH to 49 mL ddH_2O), keep the stock in a 50 mL plastic tube at room temperature.

3. Add 3.49 mL of sterile PBS in the hood. Vortex until thyroid hormone is completely dissolved.

4. Pass solution through syringe filter to make it sterile.

5. Add 30 mL of sterile PBS, in the hood.

6. Divide into 10, 4 mL aliquots, freeze at -20°C

7. Take one 4 mL aliquot, and bring volume to 50 mL of 1% FBS media (with pen-strep and l-glutamine).

THIS IS THE 10 µg/mL solution

8. To make the 1 µg/mL, take 5 mL of the 10 µg/mL solution, and add it to 45 mL of 1% FBS media (with pen-strep and l-glutamine).

9. To make the 0.1 µg/mL, take 5 mL of the 1 µg/mL solution, and add it to 45 mL of 1% FBS media (with pen-strep and l-glutamine).

Appendix E: CFSE Staining

Overview:
1. Prepare T cells and add CFSE to warm serum-free media.
2. Mix them together and put in water bath 37°C for 7 minutes.
3. Add RPMI media containing FBS and wash twice. Count cells between washes.

Materials:
-Stock CFSE is aliquoted about 10 µL/aliquot and stored at -20°C. For best reproducibility use aliquot once, do not refreeze. You can refreeze once (mark tube with X) just make sure to check the labelling (see below).
-serum free media RPMI (take an aliquot from a bottle in the fridge)
-regular serum-containing media (e.g. RPMI 5% FBS, pen-strep, l-glutamine)
-everything must be kept sterile, keep CFSE wrapped in tinfoil to reduce light exposure

Preparation:
To begin, turn on the water bath to 37°C, pre-warm your media containers.
At the beginning, set aside some cells for the negative control for flow cytometry

Labelling:
The strategy is to suspend cells in warm serum-free media and then add diluted CFSE in warm serum-free media. (Plan ahead: you will lose 30-50% of your cells in the end)

- Count your cells and spin down the desired number, try to label $5x10^6$ minimum
- 'Scratch' the tube to break pellet. Resuspend them in warm serum-free media so the final volume is $2x10^6$ cells /mL e.g.: add 2.5 mL of media to $5x10^6$ cells.
- Make diluted CFSE by adding concentrated CFSE to a separate tube of warm serum-free media.
- Recommend a 1:20,000 FINAL dilution. E.g. to do this add 1µL of CFSE to 10 mL of warm serum-free media.
- Add an equal volume of the diluted CFSE to your cells. E.g. add 2.5 mL of diluted CFSE to the cells.
- Quickly put a cap on and invert several times, put in 37°C water bath for 7 minutes.
- Take the tube from the water bath, add an equal volume of serum containing media
- Spin down in a centrifuge. 1500 rpm for 8 minutes at room temp.
- Scratch pellet, resuspend in serum media, take some for counting, and wash once more by centrifuging at1500 rpm for 8 minutes at room temp.
- Perform a viability count after with trypan while it is spinning.
- Scratch pellet and resuspend in desired media at desired concentration for the experiment.
- If using Jurkat cells, just put them in media at about 0.1-0.2 $x10^6$ cells/mL

Appendix F: 7AAD and Annexin V Staining

MATERIALS

- Jurkat cells
- 7AAD (GE fridge)
- Annexin V (GE fridge)
- Annexin V binding buffer (GE fridge)

PROCEDURE

- bring a plate from SP to GE
- keep samples on ice as much as possible
- split each well into 2 Eppendorf tubes (~$0.5x10^6$ per tube)
- OR- do this in a v-bottom plate and use the plate spinner:
- Spin down in microfuge Eppendorf tubes, 5 sec using the little table top on the bench in GE, flip the switch to make it run and count in your head.
- Remove the liquid phase
- add 45 µL PBS click to resuspend
- Add 5 µL of 7AAD, click tubes to mix
- put samples on ice for 20 minutes
- Spin down $0.5x10^6$ cells in microfuge Eppendorf tubes, 5 secs, remove the liquid phase
- resuspend in 50 µL of Annexin V binding buffer
- Add 5 µL of Annexin V
- on ice 30 minutes
- Do not wash, just add 50 µL of Annexin V binding buffer
- read on Accuri flow cytometer

Appendix G: PA Protocol

P0500 Sigma PA

PA 50 µM, 10 µM, 2 µM, 0.4 µM, 0 µM

Dissolve PA in ethanol.

Molecular weight 256.42g/mol

Solubility 20g/L

1. Measure 5mg (0.005g) of PA (in a 50 mL tube) on analytical balance.

2. Add 1.94 mL of ethanol per 5mg (recalculate based on how much you measured). Vortex solution to completely dissolve PA. This should make a 10mM stock of PA.

3. Pass solution through syringe filter to make it sterile.

4. Divide into 150 µL aliquots, freeze at -20°C

5. To make 25 mL of 50 µM media add 125 µL of 10 mM stock to 24.875 mL of media.

6. To make 25 mL of 10 µM media add 5 mL of 50 µM media to 20 mL of media.

7. To make 25 mL of 2 µM media add 5 mL of 10 µM media to 20 mL of media.

8. To make 25 mL of 0.4 µM media add 5 mL of 2 µM media to 20 mL of media.

Appendix H: PI Staining

MATERIALS

- Jurkat T cells
- PI

PROCEDURE

- Keep samples on ice as much as possible to prevent cells from dying
- Put an aliquot of each sample into 2 Eppendorf tubes (~$0.5x10^6$ cells per tube*)
- Spin down in table top microfuge Eppendorf tubes for 20 seconds using the little benchtop on the bench in GE, flip the switch to make it run and count in your head.
- Completely decant tube: remove the liquid phase using a pipette without touching the cell pellet
- add 200 µL of PBS, spin down, decant the PBS
- add 50 µL PBS click the tubes together to resuspend
- Add 5 µL of PI per sample, click tubes to mix
- put samples on ice for 20 minutes
- add 50 µl of PBS
- read on Accuri flow cytometer

Appendix I: Etomoxir Protocol

E1905 Sigma (+)-Etomoxir sodium salt hydrate

Solubility 20mM

Etomoxir solutions

Molecular Weight 338.76g/mol

- Wear protective gear when handling powdered etomoxir. Wear a face shield, lab gloves, lab coat and breathing mask.
- Measure 2.0mg of Etomoxir (in a 50 mL tube) on an analytical balance.
- Add 1 mL of PBS per 2.0mg (recalculate based on how much you measured). Vortex solution to completely dissolve powdered etomoxir.
- Add 250 µL of dissolved etomoxir stock to etomoxir condition flasks containing 14 mL of cells in 1% FBS culture media. This will give you a final etomoxir concentration of 103 µM.
- Add 250 µL of PBS to control condition flasks containing 14 mL of cells in 1% FBS culture media.