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Linoleic acid-mediated regulation of T cell cytokine-subset composition in a murine model of type 1 diabetes

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LINOLEIC ACID-MEDIATED REGULATION OF T CELL CYTOKINE-SUBSET COMPOSITION IN A MURINE MODEL OF TYPE 1 DIABETES

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ABSTRACT

Type 1 Diabetes (T1D) is a complex autoimmune disorder in which T cells destroy the pancreatic islets, leading to a loss of insulin production and hyperglycemia. The disease incidence has increased globally over the last decades, primarily in individuals with low to moderate genetic risk. There is evidence that environmental factors play a role alongside genetic risk to trigger the disease. An environmental factor that has global influence is adoption of the Western diet, characterized by increased consumption of n-6 fatty acids, including linoleic acid (LA), and decreased consumption of n-3 fatty acids. Increased n-6/n-3 ratios are associated with enhanced susceptibility to autoimmune diseases. We sought to understand how linoleic acid affects the survival and function of T cells from the non-obese diabetic (NOD) mouse, a model for T1D. We found that linoleic acid's presence during *in vitro* activation of T cells led to an increased expansion of the cells in culture. Additionally, CD4+ and CD8+ T cells activated in linoleic acid's presence produced increased levels of pro-diabetogenic cytokines, including Interleukin-21 (IL-21) and Interferon-gamma (IFN-γ). In contrast, linoleic acid

reduced IL-10-producing CD4+ T cells, which are protective in T1D, significantly changing the balance between pro-and anti-inflammatory T cell subsets. Gene expression analysis of T cells exposed to linoleic acid during *in vitro* activation revealed decreased gene expression of lipid-regulated transcription factors, peroxisome proliferator-activated receptors (PPAR), PPARα and PPARγ. These data suggest a role for these transcription factors and their associated pathways in linoleic acid-mediated T cell functions. Finally, we tested whether the T cell fatty acid response is regulated by the cytokine IL-7, which modulates T cell immunometabolism. However, our data did not reveal a prominent role for IL-7 in regulating the T cell response to linoleic acid. Together, these studies add to evidence that fatty acids present in the microenvironment can directly alter T cell functions and that changes in dietary components may contribute to enhanced T1D susceptibility.

PREFACE

"The endless cycle of idea and action,

Endless invention, endless experiment,

Brings knowledge of motion, but not of stillness;

Knowledge of speech, but not of silence;

Knowledge of words, and ignorance of the Word.

All our knowledge brings us nearer to our ignorance."

— T.S. Eliot

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LIST OF ABBREVIATIONS

AA	Autoantibody
ACC1	Acetyl-CoA Carboxylase 1
Ahr	Aryl Hydrocarbon Receptor
ALA	α-Linolenic Acid
AMPK	AMP-Activated Protein Kinase
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
Bcl-6	B-cell lymphoma 6 protein
BFA	Brefeldin A
Blimp-1	B Lymphocyte-Induced Maturation Protein-1
BMDM	Bone Marrow-Derived Macrophage
BMDC	Bone Marrow-Derived Dendritic Cell
BSA	Bovine Serum Albumin
c-maf	Musculoaponeurotic Fibrosarcoma
CD	Cluster of Differentiation
Co-IR	Co-inhibitory Receptor
CPT1a	Carnitine Palmitoyltransferase 1A
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR5	C-X-C Motif Chemokine Receptor 5
DC	Dendritic cell
DIPP	Diabetes Prediction and Prevention Study

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-Linked Immunosorbent Assay
EOMES	Eomesodermin
ETC	Electron Transport Chain
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FAO	Fatty Acid Oxidation, B-Oxidation
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FoxP3	Forkhead Box P3
GADA	Glutamic Acid Decarboxylase Antibodies
GLUT1	Glucose Transporter 1
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GWAS	Genome-Wide Association Study
HIF-1α	Hypoxia-Inducible Factor-1α
HLA	Human Leukocyte Antigen
IAA	Insulin Autoantibody
ICA	Islet Cell Autoantibodies
ICOS	Inducible T-cell Co-stimulator
IDD	Insulin Dependent Diabetes

IL-	Interleukin-
IFN-γ	Interferon-γ
IPEX	Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome
JAK	Janus Kinase
LA	Linoleic Acid
LAG-3	Lymphocyte Activation Gene 3
LPS	Lipopolysaccharide
mAbs	Monoclonal Antibodies
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP-3α	Macrophage Inflammatory Protein-3α
mL	Milliliter
mM	Millimolar
MS	Multiple Sclerosis
mTOR	Mechanistic Target of Rapamycin
n-3	Omega-3
n-6	Omega-6
NAD+/NADH	Nicotinamide Adenine Dinucleotide
NK	Natural Killer cell
NOD	Non-Obese Diabetic
OCR	Oxygen Consumption Rate

OL	Oleic Acid
OR	Odds Ratio
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate-Buffered Saline
PD-1	Programmed Cell Death- 1
PD-L1	Programmed Death-Ligand 1
PD-L2	Programmed Death-Ligand 2
PI3K	Phosphoinositide 3-Kinases
PMA	Phorbol 12-myristate 13-acetate
PUFA	Polyunsaturated Fatty Acid
Q-PCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RORγt	RAR-related orphan nuclear receptor γt
RPMI	Roswell Park Memorial Institute Media
SD	Standard Deviation
SIRT4	Sirtuin 4
SLE	Systemic Lupus Erythematosus
SRC	Spare Respiratory Capacity
STAT	Signal Transducer and Activator of Transcription
T1D	Type 1 Diabetes
TAG	Triacylglycerol

T-bet	T-box transcription factor TBX21
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
Tcm	T central memory cell
Tem	T effector memory cell
Tfh	T follicular helper cell
TGF-β	Transforming growth factor- β
Th-	T helper cell
TIGIT	T cell Immunoreceptor with Ig and ITIM domains
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3
TNFα	Tumor necrosis factor α
TNFβ	Tumor necrosis factor β
Tr1	T regulatory-1 cell
Treg	T regulatory cell
Trm	T resident memory cell
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER ONE: INTRODUCTION

Type 1 Diabetes

Epidemiology and Treatments

The autoimmune disorder Type 1 Diabetes (T1D) is a chronic T cell-mediated immune disorder that leads to a loss of endogenous insulin production over time (1). Recent studies have demonstrated that more than 96,000 youths under 15 years of age are diagnosed with T1D globally every year in developed and developing countries (2). Within children T1D is the most common form of diabetes and represents 80% of all cases in the United States, while only 10% of all adult diabetes cases in the U.S. are T1D (3). The T1D incidence rate has risen globally, and statistics indicate that the relative annual increase in T1D incidence is 1.8% based on data from the SEARCH multicenter observational study of new-onset youths who have been diagnosed with T1D within a ten year (2002-2012) period in the United States (4). When looking into specific racial and ethnic populations within the SEARCH study, there is a noticeable rise in the incidence of T1D among Hispanic youths (4.2%), Black youths (2.2%), and other minority/ethnic groups (4,5). The rise in incidence among racial and ethnic minority populations can present a challenge due to barriers to healthcare access and quality (6).

The onset of T1D is indicated by a gradual loss of endogenous insulin production as pancreatic β-cells are destroyed by autoreactive T cells (7). Loss of insulin production leads to polyuria, polydipsia, and hyperglycemia (7). Complications of T1D include nephropathy, ketoacidosis, cardiovascular disorders, difficulties managing blood glucose

levels, and decreased life expectancy by as much as 11-13 years compared to individuals without T1D (8). Autoantibodies (AAs) against islet antigens formed before and during pathogenesis are used as biomarkers of disease and have helped to study T1D patient cohorts (9,10). The TRIALNET study of siblings, including twins who have a family member with T1D, screened children for risk genes and tracked children until the presence of AAs or development of diabetes (11). This study revealed that AA-positive twins had a 69% risk of developing T1D by three years compared with AA negative twins, whose risk is 1.5% (11) and indicates that genetics alone does not explain the risk of disease development. In non-identical twins, those who were positive for multiple autoantibodies had a 72% risk of T1D by three years compared to 13% for single antibody positive and 0% for autoantibody negative (11) and shows that islet autoantibodies can be used to track disease progression. Islet autoantibodies include Glutamic Acid Decarboxylase AAs (GADA), Insulin AAs (IAA), Islet Cell Cytoplasmic AAs (ICA), and AAs against many other target proteins (12). The presence of AAs to pancreatic islet antigens often precedes the onset of dysglycemia, which over a period of time (months/years) converts to diabetes due to insulin deficiency (13).

Standard of care for T1D includes diagnosis by C-peptide and blood glucose measurements and exogenous insulin supplementation to maintain euglycemia (14). Many immune-based therapies to prevent or reverse T1D have made it to clinical trials but have had low success rates. Often, issues with these therapies lie in the therapy's timing and whether a therapeutic is better suited for the prevention of disease in high-risk individuals since reversal upon disease onset is often difficult. Potential therapeutics

being investigated include immune modulation methods using antibodies such as anti-IL-7Rα, adoptive immunotherapy with T regulatory cells, stem cell implantation to restore β-cells, and cytokine/cytokine complex supplementation (14–16). Of the more successful investigative therapeutics, antibody blockade of cluster of differentiation (CD) – 3 (Teplizumab and Otelixizumab), which is important for T cell activation, has shown promise in restoring a level of lasting immune tolerance and maintenance of β-cell mass (17–19). Teplizumab has reached phase 3 clinical trials with new-onset patients (<6 months diagnosed), but a similar therapeutic Otelixizumab has had no significant outcomes in its phase 3 clinical trials. (17,18,20,21). While there is an effort to try different therapeutic strategies to prevent and reverse diabetes, most investigative therapeutics have not produced promising results to expand their use in patients who can progress to T1D. Therefore, the only therapeutic option approved for T1D patient use is insulin supplementation, which presents its own set of challenges due to variable access and affordability of insulin in countries with unstable health insurance and health care systems (22) and suboptimal blood glucose regulation.

Genetic Factors

T1D is a polygenic disorder with a variety of risk genes that can contribute to disease pathogenesis. The Major Histocompatibility Complex (MHC) locus in humans is subdivided into three Human Leukocyte Antigen (HLA) subclasses which include class I HLA, non-classical HLA, and class II HLA (22,23). The role of HLA is to present antigens to T cells; class I and II HLA are highly polymorphic and are thought to drive

associations with human immune diseases (22,23). The most characterized and important risk gene for T1D is the Human Leukocyte Antigen or HLA gene which accounts for nearly 50% of familial T1D risk (24). The strongest associations with T1D risk are the class II HLA gene loci DR and DQ (25). There is evidence that polymorphisms in the DNA of HLA genes can affect the binding affinity and repertoire of antigens bound (25). The HLA DR and DQ haplotypes most associated with the disease include DR4-DQ8 and DR3-DQ2, 30% of patients carry both haplotypes, and this subset has the most significant risk of T1D (OR=16) (26,27). HLA protective gene loci exist in non-Hispanic whites (28). HLA-DQB1*06:02 is present in 20% of the study population but only in 1% of T1D children demonstrating that this loci may offer some protective effects (28).

Many non-HLA genes have associations with T1D risk. The non-HLA gene with the strongest association is the insulin gene (29). Polymorphisms in the insulin gene are thought to cause variations in insulin RNA regulation in the thymus and influence immune tolerance to insulin (30). There have been over 50 non-HLA loci found by GWAS associated with T1D and other immune diseases (31). Some of these genes include the IL-2 receptor α subunit, which is thought to cause abnormalities in T cell sensitivity to IL-2 and disrupt the balance of T effector (Teff) and T regulatory cells (Tregs) (32,33). The PTPN22 gene encodes a lymphoid-specific phosphatase that can suppress T cell activation and has been associated with T1D risk along with Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a receptor that negatively regulates T cells (34,35). In recent decades, there has been a reduction in the frequency of high-risk HLA patients with T1D (31,36,37). However, the incidence is increasing in people with low

and moderate risk genes, which points to environmental factors playing a role in triggering disease development (31,36,37). These observations indicate a shift in contributions of genetic and environmental factors in the pathogenesis of T1D (27,36,37).

Environmental Factors

Genetic risk alone does not trigger the pathogenesis of T1D, and there are a variety of environmental factors linked to disease pathogenesis. Some of these environmental factors include viral infections, microbiome changes, and dietary factors (13,38). The viral infection hypothesis has many mechanisms by which different viruses are thought to induce T1D in those with genetic risk, and one hypothesis is that the Coxsackie B virus has strong tropism towards pancreatic β-cells and can induce inflammation that promotes autoimmunity (39). Other studies confirm that pancreatic β-cells express the Coxsackie adenovirus receptor, a major receptor for the Coxsackie B virus for viral entry (40). The Diabetes Prediction and Prevention Study (DIPP) looked at Enterovirus serotypes by screening children with genetic risk of T1D for neutralizing antibodies against forty or more Enterovirus types (41,42). They found an increased risk of an AA-positive sample if Coxsackie B1 virus infection precedes the autoantibody appearance (41,42). The same association was seen in a group that proceeds to develop T1D, while if Coxsackie B3 or B6 virus infection was first, the risk of an autoantibody-positive sample is lower (41,42). From maternal cord blood samples, the risk of T1D is greatest in the group of children that experienced Coxsackie B1 virus infection without the presence of maternal Coxsackie B1 viral antibodies (41).

Changes to the gut microbiome have also been implicated in the triggering of T1D pathogenesis when combined with genetic risk (43). An infant acquires their microbiome through vaginal delivery and being fed breastmilk; afterward, environmental exposures, hygiene, and diet influence the development of the microbiome (44). The gut microbiota has a symbiotic relationship with the host GI tract: commensal microbiota helps the host by preventing pathogenic species colonization and producing metabolites beneficial to the body, such as short-chain fatty acids (45). Specific gut microbiota species such as *Bifidobacteria* ferment undigested fibers into short-chain fatty acids like butyrate, which can activate specific G-protein coupled receptors (free fatty acids receptors 2 and 3), inhibit histone deacetylases, and have an impact on immune cells such as T cells and their functions (45,46). The gut microbiota is easily influenced by antibiotic use and variations of foods in the diet that support the growth of specific bacteria species (47). Stool samples from children at risk for T1D in the TEDDY study were collected starting at three months until the clinical endpoints of islet autoimmunity or diabetes to study the natural history of the early infant gut and associations with T1D (47). This study found that the microbiomes of controls had a greater number of genes related to processes such as fermentation and the biosynthesis of short-chain fatty acids when compared to children who progressed to diabetes (47)(48). Comparing AA-positive and -negative children with T1D genetic risk, they found that there are increased *Bacteroides* species in fecal samples from those who progress to islet autoimmunity, and there is a low quantity of lactate- and butyrate-producing bacterial species such as *Bifidobacteria* (49). Short-chain fatty acids produced by specific commensal species of

bacteria are anti-inflammatory and promote tolerogenic effects on the immune system, such as expanding T regulatory (Treg) populations (48,50,51). There is an abundance of evidence that suggests dysregulated microbiomes can contribute to T1D pathogenesis in those with known HLA genetic risk. However, differences in microbiomes still do not explain the increased incidence of disease in populations with low and moderate risk genes. There is confusion about which gut microbiota species are considered beneficial or pathogenic and how they contribute to autoimmunity (38,52–54).

The diet's influence on disease development includes factors such as the variations of foods in the diet and when they were introduced during infancy, and the composition of nutrients (55–58). The DAISY study on children with low to moderate T1D risk genotypes revealed that cow's milk protein is associated with an increased risk of islet immunity. This association was not seen in children with high-risk HLA genotypes (55). A different study, the Type 1 Diabetes Prediction and Prevention study (DIPP), found that exposure to cow's milk protein in the formula before three months of age was not associated with risk of islet immunity or T1D (56). Gluten intake has also been considered a trigger for T1D pathogenesis (58). The BABYDIET study delayed gluten intake until 12 months of age in children at risk, but the delaying of gluten in the diet was not associated with reduced islet autoimmunity risk (58). The TEDDY study revealed that early introduction of gluten into the diet before four months of age was associated with reduced islet autoimmunity, while late gluten introduction was not associated with increased islet autoimmunity risk (57). There is no clear consensus that any particular dietary factors are associated with increased islet autoimmunity risk;

studies looking at the intake of specific foods often rely on self-reported data, which adds to the variable nature of results.

There have been human studies displaying a trend towards developing T1D in association with obesity and overnutrition. It has been shown that 22.1% of youth that develop T1D are overweight compared to 16% of non-T1D youth (59,60). This chronic exposure of immune cells to glucose and other dietary components such as lipids combined with reduced insulin production overtime can influence the activation state and differentiation of T cells central to T1D pathogenesis. There are limited studies that characterize autoreactive T cells' environment during T1D pathogenesis and how that can influence T cells and their pathogenicity. Other dietary factors that have been looked at are the intake of n-3 and n-6 fatty acids. One study found that the use of cod liver oil, which is rich in n-3 fatty acids, during the first year of life significantly reduced the risk of T1D in children (61). Another study looked at the early serum composition of fatty acids in association with the risk of T1D in HLA susceptible infants: this study found that the serum fatty acid compositions were different in infants who were breastfed compared to those who received formula (62). Moreover, it was found that higher n-6 fatty acids compared to n-3 fatty acids were associated with islet autoimmunity (62). These studies reveal that fatty acid composition and status during infancy may play a role in T1D pathogenesis. To support that fatty acid composition may play a role in disease development, other studies in children with genetic risk for T1D reveal an inflammatory serum lipidome and reduced serum phospholipids in children who progress to disease versus non-progressors (63). Similar results showing an altered lipidome have been

revealed in the Non-obese Diabetic (NOD) mouse model of T1D (64). Since T1D is a complex autoimmune disease, it is expected that there are many different genetic and environmental factor combinations that can trigger disease pathogenesis.

Pathogenesis

Disease pathogenesis occurs over months to years, usually in younger children, leading to diabetes. Most of the pathogenic events of T1D occur before the diagnosis where a patient usually has diabetes, and β-cells are depleted (10,65). T1D pathogenesis has basic steps where an immune response against β-cells is elicited by autoimmune cells, this response needs to develop into a pro-inflammatory response, and there must be a loss of immune tolerance, which regulates autoimmune responses (65). In T1D, autoreactive T cells that escape thymic deletion are activated. This activation process is unknown as there are many genetic and environmental factors involved in triggering disease (66).

In general, T1D is a T cell based disorder where the direct damage to β-cells is caused by CD8+ T cells and the inflammatory conditions created by CD4+ T cells. Other T cell subtypes involved are the CD4+ T cells which produce different cytokines depending on subset to promote a pro-inflammatory environment and help CD8+ T cells with the β-cell attack (66–69). A specific subset of CD4+ T cells, the T regulatory cell (Treg), which is tasked with preventing and inhibiting autoimmune responses, is less suppressive in murine and human T1D and fails to inhibit autoreactive effector T cells (70–72). There is also evidence in the NOD mouse model of T1D that Interleukin-2 (IL-2), a critical cytokine Tregs need for proliferation and function, is produced in reduced

quantities, which is another mechanism of loss of tolerance along with a reduced expression of IL-2Rα and overall weaker signaling in IL-2R pathway (72,73). It has been shown in human studies that islet antigen-specific CD4+ T cells from diabetic patients generate high levels of the pathogenic Th1 cytokine interferon γ (74). In contrast, islet antigen-specific CD4+ T cells from healthy controls recognizing the same antigen produce IL-10, an anti-inflammatory cytokine (74). Before an autoreactive T cell is directed to the pancreatic β-cells, islet antigens must be presented to T cells by antigen-presenting cells which include cell types such as dendritic cells or macrophages to activate T cells which initiates islet autoimmunity (68,75). B cells in T1D have been shown to produce autoantibodies (AAs) against β-cell islet antigens, and the AAs are biomarkers of disease progression in patients and the NOD mouse model (76). AAs, however, do not play a role in pathogenesis, in contrast to other autoimmune disorders such as lupus (75–78). The role of macrophages in T1D is still not fully known. Macrophages have been found in immune infiltrates in the pancreatic islets and are thought to help remove dead cells (79). Dendritic cells are involved in disease pathogenesis since they are required to start the anti-islet immune response and present antigens to T cells; thus, if dendritic cells are removed in NOD mice, there is no diabetes development (80). There is still a lot unknown about the direct contributions of immune cell types other than T cells to T1D pathogenesis; also, it is unknown how tolerance is broken and why autoreactive T cells fail to be suppressed.

T1D Mouse Model

The NOD mouse is typically the standard model used to study T1D. The NOD mouse is comparable to T1D human patients in quite a few aspects, making them a reliable model for most studies involving immune dysfunction in T1D (81–84). The NOD mouse is an inbred mouse strain developed while attempting to establish a cataract-prone subline. It was realized that the NOD mice were not cataract-prone but had spontaneous diabetes after 10-20 weeks of age; the penetrance of disease is 60-80% in female mice compared to about 30% in male mice (81,84).

Symptoms of diabetes in the mice are similar to humans and include hyperglycemia, polydipsia, and polyuria. NOD mice also produce autoantibodies like human T1D autoantibodies and include GAD- and insulin-specific antibodies, meaning they have similar antigen specificity (68,85). There also is a period of temporary remission from β-cell attack by T cells in the NOD mice and in humans found shortly after diagnosis of diabetes (82,84). The process is similar between human patients and NOD mice; insulitis and leukocyte infiltrates are also present in pancreatic islets (82,84).

The highest genetic contributor to the risk of T1D in NOD mice is the MHC haplotype $H2^{g7.}$ In addition, there are more than 20 non-MHC *Insulin-dependent diabetes* (Idd) loci that can contribute to disease susceptibility in NOD mice, including loci in genes of IL-2, IL-2R, CTLA-4, and others (26,86,87). . In humans, HLA risk gene loci, as well as genes found in non-HLA loci, also contribute to disease susceptibility (9,81,85,88). Other evidence supporting NOD mice as a suitable model of T1D is that

there is defective IL-2 production and signaling, which is similar to humans with T1D. It is thought this defective IL-2 production leads to the breakdown of immune tolerance (72,73) by limiting Treg survival and function. Environmental factors are also involved in triggering disease in NOD mice. There is evidence that restricted gut flora contributes to pathogenesis in NOD mice, supporting the theory that altered gut microbiomes contribute to disease pathogenesis in humans with T1D risk (43,89). Studies have shown that metabolic perturbations exist in NOD mice of different ages and sex who progress to disease; some of these metabolic perturbations include markers of oxidative stress and reduced unsaturated fatty acids (90). This study's results are similar to the prospective human metabolome study in children at risk for T1D, where alterations in lipid metabolism exist before autoantibody production in children who progress to T1D (91,92). Other studies in NOD mice confirm the findings that NOD mice who progress to T1D have a similar metabolome to children with T1D risk that progress to disease (64). Moreover, when NOD mice are compared to healthy control C57BL/6J mice, they demonstrated an altered metabolome. (93). It has also been shown that NOD mice fed n-3 fatty acids progress to disease more slowly than mice fed n-6 fatty acid arachidonic acid (94). These studies demonstrate that NOD mice are a suitable model for human T1D due to the similarities in genetic and environmental risk factors.

T-cell subsets and cytokines in T1D

Introduction

T cell subsets in T1D play different roles in the pathogenesis of disease (74,95,96). The general T cell compartments involved in disease are CD4+ and CD8+ T cells (67). Within the CD4+ T cells, subsets such as Th1 are generally involved in the clearance of intracellular pathogens, Th2 involved in the allergic response, Th17 also involved in autoimmunity, Tfh which have a role in promoting B cell functions (95,97–104). Dysfunctional Tregs and memory T cells are involved in disease pathogenesis by Tregs having compromised regulatory activities or memory T cells producing inflammatory cytokines (95,97–104). CD8+ T cells produce pathogenic cytokines and destroy pancreatic β-cells, promoting the loss of insulin production (105–107). The whole story of how these T cell subsets coordinate to promote pathogenesis is not fully known yet. Also, the plasticity of T cells, especially CD4+ T cells, allows for these defined T cell subsets to switch their lineage transcription factors and cytokine production depending on environmental cues such as dietary factors that change T cell environments, metabolic changes, and exposure to cytokines (95,108–110). The variety of T cell subsets involved in T1D pathogenesis and T cells' ability to change their differentiation state adds difficulty in determining suitable therapeutic options to restore tolerance and inhibit autoreactive T cell subsets.

T helper-1 cells

Th1 cells express the lineage transcription factor T-bet and are induced by the cytokine IL-12 (111,112). Their general role in the immune system is to clear intracellular pathogens, and they are involved in macrophage activation (113,114). In T1D Th1 cells coordinate the attack of pancreatic β-cells by producing cytokines including IFN-γ and IL-2, which help with effector functions and proliferation of CD8+ T cells. (97,115,116). IFN-γ is a proinflammatory cytokine that has been shown to promote homing of autoreactive T cells to islets in NOD mice and has been associated with the progression to T1D (117,118). Also showing how important Th1 cells are to T1D, T cells that have diabetogenic T cell receptors can promote diabetes in neonatal mice when differentiated as Th1 cells but not Th2 (97).

T helper-2 cells

The Th2 subset of T cells has been shown to be protective in T1D but usually are responsible for the response against parasites/extracellular pathogens (74,119). Th2 cells express the transcription factor GATA3 and are important for humoral immunity development and produce cytokines such as IL-4, IL-5, IL-13, and others (112,120). It has been demonstrated that when IL-4, a Th2 cytokine, was administered to NOD mice, T1D development was inhibited (121). Also, helminth infection, which promotes a Th2 response, has been shown to protect mice from diabetes (122).

T helper-17 cells

How Th17 cells contribute to pathogenesis in T1D is unclear, but studies suggest T cells that express both IFN-γ and IL-17 are expanded in T1D patients (95,99). Apart from IL-17A and IL-17F, Th17 cells can produce cytokines such as IL-21 and IL-22 (112,123). The lineage transcription factor expressed by Th17 cells is RORγt, and these cells require TGF-β for differentiation (112,123). Th17 cells and their cytokines have been associated with autoimmune disease progression in the experimental autoimmune encephalomyelitis (EAE) mouse model of Multiple Sclerosis (MS) and mouse models of arthritis (124–126). Increased levels of serum IL-17 was found in TCR transgenic NOD mice and are associated with accelerated disease progression (127). In T1D, Th17 cells play a role in early inflammation, but the treatment of NOD mice younger than 5 weeks of age with αIL-17 revealed that treated NOD mice had a decrease in autoantibody levels (GAD) and an increase in Treg frequency but no effects on inhibiting diabetes (128). Also, αIL-17 treatment of NOD mice reduced the percentages of Th17 cells in insulitic lesions (127).

T follicular helper cells

T follicular helper cells are similar to Th17 in that they both produce IL-21, but Tfh have a vastly different role in immunity, and there is evidence that they contribute to T1D pathogenesis (100,104,129). The role of Tfh in immunity is to contribute to the humoral immune response by assisting B cells through cytokine production and assist in the formation of germinal centers that can contribute to autoantibody production by B

cells in T1D (100,104,126,129). IL-21 produced by Tfh promotes the proliferation of B cells (130). The lineage transcription factor for Tfh is B-cell lymphoma 6 protein (Bcl-6) (129). However, Tfh are also identified by the expression of surface markers C-X-C Motif Chemokine Receptor 5 (CXCR5), which is a chemokine receptor used for migration to B cell zones, Programed cell death -1 (PD-1), a co-inhibitory receptor, and inducible co-stimulator (ICOS) a co-stimulatory molecule (129). As for Tfh contributions to T1D pathogenesis, it has been shown that there is an increase in T cells with a Tfh signature in peripheral blood in patients with T1D and new-onset patients (100,104,114,131). It is thought that Tfh cells are responsible for the increased IL-21 levels found in T1D patients and NOD mice (104,126,131).

T regulatory cells

T regulatory cells are typically responsible for suppressing effector T cells by producing anti-inflammatory cytokines like IL-10 (132). In T1D, however, not only are Tregs less suppressive due to deficiencies in IL-2, but effector cells are resistant to their suppression, dysfunctional Tregs promote defects in immune tolerance which a driver of disease pathogenesis (32,33,71,73,96,133,134). Tregs express the lineage transcription factor FoxP3 and surface marker CD25 (73,135). Humans that genetically lack FoxP3 have a multi-system autoimmune disorder, X-linked, immune dysregulation, polyendocrinopathy, enteropathy (IPEX) (136,137). IPEX results in immune dysregulations due to a deficiency in Tregs. These patients often have multiple autoimmune disorders, including autoimmune diabetes (136,137). NOD mice treated with

αCD25, also known as the IL-2Rα chain that is important for Treg function (33,138,139), progressed to diabetes faster than controls (140). Studies show that the transfer of Tregs to NOD mice prevents autoimmunity if T1D antigens are present (101,139). Also, adoptive transfer of Tregs stops T1D pathogenesis through the inhibition of IFN-γ produced by autoreactive CD4+ and CD8+ T cells and inhibiting CD8+ T cells from infiltrating pancreatic islets (141). These studies demonstrate the crucial role Tregs have in preventing T1D autoimmunity.

T regulatory-1 cells (Tr1) are similar to both Th1 and Tregs (142–145). These Tr1 cells are CD4+ and produce IL-10 (146–148), which inhibits antigen-presenting cells and antigen-specific T effector cells (143,149–151). Tr1 cells are mainly responsible for maintaining tolerance and controlling excessive inflammatory responses and autoimmunity (142,143,145,151). Tr1 cells typically have transient FoxP3 expression or lack it and can be induced with IL-27 alone or in combination with Transforming Growth Factor *Beta* (TGF-β) (142–144,147,148,151). In general, Tr1 cytokines produce IL-10, TGF-β, IFN-γ, and IL-5 (142–144,147,148). Some markers commonly used to identify Tr1 cells include CD49, Lymphocyte Activation Gene 3 Protein (LAG-3) (144), and the transcription factors: Maf-musculoaponeurotic fibrosarcoma (c-maf) (152), Aryl Hydrocarbon Receptor (Ahr) (153,154), and B Lymphocyte-Induced Maturation Protein-1 (Blimp-1) (145,151,155).

Cytotoxic CD8+ T cells

The role CD8+ T cells have in T1D is to directly attack the pancreatic β- cells which leads to insulin loss (105,156). CD8+ T cells have been found in the islets of NOD mice (105) and insulitic lesions in biopsies from recent-onset T1D patients (157,158). CD8+ T cells receive cytokine messages from CD4+ T helper cells which help fuel their attack on pancreatic islets for which they have antigen specificity (159,160). Some cytokines involved in promoting their effector functions are IL-2 and IL-12 (159,160). Also, transcription factors that are important for controlling their effector functions include T-bet and Eomes (159,160). The attack on pancreatic islets consists of the release of perforins and granzyme B, which kill pancreatic β-cells (106,161,162). Cytotoxic CD8+ T cells also produce inflammatory cytokines such as IFN-γ, which adds to the inflammatory response found in T1D (106,161,162). Also, and IFN-γ is also toxic to pancreatic β-cells (106,161,162). Studies have also revealed that T1D pathogenesis in humans is delayed if exhausted islet antigen-specific CD8+ memory T cells are present since T cell exhaustion through co-inhibitory receptor expression limits their production of pathogenic cytokines IL-2 and IFN-γ (107). These studies reveal the role CD8+ T cells have in the pathogenesis of T1D, where this specific T cell subset is responsible for the majority of β-cell death leading to diabetes.

Memory T cells

Both CD4+ and CD8+ memory T cells are significant contributors to pathogenesis in T1D (103,163). It is thought that these memory cells are the reason diabetes development comes in stages. Typically, after the first symptoms of diabetes, there is a period where T cells pause in attacking islets which allows the β-cells to recover somewhat and resume some insulin production (164–167). However, memory T cells can be activated and resume the destruction of islets over time until inducing full diabetes (164,165). Islet antigen-specific memory T cells contribute to difficulties in preventing pathogenesis in new-onset patients (163) and maintaining allogenic islet transplantation even with immunosuppressive medications (168). Memory T cells develop in the contraction phase of an immune response where T cells undergo the decision to undergo apoptosis or become memory cells (169,170). Within the memory cell subset, they can be further subdivided into T central memory cells that reside in lymphoid tissues (120,171), T effector memory cells that home to inflamed tissues (103,120,171), and T resident memory which homes and reside in specific peripheral tissues (157,172). Memory T cells have strong proliferative responses to IL-7 and IL-15 (173,174), especially since memory T cells have increased expression of the IL-7 receptor for survival and homeostasis (174–177). Blockade of the IL-7 receptor prevents and reverses diabetes through memory T cell inhibition in NOD mice (15,16). T effector memory cells may be dominant in T1D and other autoimmune diseases (178).

T cell Subset Metabolism and Metabolic Pathways

Introduction

T cell function and differentiation are dependent on the cell's metabolic program (179). T cells transition metabolic program as they go from one differentiation state to another and upon activation (179,180). Naïve states dependent on oxidative phosphorylation (OXPHOS), and upon activation there is a switch to glycolysis (aerobic and anaerobic) with less dependence on OXPHOS (179,180). T cells that also undergo memory formation transition their metabolic programs to depend on fatty acid oxidation and oxidative phosphorylation for their long-term functions (171,173,181). The plasticity of T cell differentiation is linked to changes in metabolic function (110,182).

Metabolic Pathways Involved in T cell Functions.

Naïve T cells that circulate are quiescent and depend on OXPHOS breaking down nutrients to fuel cell survival (183,184). Upon TCR stimulation along with CD28 co-stimulatory signals and IL2 receptor signaling, T cells are activated, and these activation signals switch the metabolism of naïve T cells from OXPHOS dependence to anaerobic glycolysis for proliferation and effector functions (183–185). This metabolic switch upon activation promotes energy production through anaerobic glycolysis, where the end products are glucose converted to pyruvate, ATP production, and the release of lactate which is excreted from the cell (183–185). CD28 co-stimulation and IL- promote the metabolic switch to glycolysis through the mechanism of PI3K activation of Akt, promoting the mechanistic target of rapamycin (mTOR) pathway to utilize glucose and

amino acids (183,185–188). If T cells cannot undergo glycolysis or are deprived of glucose, they exhibit reduced survival and proliferation (185,189,190). Cytokine production is also influenced by glycolysis. Inhibition of glycolysis has been shown to reduce the IFN-γ cytokine production by Th1 cells, and transgenic expression of GLUT1 enhances IL-2 and IFN-γ production (183,191,192). After effector T cell activation, there is a switch from using anaerobic-glycolysis to aerobic-glycolysis coupled with OXPHOS (183,184). The pyruvate created during glycolysis is transformed to acetyl-CoA, which then goes into the TCA cycle (183,184). The TCA cycle generates electron donors for the electron transport chain (ETC), allowing ATP production through OXPHOS (183,184).

Other metabolic pathways that that can influence the function of T cells include FAO coupled with OXPHOS to produce ATP. In FAO, fatty acyl-CoA is transported into the mitochondria across the outer mitochondrial membrane using CPT1 to undergo β-oxidation and supply acetyl-CoA for the TCA cycle, which provides electron donors for OXPHOS and leads to ATP production (193,194). Treg and memory T cell functions are dependent on FAO metabolic program (181,195). FAO is induced in memory CD8+ T cells and promoted by the cytokine IL-15. Treating memory CD8+ T cells with IL-15 led to increased mitochondrial biogenesis and CPT1a expression (173). It is thought that FAO in memory T cells promotes long-term survival (196). CD8+ T resident memory (Trm) cells are dependent on exogenous free fatty acids (FFA) and FAO for long-term survival (172). In this study, deficiency of fatty acid-binding proteins 4/5 (FABP) impaired exogenous free fatty acid uptake in CD8+ Trm. Also, culturing CD8+ Trm cells

with exogenous FA's increases FAO rates compared to CD8+ Tcm (172). In Tregs, it has been shown that blocking FAO can impede the development of Tregs while inhibiting the mTOR pathway in T cells increases the frequency of Tregs (197). Also, increasing AMPK activation with AMPK activator metformin increases the frequency of Tregs (198). This treatment leads to reduced GLUT1 expression, which is vital for glycolysis, promotes FAO in Tregs, and increases their numbers and frequency (199–201). These examples highlight the importance of FAO for specific T cell subset functions. However, exposure to FAs can have many other effects on individual cell physiology (202,203). Some of these effects include triacylglycerol accumulation and storage of FAs. Conversion into lipid mediators can influence T cell functions and cytokine production (202,203). Also, FAs also serve as ligands for nuclear signaling and affect metabolic pathways in that manner (202,203). The type of fatty acids, nutritional requirements of T cells, and T cell environment all determine the outcome of specific fatty acids in terms of how the cell handles the FA and what purposes further complicate fatty acid metabolism (204,205).

Autoreactive T cell Metabolism

There is evidence that metabolic aberrations and altered energy metabolism in those with a genetic risk of T1D may promote autoimmunity (77,90–92,206–210). There is an altered lipid metabolome in human and NOD mouse T1D progressors (77,91). In the NOD mouse model, mice had increased expression of genes related to the TCA cycle, glycolysis, and gluconeogenesis (77). Metabolomic analysis of the high-risk mice

confirmed dysregulation of energy and amino acid metabolism, indicating increased energy demands before T1D progression (77). Studies of metabolites in humans at risk of T1D revealed that in early infancy, before T1D progression, there is a reduction in metabolites related to sugar derivatives and fatty acids, and LA metabolism is altered (92). These studies indicate that altered energy metabolism may trigger T1D autoimmunity and that autoreactive T cells may have heightened energy demands. Furthermore, in the progression to full diabetes, the lack of insulin also leads to irregularities in glucose, lipid, and protein metabolism (212), affecting T cell function and differentiation due to these functions being linked to metabolic pathways (208). There is a lack of information about how the reduction in insulin over time and the irregularities in metabolic pathways influence T cells over the timeline of T1D pathogenesis. Also unknown is how the autoreactive T cell environment changes and how they adapt, which may influence pathogenesis. Also not well understood are how T cell local environments can manipulate and influence the metabolic programs of T cells to either promote pathogenicity or tolerance. If metabolic pathways and environments of T cells are better characterized, we can understand how changes to the metabolic programs of T cells can promote pathogenic functions which lead to T1D and other T cell-mediated autoimmune diseases.

T cell Cytokines

Introduction

The cytokines produced by T cells are diverse and heavily dependent on subset phenotype, function, and environmental context. Since different T cell subsets are involved in T1D pathogenesi, there are several cytokines produced that can contribute to pathogenesis (207). Pathogenic cytokines include IFN-γ, IL-2, IL-21, and IL-7 (211), while IL-10 is considered anti-inflammatory (212). While many of these cytokines are T cell subset-specific, some, including IFN-γ, are produced by Th1 and CD8+ T cells (141), while IL-10 is produced by many immune cells but Tregs and Tr1 cells have increased production (132,151). Homeostatic cytokines such as IL-7 support the persistence and survival of autoreactive memory T cells, contributing to disease development (174,178). The anti-inflammatory cytokine IL-10 has a role in inhibiting effector T cell functions (132). The main producers of IL-10, Tregs, fail to suppress autoreactive effector cells and restore tolerance in T1D (71). Cytokines and their receptors have been studied as therapeutics and as targets to control autoimmunity (15,16,213) but require more studies of different cytokines' functions on T cells and other immune cells.

Interferon-γ

Interferon-γ, a pathogenic cytokine in T1D, is a homodimeric cytokine member of the type II class of interferons and has pleiotropic immune functions in tissue homeostasis and immune and inflammatory responses (214). It is typically produced by Th1 cells, CD8+ T cells, NK cells, and Innate Lymphoid Cells (ILCs) (215). IFN-γ binds and

signals via IFN-γ receptors (IFN-γ R1 and IFN-γ R2) which activate the JAK 1/2 and STAT1 pathway (214). STAT1 translocates into the nucleus and binds to a specific conserved IFN-γ activation site DNA elements that directly activate the transcription of interferon-stimulated genes (214). These interferon-stimulated genes encode products that have direct effects on effector immune functions (214,215). IFN-γ amplifies Th1 responses, which can inhibit Th2 and Th17 subset differentiation (214). For example, IFN-γ can suppress the IL-4-STAT6 pathway necessary for Th2 differentiation (216). IFN-γ can impact the progression of T1D at multiple points during disease pathogenesis (217). Blocking IFN-γ in NOD mice with αIFN-γ antibodies or soluble IFN-γ receptors reduces the incidence of diabetes and prevents the transfer of disease by splenocytes from diabetic NOD donor mice (217). Also, IFN-γ receptor deficiency prevents the development of T1D by CD4+ T cells but not CD8+ T cells (118).

Interleukin-2

IL-2 is an autocrine growth factor that can stimulate T cell proliferation (218,219). The IL-2 structure consists of four α-helices. IL-2 binds the IL-2R, which signals using three subunits including IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ (CD132) (219,220). When IL-2 binds its receptor it leads to the phosphorylation and activation of STAT proteins, specifically STAT5A/B and STAT1/3 (219,220). Production of IL-2 is regulated by TCR and CD28 signaling (219). IL-2 promotes the expansion and proliferation of activated T cell populations (221). IL-2 is primarily produced by CD4+ T cells excluding Tregs (221). The IL-2Rα gene contains T1D and other autoimmune risk

variants conserved between mice and humans (222,223). IL-2Rα deficiency leads to chronic immunodeficiency. Humans and mice that lack IL-2Rα have autoimmunity that results from decreased numbers of Tregs (136,137,222). Since a lack of IL-2 and Treg dysfunction are connected to a lack of immune tolerance, IL-2-based therapeutics have been studied to target Tregs for expansion to induce immune tolerance (224). Tregs express the high-affinity IL-2Rα chain to outcompete other T cells for IL-2 (224). An example of which are IL-2 complexes consisting of low IL-2 doses combined with αIL-2 antibodies, which promote Treg expansion (134,225). In NOD mice, IL-2 complexes have been shown to inhibit diabetes by promoting antigen-specific Treg expansion (225). While successful in mice, the main issue with IL-2 based therapeutics for T1D is that while Tregs can be expanded by low doses of IL-2 (139), there is a risk that effector T cells can be expanded as well (218) and contribute to pathogenesis.

Interleukin-10

IL-10 is an anti-inflammatory cytokine responsible for inhibiting host immune responses (132). IL-10 dysregulation has been associated with increased autoimmunity risk (132). IL-10 can be produced by many different immune cell types such as T helper cells (Th cells), monocytes, macrophages, dendritic cells, B cells, and CD8+ T cells (132). Effects of the IL-10 cytokine are mediated through the heterodimeric IL-10R1 and IL-10R2 (212). IL-10R2 is ubiquitously expressed, while IL-10R1 is mainly expressed on leukocytes (212). When IL-10 binds its receptor, it activates JAK/STAT signaling, changing immunomodulatory genes' expression (226). These immunomodulatory gene

changes inhibit pro-inflammatory cytokine production, decrease antigen presentation, and decrease phagocytosis (149,226). STATs are important for IL-10 induction in myeloid and lymphoid cell types. STAT1 and 3 are critical for IL-27-induced IL-10 gene expression in T cells, while STAT3 promotes IL-6-mediated IL-10 production (149,226). The c-maf transcription factor has been implicated in IL-10 expression by T cell subsets Th1, Th2, and Th17 (149). In Th1 and Th17 populations, c-maf dependent expression of IL-10 is reliant on the activation of ERK (149). In T cells, c-maf induces the production of IL-10 by binding MAF recognition element motif (MARE), in the IL-10 promoter (149). Apart from c-maf other transcription factors that participate in IL-10 regulation include SP1, C/EBPs, IRF, AP-1, NFκB, CREB, Blimp-1, Ahr, and others (149). One of their central cytokines produced for Treg suppressive functions is IL-10 (135). Early studies that injected NOD mice with IL-10 demonstrated that IL-10 was delayed disease and provided long-lasting protection (227). However, transgenic IL-10 production in pancreatic islets led to diabetes development, and genetic deletion of IL-10 led to no noticeable effects on disease pathogenesis (228,229). Neutralization of endogenous IL-10 in NOD mice at three weeks of age inhibited insulitis development, but NOD mice injected at later stages displayed no significant effects on disease development (230,231). In first-degree T1D relatives and new-onset T1D patients, peripheral blood T cells have similar IL-10 responses, both of which are lower than IL-10 responses from healthy controls, and show more IFN-γ production than in healthy controls (119). As mentioned, the role of IL-10 in T1D is inconclusive, and the extensive regulation of IL-10 adds to the complexity of the role it plays in T1D pathogenesis.

Interleukin-21

Produced by T cells and NK cells, IL-21 is a type I cytokine with pleiotropic effects on immune and non-immune cells (126,232,233). IL-21 binds to its IL-21 receptor, which consists of IL-21R and the common γ chain. IL-21 signals through JAK 1/3 in T cells, and IL-21 more strongly activates STAT3 compared to STAT1, 5A/5B (232). Genes targeted by IL-21 are regulated using the transcription factors and regulators BATF, JUN, IRF4, and STAT3 (232). Th17 cells produce IL-21, which stabilizes and expands this population, and induces RORγt expression which is the lineage transcription factor, and regulator of Th17 cells (125). IL-21 is also produced by T follicular helper (Tfh) cells and upregulates Bcl-6 and c-maf in Tfh cells (126,234). IL-21 also promotes proliferation and functional responses in CD8+ T cells and can regulate NK cells, macrophages, and DCs (235). Effects of IL-21 are complex and tend to vary. The cytokine is induced by TCR signaling, co-stimulation, and other cytokines which include IL-1β, IL-6, IL-27, and IL-21 itself (232). IL-21 activates PI3K-Akt and MAPK signaling pathways that have a role in the proliferation of CD8+ T cells promoted by IL-21 (126,232,233). It also suppressed the differentiation and expansion of Tregs, inhibit IL-2 production by non-Tregs, and promote the differentiation of Th17 and Tfh (126). Culturing T cells with IL-21 skewed metabolism from aerobic glycolysis to FAO along with increased mitochondrial fitness and biogenesis and increased proliferation regardless of glucose concentrations (236). Also found were a higher CPT1a (FAO rate-limiting enzyme) expression in IL-21-treated T cells and greater mitochondrial spare respiratory capacity (SRC) which is the extra

ATP a cell can produce from OXPHOS to respond to a sudden increase in energy demand (236). In T1D, there are high levels of IL-21 in NOD mice and T1D patients and a Tfh signature in T1D patients (104,131). Tfh cells co-express TNFα and IFN-γ, which are pro-inflammatory cytokines implicated in T1D pathogenesis (114,223). It has been found that NODs lacking IL-21 signaling do not develop diabetes and the susceptibility loci for T1D (Idd3) contains the gene encoding IL-21 (131). IL-21 is necessary for the development of T1D in NOD mice, and treatment of NOD mice with IL-21R-Fc fusion protein led to reduction of immune infiltrates into the pancreas in early disease stages, but not once diabetes had developed (131,237). The role of IL-21 in T1D has yet to be fully understood, but there are promising developments in the understanding of how IL-21 can influence T cell subsets involved in pathogenesis and in therapeutic strategies that involve targeting IL-21 to prevent autoimmunity.

Interleukin-7

The cytokine interleukin-7 is vital for B and T cell development along with lymphoid homeostasis and the differentiation, survival, and generation of memory T cells (238–240). IL-7 is primarily produced by non-lymphoid cells like keratinocytes, epithelial, and stromal cells found in lymphoid organs (240). IL-7 is a globular protein that binds to its receptor IL-7R, a heterodimeric complex which includes IL-7Rα and the common γ chain (239,240). IL-7 binding its receptor promotes IL-7Rα and common γ heterodimerization, leading to JAK1 and JAK3 activation and downstream signaling with STAT1, STAT3, STAT5, PI3K-Akt-mTOR, and MEK-ERK pathways (239,240). IL-7 signaling promotes

lipolysis in memory CD8+ T cells, supporting long-term survival and maintenance (176,196). The cytokine can promote cell survival by modulating apoptosis pathways (240). Susceptibility to autoimmune diseases like T1D are linked to several SNPs in the IL-7R gene loci (241,242). Mechanisms of how IL-7 regulation leads to increased autoimmune susceptibility are unclear. It is thought to promote autoreactive T cells' increased proliferation when responding to self-antigen, and reduced expression of co-inhibitory receptors such as PD-1 (15,243). Therapeutics involving the IL-7R pathway include the IL-7R blocking antibody have been shown to prevent and reverse T1D in NOD mice (15,16). In human T1D trials, IL-7Rα blockade increased Treg frequencies (20). In phase I clinical trials testing anti-IL-7Rα monoclonal antibodies (mAbs) in T1D, it was found that the anti-IL-7Rα mAb inhibit the activity of memory T cells and preserves Tregs and naïve T cells (20). These are promising results for therapeutics involving the IL-7R pathway in T1D, but there is still much to explore of when and from which cells IL-7 is produced to promote pathogenesis in T1D.

Specific Aims and Hypothesis

As described, T1D is a complex autoimmune disorder in which multiple T cell subsets organize an attack against pancreatic β-cells (1). The ensuing loss of endogenous insulin production over time leads to hyperglycemia and complications in multiple organ systems due to improper glucose management (1). It is clear that T1D has a genetic component where many risk genes have been defined, and in the last 30 years, the incidence of T1D has been increasing globally (2,4,5). The increased incidence points to environmental factors contributing to enhanced disease development in genetically susceptible individuals that do not necessarily have high-risk T1D genes (13,36). Several environmental factors have been studied and proposed to influence T1D pathogenesis, including viral agents, the microbiome, and dietary changes that alter the metabolome (13,77,92).

One of the main environmental factors we chose to focus on is polyunsaturated fatty acids (PUFA) and their effects on pro- and anti-inflammatory T cell subset phenotype, function, and cytokine production in the context of T1D. Consumption of PUFAs has been suggested to affect disease risk (61,62), and PUFAs have many cellular roles. Some of these include incorporation into the cell membrane, signaling mediators, impacting gene transcription, and affecting mitochondrial function and energy status (244–246). Clarifying the crosstalk between altered exposure of T cells to PUFAs and altered responsiveness to these PUFAs in autoimmune-prone mice will further our understanding of the role PUFAs may play in the etiology of T1D.

We hypothesized that altered lipid environments affect T cell differentiation and function in T1D pathogenesis. To explore how different PUFAs impact autoreactive T cells, we developed a PUFA treatment assay to test the individual effects of PUFAs on T cells from the T1D mouse model non-obese diabetic (NOD) mice. This assay helped us to acquire information on how changing the lipid environment influenced the phenotype and function of T cells from pre-diabetic NOD mice. This dissertation is organized into two main research aims with a related side-project discussed in **Chapter 5** that looks into how IL-7Rα blockade, a potential therapeutic for T1D (20), influences lipid metabolism in T cells from anti-IL-7Rα treated NOD mice. In the first aim, we investigated the changes to T cell phenotype and function after changing the fatty acid environment at relevant physiological concentrations. In the second aim, we examined the changes to metabolic and lipid gene expression in T cells after altering the environment with the PUFA LA to understand how specific genes could contribute to T cell functional changes.

CHAPTER TWO: MATERIALS AND METHODS

Common Buffers and Reagents

Phosphate Buffered Saline (PBS 1X)

pH 7.4, Sodium Chloride (NaCl)155.17 mM, Potassium Phosphate monobasic (KH_2PO_4)1.06 mM Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$) 2.97 mM (Gibco® Life Technologies)

Fatty Acid-Free Complete Media (C10)

RMPI 1640x Cell Culture Media with L-glutamine and phenol red, without HEPES (Gibco®), supplemented with 10% heat-inactivated Charcoal-Dextran stripped Fetal Bovine Serum (Omega), 10mM HEPES (Gibco) 1 x MEM Non-essential amino acids (Gibco) 1mM Sodium Pyruvate (Gibco), 55nM 2-Mercaptoethanol (Gibco), and 1% Penicillin-Streptomycin

Complete Media (C10)

RMPI 1640x Cell Culture Media with L-glutamine and phenol red, without HEPES Gibco®, supplemented with 10% heat-inactivated Fetal Bovine Serum (N), 10mM HEPES (Gibco®) 1 x MEM Non-essential amino acids (Gibco®) 1mM Sodium Pyruvate (Gibco®), 55nM 2-Mercaptoethanol (Gibco®), and 1% Penicillin-Streptomycin

Glucose-free Complete Media (C10)

Glucose-free RMPI 1640x Cell Culture Media with L-glutamine and phenol red, without HEPES Gibco®, supplemented with 10% heat-inactivated Fetal Bovine Serum (Omega), 10mM HEPES (Gibco®) 1 x MEM Non-essential amino acids (Gibco®) 1mM Sodium Pyruvate (Gibco®), 55nM 2-Mercaptoethanol (Gibco®), and 1% Penicillin-Streptomycin

FACs Staining Buffer

1x PBS (Gibco®) with 2% FBS (Omega)

Intracellular Fixation and Permeabilization Buffer Set (Cell Signaling)

1X PBS (10% 10X PBS, 90% Purified Water)

Fixation Buffer

Permeabilization Buffer

Antibody Dilution Buffer

FoxP3 staining Buffer Set (Invitrogen)

Fix/Perm Buffer (75% Fix/Perm Diluent, 25% Fix/Perm Concentrate)10x

Permeabilization Buffer (1x Perm buffer, 10% 10x Permeabilization Buffer 90% Purified Water)

2-4% Paraformaldehyde

2% Paraformaldehyde Diluted FACs Buffer

FA Conjugation to BSA

LA, Oleic Acid, and α-Linolenic Acid (Sigma/Millipore) were individually dissolved with DMSO (0.1%DMSO final concentration in culture) and separately conjugated in Fatty Acid-Free Complete Media (FA free C10) at a 2:1 molar ratio (2 FA: 1 BSA) with fatty acid-free BSA (Sigma) using methods previously described (247,248). Fatty acid-free BSA was dissolved into FA-free C10 media to create 1 13.6% solution, fatty acid dissolved in DMSO was dripped into the BSA/C10 solution, and solutions were placed at 37°C until fully dissolved and stored at 4°C until use. BSA vehicle control is created in the same manner using DMSO only.

Mice and In Vivo Methods

NOD/ShiLtJ (NOD) mouse breeding pairs were purchased from The Jackson Laboratory (#001976) and bred homozygous in a specific pathogen-free facility at Boston University School of Medicine. To compare the role of polyunsaturated fatty acids on T cells from diabetes-sensitive NOD mice were compared with a non-autoimmune mouse strain C57BL/6J mice. C57BL/6J mice were purchased from The Jackson Laboratory (#000664) and housed in a specific pathogen-free facility at Boston University School of Medicine.

Tissue Collection

Spleen and Lymph Nodes

Spleens and lymph nodes were collected from mice and placed in 2% FBS PBS in a 6-well plate (Corning) on top of a 40µm filter (ThermoFisher). Spleens and lymph nodes were crushed through a 40µm filter and washed (1500rpm, 4°C, 5mins, decant supernatant) with 10ml of 2% FBS PBS. Splenocytes were re-suspended with Ack (Ammonium-Chloride-Potassium) lysis buffer (Gibco®) and incubated 3 minutes at room temperature afterward washing twice with 10mls of C10 media. Lymph node cells were washed twice (1500rpm, 4°C, 5mins, supernatants decanted) with 5mLs of C10 media. Splenocytes are resuspended in 5mls of C10 media for counting in Trypan blue (Gibco®) (1:100 dilution). Lymph node cells are resuspended in 1ml of C10 media for counting in Trypan blue (1:20 dilution).

Bone Marrow

Femurs and tibias were harvested from NOD mice. Ends of bones were cut, and bone marrow was flushed out of the core into C10 media in a small cell culture dish (Corning) using a small gauge needle (#26 or #30, BD Biosciences). The bone marrow was homogenized with a needle (#18 gauge, BD Biosciences) and filtered through a 40µm filter to remove clumps. Cells are washed with 10-15 ml C10 media (spin at 1000 RPM for 5 minutes) and resuspended in ACK lysis buffer for 3 minutes at room temperature. Cells were washed twice (spin 1500RPM, 5 minutes, 4°C) with C10 media and

resuspended in 10mL C10 media for counting with Trypan blue (1:100 dilution, Gibco®) and plated on non-TC treated Petri dishes (Corning).

Tissue Culture

Splenocyte Culture with FAs

Harvested spleens were crushed into single-cell suspensions using 40u cell strainers (USA Scientific). Splenocyte suspensions were treated with ACK lysis buffer to remove red blood cells. (Gibco®) LA (or other FA) conjugated to BSA or BSA only (control) was added to splenocyte cultures at low and high concentrations (100μM to 1mM). T cells underwent TCR activation in culture with 1μg/mL of αCD3 (clone) and 2μg/mL of αCD28 (clone) antibodies (Invitrogen) in a 96-well round-bottom plate (Corning). Splenocyte cultures were incubated for 48 hours at 37°C. Supernatants were collected and frozen at -80°C, and cells were harvested for flow cytometric staining using the panel in **Table 3** and **Table 6.**

Treatment of NOD mice with anti-IL-7Rα antibodies and splenocyte culture with FAs

Pre-diabetic NOD mice were injected intravenously in the tail vein with 200μl of anti-IL-7Rα antibodies (BioXcell) or control rat IgG (Company). Three days after injection, mice were euthanized, and spleens were harvested. Harvested spleens were crushed into single-cell suspensions using 40μm cell strainers (USA Scientific). Splenocyte suspensions were treated with ACK lysis buffer to remove red blood cells. (Gibco®) LA (or other PUFA) conjugated to BSA or BSA only (control) was added to splenocyte cultures at low and high concentrations (100μM to 1mM). T cells underwent TCR

activation in culture with 1 µg/mL of αCD3 (clone-:145-2C11) and 2 µg/mL of αCD28 (clone:37.51) antibodies (Invitrogen) in a 96-well round-bottom plate (Corning). Splenocyte cultures were incubated for 48 hours at 37°C. After T cells were re-stimulated with PMA and Ionomycin for 4 hours total with BFA added after an hour. Supernatants were collected and frozen at -80°C for ELISA. Cells were harvested for flow cytometric staining using the flow panels in **Table 3** and **Table 8**.

Th17 Polarization of NOD mouse T cells

Using methods described previously (249), naïve CD4+ T cells were isolated using the naïve CD4+ STEMCELL negative selection kit following the kit instructions. Cells were counted, washed, and re-suspended in C10 media at $1x10^6$cells/500ul. A 24 well plate (Corning) was coated with αCD3 antibody (Invitrogen) at 2ug/mL in 500uL of 1X PBS. The plate was incubated at 37°C for 1 hour. After 1 hour, the antibody/PBS solution was discarded, and wells were rinsed with 1mL of 1X PBS. Cells were added to the αCD3 coated plate at $1x10^6$cells in 500uL C10 media. ACD28 and TH17 polarizing cytokines and blocking antibodies were added to the wells in 500uL C10 for a final volume of 1mL, including an "αCD28" only no polarization control. Th17 Polarizing cytokines and blocking Abs include 2ug/mL αCD3 (coated on 24 well plate), 5ug/mL αCD28, 80ng/mL IL-6 , 20ng/mL IL-23 , 10ng/mL human TGF-beta, 10ug/mL αIL-4, and 10ug/mL αIFN-γ antibodies. Cells were incubated for 4 days at 37°C, and T cells were re-stimulated with PMA and Ionomycin for 4 hours with BFA added an hour in and immunostained using the panel in **Table 9**.

Proliferation and Survival Assay

Splenocytes from NOD mice were plated with fatty acid or BSA, activated, and incubated for 48 hours at 37° C in a 96-well flat-bottom plate (Corning). After 48 hours, T cells were isolated using the STEMCELL negative selection T cell isolation kit and re-plated only with media. T cell samples were taken at 24, 48, and 72 hours to stain for surface markers and proliferation markers using the panel in **Table 7**.

Purified T cell culture with FAs

NOD mouse T cells were purified using a magnetic T cell isolation kit (STEMCELL) and plated at $1x10^6$ cells/500µl in C10 media, activated with 1µg/ml of αCD3 and 2µg/ml of αCD28 antibodies (Invitrogen) in 50µL and treated with LA conjugated to BSA or controls (media only and BSA only). Cells were incubated for 48 hours at 37°C.

Generation of Bone-marrow derived Macrophages and Dendritic Cells

Using methods described previously (250,251), starting from bone marrow harvest, for Bone marrow-derived macrophages (BMDM) generation bone marrow cells were plated with C10 media containing 20% L-929 media, while Bone marrow-derived dendritic cells (BMDC) generation required 20ng/ml of Granulocyte-macrophage colony-stimulating factor (GM-CSF) added to C10 media. On day three, BMDMs were fed by adding 5mls of C10 media with 20% L-929 media, BMDCs were fed 5mls of C10 with 20ng/ml of

GM-CSF, and cultures were incubated at 37°C. On day five, the APCs were matured by BMDMs re-plating with 1ug/ml LPS, and BMDCs received 1ug/ml. On day seven, BMDCs were isolated and enriched using a mouse DC enrichment kit (Dynabeads®), and BMDMs were isolated by scraping cells adhered to plates. Cells were washed by spinning at 1500RPM for 5 minutes at 4°C and were counted with Trypan blue for plating in APC/T cell co-culture. The purity of cell types (BMDMs, BMDCs, and magnet-purified T cells) was established using the following markers in **Table 2**.

APC and T cell Co-culture

Purified NOD mouse T cells were plated at $1x10^5$/100µl in C10 media, activated with 1ug/ml of αCD3 and 2ug/ml of αCD28 antibodies and co-cultured with LA pre-treated APCs, 100µM and 500µM of LA conjugated to BSA or controls (media only and BSA only). To pre-treat the APCs, APCs were sorted by flow cytometry (CD3- cells) were added to the fatty acid assay culture system for pre-treatment, then, after 48 hours, were re-plated at a 3:1 ratio (APC:T cell) with purified T cells. A sample of cells was immunostained for flow cytometry analysis for cell purity using the antibody-staining panels **Table 2**. Cells were incubated for 48 hours at 37°C, and afterward, T cells were stimulated with PMA and Ionomycin for 4 hours along with Brefeldin A (BFA) for intracellular cytokine capture. A sample of cells was immunostained for flow cytometry analysis using combined flow panels with markers found in **Table 2** and in **Table 3**.

For co-culture with BMDMs and BMDCs, NOD mouse purified T cells were plated at $1x10^5$/100µl in C10 media, activated with 1ug/ml of αCD3 and 2ug/ml αCD28 antibodies in 25µl and treated with 100µM and 500µM of LA conjugated to BSA or controls (media only and BSA only) in 25µl and plated at a 2:1 ratio (T cell: APC) with BMDMs or BMDCs in 50ul. Cells were incubated for 48 hours at 37°C.

Enzyme-Linked Immunosorbent Assay

Cytokine detection with ELISA

ELISAs were all run using Invitrogen Cytokine ELISA kits. 96-well plates were coated with capture antibody in 1X coating buffer (100µl/well) and incubated overnight at 4°C. Using a 96-well plate washer, wells were washed three times with wash buffer made of 0.025% Tween in 1X PBS. After discarding the remaining wash buffer, wells were blocked with ELISA diluent and incubated at room temperature for 1 hour. Lyophilized cytokine standards were resuspended with DI water and incubated for 15 minutes at room temperature before use. Wells were washed three times with wash buffer, and after discarding the remaining wash buffer, a cytokine standard curve was created (100µl/well) using a two-fold serial dilution. Samples of 100µl were added to the remaining wells, and the plates were covered and stored at 4°C overnight. The following day plate wells were washed three times with wash buffer made of 0.025% Tween in 1X PBS. Afterward, 100µl of detection antibody in 1X ELISA diluent was added to all wells, and samples incubated at room temperature for 1 hour. Wells were washed six times with 0.025% Tween in 1X PBS, and 100µl of Avidin-HRP diluted in ELISA diluent was added to the

wells and incubated for 45 minutes at room temperature. Wells were washed six times with wash buffer made of 0.025% Tween in 1X PBS, making sure to soak wells for 1-2 minutes between each wash. After discarding the remaining wash buffer, 100µl of room temperature 1X TMB solution was added to all wells. When the color developed to blue, the reaction was stopped with 50µl of 0.16M H_2SO_4. ELISA plate was read at 450nm using a 96-well plate reader (Company).

Cytokine detection with Multiplex ELISA

Supernatants were obtained from re-plated magnet isolated T cells (100k cells/well) from 48 hours splenocyte cultured with fatty acids (LA, ALA, OL, control) that were re-stimulated for 4 hours with PMA and Ionomycin. Supernatants from enriched (STEMCELL) T cells were analyzed using a mouse Th17 magnetic bead panel kit (MTH17MAG-47K),(Milliplex^{TM} MAP Kit, Millipore Corp, USA) on the BIORAD, Bio-Plex $^{®}$ 3D Suspension Array System to detect and quantify Th17 cytokines, which include IL-17A, IL-17F, GM-CSF, IFN-γ, Macrophage Inflammatory Protein-3α (MIP-3α), IL-1β, IL-2, IL-4, IL-6, IL-21, IL-22, IL-10, IL-23, IL-12, IL-27, IL-13, IL-15, IL-33, IL-31, TNFβ, TNFα, CD40L, and other cytokines. Samples were run by the Nikolajczyk Lab at the University of Kentucky. The data were analyzed using the Bio-Plex Manager 6.1 Software. Standard curves were determined using corresponding standards, and there were no cytokine exclusions from the analysis.

Gene Expression Analysis

Q-PCR of NOD Mouse T cells

T cells were isolated from culture using a magnetic negative selection T cell isolation kit (STEMCELL) following kit instructions. After isolating and pelleting T cells, the cells were lysed with buffer RLT containing 2% 2-mercaptoethanol, and RNA was isolated using the Qiagen mini spin-kit following kit instructions. RNA was quantified with a nanodrop machine. RNA was reverse transcribed to cDNA. For quantitation of lipid metabolism-related gene expression, the following primers in **Table 1** were used. Q-PCR was performed using SYBR green master mix and a StepOnePlus^{TM} Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc). Relative expression of target genes was normalized to 18S using the ΔΔCT method.

Table 1: Primers for Q-PCR.

Target	Sequence	Source
CD36	Forward TCCTCTGACATTTGCAGGTCTATC Reverse AAAGGCATTGGCTGGAAGAA	(252)
PPARα	Forward AGAGCCCCATCTGTCCTCTC Reverse ACTGGTAGTCTGCAAAACCAAA	(253)
PPARγ	Forward CCACCAACTTCGGAATCAGCT Reverse TTTGTGGATCCGGCAGTTAAGA	(252)
CPT1a	Forward CTCCGCCTGAGCCATGAAG Reverse CACCAGTGATGATGCCATTCT	(254)
ACC1	Forward TGACAGACTGATCGCAGAGAAAG Reverse TGGAGAGCCCCACACACA	(255)
Aquaporin 9	Forward TTGTGATGGCTCTTTATGCG Reverse CAGAGTTGAGTCCGAGAGAA	(256)

Flow Cytometry

Intracellular Cytokine and Transcription Factor Staining

Before cytokine staining to determine the level of cytokine production, cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma/Millipore) and ionomycin (Sigma/Millipore) to induce cytokine production for 1 hour at 37°C followed

by the addition of Brefeldin A (BFA) (Invitrogen) to trap intracellular cytokines. Cells were incubated for an additional 3 hours at 37°C. Cells (10^6) were harvested and stained with cell viability dye (Invitrogen) for 15 min at 4°C. Cells are washed with 1X PBS by centrifuging for 5 minutes, 1500 RPM at 4°C. 50µl of Fc receptor block was added to cells, and cells were incubated at room temperature for 5 minutes. Afterward, antibodies for T cell surface markers were added to cells in 50µl of 2%FBS-PBS, and cells were incubated in the dark for 25 minutes at 4°C. Cells were washed with 1X PBS by centrifuging for 5 minutes, 1500 RPM at 4°C. After viability staining and surface marker labeling, cells were fixed and permeabilized overnight at 4°C using the FoxP3/Transcription Factor Staining Buffer Set (Invitrogen). Cells were washed twice with permeabilization buffer, and cells were labeled for intracellular cytokines and transcription factors with the indicated antibodies for 30 minutes at 4°C. Cells were washed once with 2% FBS-PBS by centrifuging for 5 minutes, 1500 RPM,4°C, and before analysis on the BD LSR-II, cells were resuspended in 200µl FACS buffer, and 10µl of 123count beads (Invitrogen) were added to samples to obtain absolute cell counts.

Bodipy Staining of Neutral/Nonpolar Lipids

To measure lipid droplet accumulation, splenocytes were harvested and resuspended in 1X PBS containing 0.5ug/mL of Bodipy 493/503 (Invitrogen). Cells were incubated at RT for 15 mins and washed with 1X PBS (1500rpm, 37°C, 5mins, decant supernatant) followed by viability and surface marker staining with fluorophore-labeled antibodies. The flow panel in **Table 4** was used to immunostain and dye cells. Live cells were

washed and resuspended in 200µl 1X PBS. Samples were analyzed by flow cytometry the same day.

Mitotracker Staining

To measure mitochondrial mass, splenocytes were harvested and resuspended in warm 1X PBS containing 100nM of Mitotracker Green FM (Invitrogen). Cells were incubated for 15 mins at 37°C. After Mitotracker staining, cells were washed twice with 1X PBS (Gibco®) (1500rpm, 37°C, 5mins, decant supernatant) followed by viability and surface marker staining with fluorophore-labeled antibodies. The flow panel in **Table 5** was used to immunostain and dye cells. Live cells were resuspended in 200µl 1X PBS and were analyzed by flow cytometry the same day.

Analysis of Samples and Data

All flow cytometry samples were analyzed on the BD LSR-II or sorted using the BD FACS Aria, part of the Boston University School of Medicine Flow Cytometry Core. Data was analyzed using FlowJo™ v10 Software.

Panels for Flow Cytometry

Table 2: Flow cytometric panel for T cell purity.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
CD3	BV510	145-2C11
CD11b	PE-Cy7	M1/70
CD11c	APC	N418
Viability Dye	UV	-

Table 3: Flow cytometric panel for T cell cytokines and phenotype.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
IFN-γ	PE-Cy7	XMG1.2
IL-10	PE	JES5-16E3
IL-21	APC	FFA21
FoxP3	FITC	FJK-16s
Viability Dye	Aqua	-

Table 4: Flow cytometric panel for neutral lipid staining.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
Bodipy (neutral lipid dye)	493/503	-
Viability Dye	Aqua	-

Table 5: Flow cytometric panel for mitochondrial mass staining.

Specificity	Fluorophore	Clone
CD4	BV421	RM4-5
CD8a	PerCP	53-6.7
Mitotracker	Green FM	-
CD62L	APCe780	MEL-14
KLRG-1	APC	2F1
Tim3	PE	RMT3-23
Viability Dye	Aqua	-

Table 6: Flow cytometric panel for Tfh and Th17 cells.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
CXCR5	FITC	SPRCL5
Bcl-6	APC	K122-91
PD-1	PE-Cy7	J43
RORγt	PE	Q31-378
Viability Dye	Aqua	-

Table 7: Flow cytometric panel for survival and proliferation assay.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
Ki-67	PE-Cy7	SolA15
Annexin V	PE	-
Viability Dye	Aqua	-

Table 8: Flow cytometric panel for co-inhibitory receptors.

Specificity	Fluorophore	Clone
CD4	APC	RM4-5
CD8a	FITC	53-6.7
Tim-3	PE	RMT3-23
TIGIT	PE-Cy7	GIGD7
PD-1	APC-Cy7	J43
Lag-3	PerCPe710	C9B7W
Viability Dye	Aqua	-

Table 9: Flow cytometric panel for Th17 polarization.

Specificity	Fluorophore	Clone
CD4	APCe780	RM4-5
CD8a	PerCP	53-6.7
IL-17A	eF450	eBio17B7
IL-10	PE	JES5-16E3
IL-21	APC	FFA21
RORγt	PE	Q31-378
FoxP3	FITC	FJK-16s
Viability Dye	Aqua	-

Statistical Analysis

Statistical analysis was completed using Graphpad Prism 9 Software. See figure legends for specific statistical tests completed.

CHAPTER THREE: LINOLEIC ACID ENHANCES VIABILITY AND PROLIFERATION, AND ALTERS CYTOKINE PRODUCTION, IN T CELLS FROM NOD MICE

Rationale

T1D occurs due to a loss of T cell tolerance, enabling βcell-specific autoreactive T cells to activate and lead an inflammatory response that destroys the insulin-producing pancreatic β-cells (1). While there is an association between disease susceptibility and high-risk genes (9,11), genetics alone cannot explain why there is an increased incidence of disease in individuals with low or moderate risk genes. These facts point to environmental factors influencing disease status (2,4,5,13). One specific environmental factor that has changed globally in recent decades is the adoption of the Western diet, which contains high levels of the n-6 PUFA (257,258), linoleic acid (LA). LA (C18:2) is an essential fatty acid that can only be obtained through dietary plant and animal sources (259). Like other PUFAs, LA can impact cells in various manners, such as promoting the synthesis of signaling lipids, incorporating it into the membrane, and entering the mitochondria to undergo beta-oxidation (246,258,260,261).

T1D patient cohort studies have observed an altered lipid metabolic profile before autoantibody production in children who eventually develop T1D (91). Some of these altered lipids and pathways include triacylglycerols, multiple phospholipids containing PUFAs, and LA metabolism (64,77,92). These lipid metabolism pathways are altered between non-diabetic patient controls and T1D progressors at three months of age (64,77,92). Moreover, feeding studies have demonstrated that the introduction of n-3

PUFA dietary sources into the diets of pregnant women with a family history of T1D reduces the risk of disease development in their offspring (61,262). Also, feeding studies involving the supplementation of NOD mice with n-3 PUFAs demonstrated delayed T1D development and decreased pro-inflammatory T cell subsets (94).

Although there is an abundance of evidence demonstrating *in vivo* effects of PUFA supplementation on autoimmunity and inflammation (258,263,264), very few studies characterize the direct effects of PUFAs on T cells from autoimmune mice. Also unknown are the mechanisms by which metabolites and factors can influence autoreactive T cells in their local environments. These local fatty acid-based environment changes can promote changes to T cells' differentiation state and functions since T cell differentiation and functions are linked to specific metabolic pathways (208,265). Hence, diet-derived metabolites present in tissue environments likely impact survival, proliferation and contribute to skewing cytokine production profiles of antigen-stimulated autoreactive T cells. Determining how dietary components can affect autoimmune disease states by regulating T cell subsets helps increase the understanding of the diet-based sources of triggers and inflammation in T1D. Studies looking into these factors could reveal novel strategies to use dietary interventions and immunotherapies for the prevention and reversal of T1D.

Results

3.1 LA enhances T cell viability and proliferation.

To determine the impact of LA on activated T cells, splenocytes from pre-diabetic NOD mice were cultured *in vitro* with αCD3 and αCD28 mAbs in the presence of high and low physiological concentrations of LA conjugated to BSA or with BSA alone as a control. T cells were incubated with LA for 48 hours. To measure LA's effects on T cell viability after re-stimulation, cells were stained with viability dye. Viability dye labels dead cells by binding to amine groups within dead cells, which are more permeable. Cells were also labeled with antibodies for CD4 and CD8, counting beads were added to obtain absolute cell counts, and samples were analyzed by flow cytometry. The gating strategy used to gate viable cells and CD4+ and CD8+ T cells are shown in **Figures 1C and 1D**.

Culturing NOD mouse splenocytes in the presence of LA allowed greater T cell viability. This observation is demonstrated by the increase in the number of live CD4+ T cells **(Figure 2A)** at both high and low LA concentrations. The same trends can be seen for CD8+ T cells **(Figure 2B)**, but in both scenarios, the higher concentration of LA promotes a more significant number of viable CD4+ and CD8+ T cells. However, while there are greater numbers of viable T cells, there are no differences in the frequency of live CD4+ and CD8+ T cells in the LA-exposed group compared to controls. Data suggest that increased cell numbers could be due to enhanced viability or increased T cell proliferation.

To distinguish between the possibilities of LA promoting proliferation and enhancing survival in T cells, a proliferation/survival assay was designed to address the enhanced viability seen in LA-treated T cells. NOD mouse splenocytes were exposed to

LA bound to BSA or BSA at high and low concentrations, T cells were activated with αCD3 and αCD28 mAbs, and after 48 hours, T cells were isolated from splenocytes using a magnetic T cell enrichment kit. T cells were replated in FA-free C10 media and incubated at 37°C. Samples were taken at 24- and 48-hours post-replating for flow cytometry staining, including staining for T cell surface markers, the cellular proliferation marker Ki-67, Annexin V, and viability dye. Annexin V can detect apoptotic cells by binding to phosphatidylserine when located on the outer plasma membrane. After gating, non-apoptotic cells are designated as Annexin V and viability dye negative cells. T cells were gated using surface markers CD4+ and CD8+ T cells. **Figure 3** demonstrates representative plots of the gating strategy used to determine proliferative and non-apoptotic cells. The results of the proliferation/survival assay demonstrate that 24 hours after T cells are removed from LA conditions and re-plated in FA-free C10 media, there is still enhanced proliferation displayed by the increased frequency of Ki-67+ non-apoptotic (Annexin V-) CD4+ T cells in the LA groups **(Figure 3B and 3D)**, compared to controls **(Figure 3A and 3C)**. Also seen after 24 hours, post-re-plating is a greater frequency of non-apoptotic CD4+ T cells in both high and low LA concentrations compared to controls **(Figure 4A)**. At 24 hours post-re-plating, there is an increase in proliferation at the higher concentration of LA **(Figure 4C).** At 48 hours post-re-plating, CD4+ T cells in the 100μM LA group also demonstrated enhanced survival **(Figure 4B)** but not proliferation **(Figure 4D)** as compared to BSA controls. The increase in CD4+ T cell proliferation 24 hours after re-plating may be advantageous for autoreactive T cells to increase the number and skew T cell subset ratios, promoting pathogenicity in T1D (266).

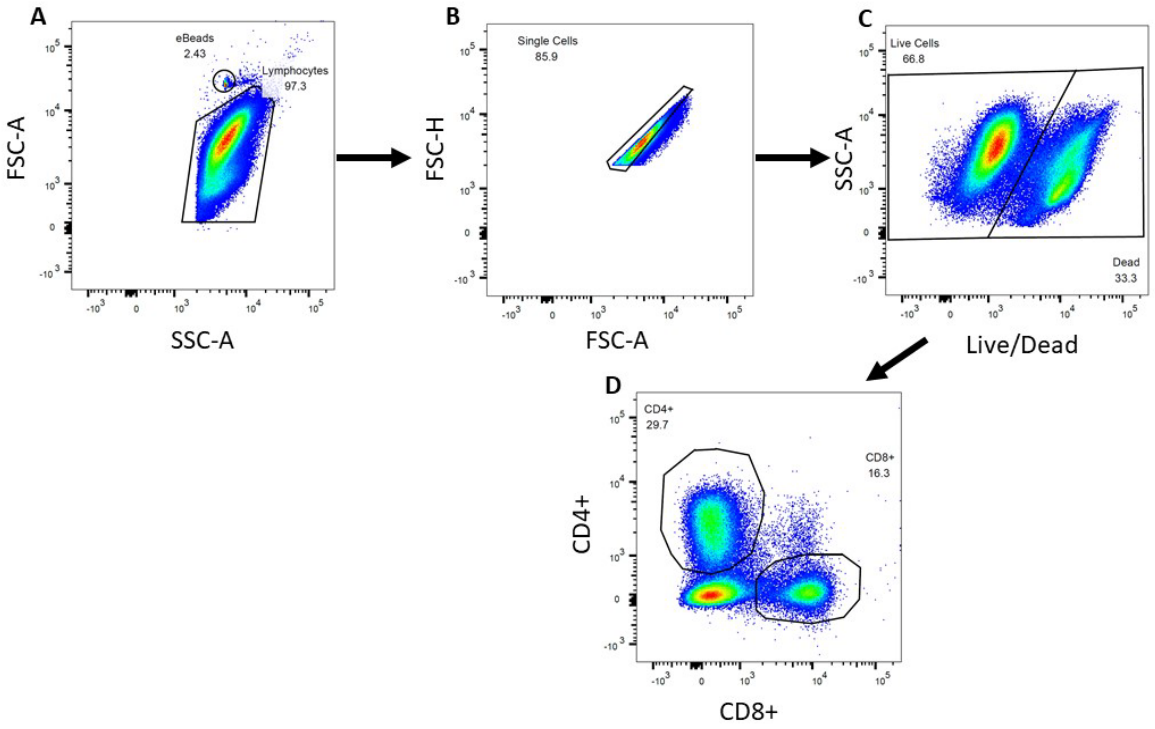

Figure 1. Gating strategy for T cell surface markers and viability.

Flow plots represent the gating strategy utilized to gate **A)** lymphocytes and counting beads (for absolute cell counts), **B)** single cells, **C)** live cells gated by gating viability dye- population, and **D)** CD4+ and CD8+ T cells**.** CD4+ T cells in **(D)** were selected by gating CD4+, CD8- population, and CD8+ T cells in **(D)** were selected by gating the CD8+, CD4- population.

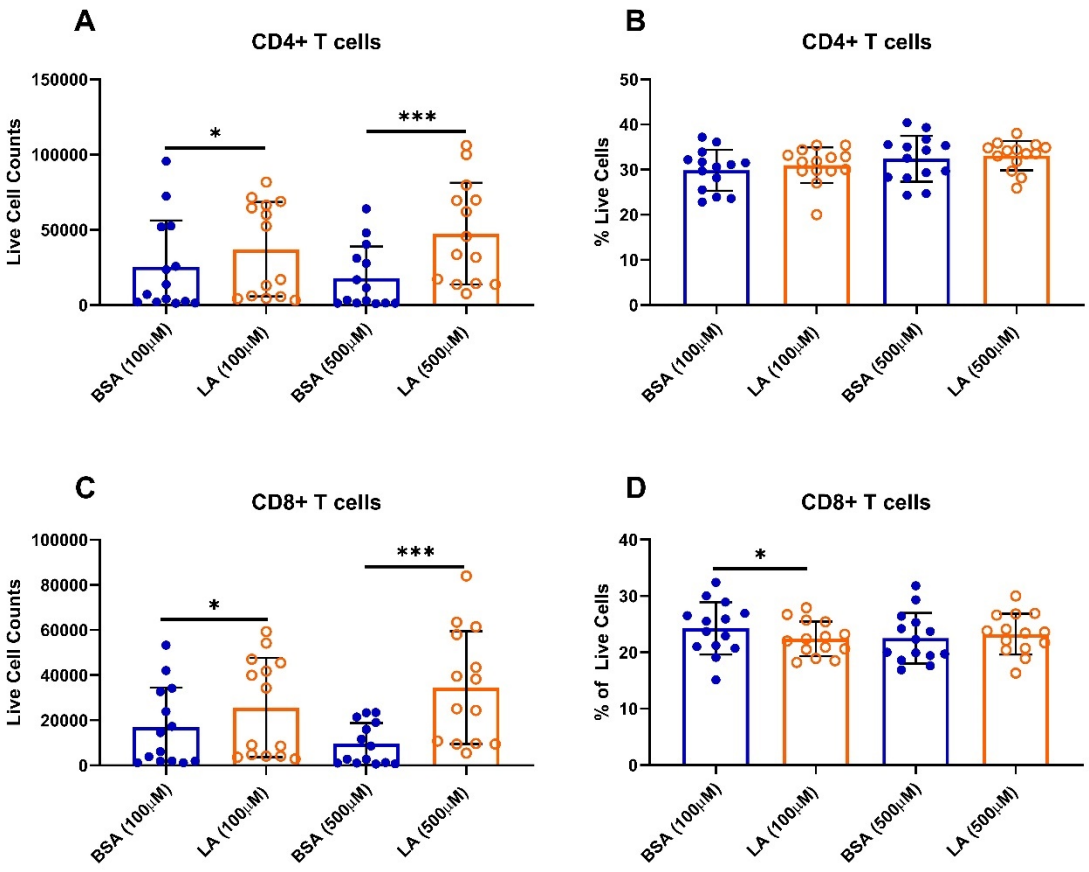

Figure 2. Live T cell counts and frequencies after LA splenocyte culture.

The number of viable **(A)** CD4+ and **(C)** CD8+ T cells after culturing with LA or BSA control. Cell counts were obtained using 123ecount beads and flow cytometric analysis. Graphs show the frequency of live **(B)** CD4+ and **(D)** CD8+ T cells after LA splenocyte culture. Live cells were gated as viability dye negative and T cell marker (CD4 or CD8) positive. Each symbol represents one mouse, n=14, graphs displayed as mean ± SD, paired t-tests were used for statistics ***$p<0.001$ *$p<0.05$.

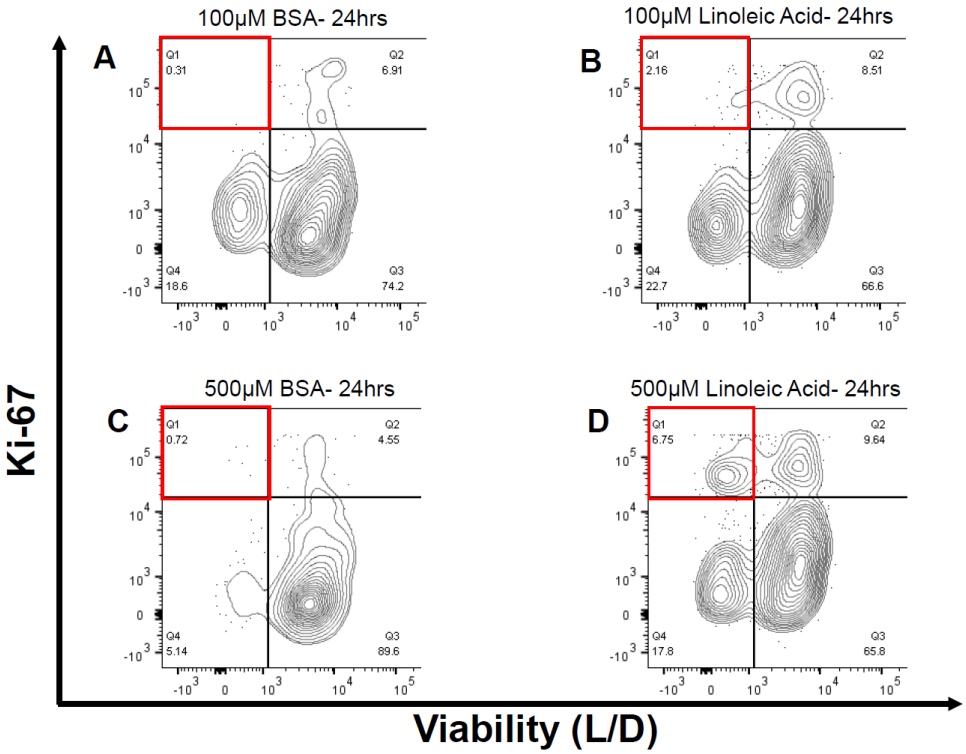

Figure 3. Representative plots showing CD4+ T cell proliferation and survival are enhanced by LA.

Representative flow cytometry plots displaying an increased frequency of proliferation and survival in **(B)** 100µM and **(D)** 500µM LA treated CD4+ T cells 24 hours after re-plating. There is increased cell death and reduced proliferation in BSA-treated CD4+ T cells **(A and C)**. Live and proliferating cells were gated as Ki-67 positive, Viability dye negative, marked in the red quadrant.

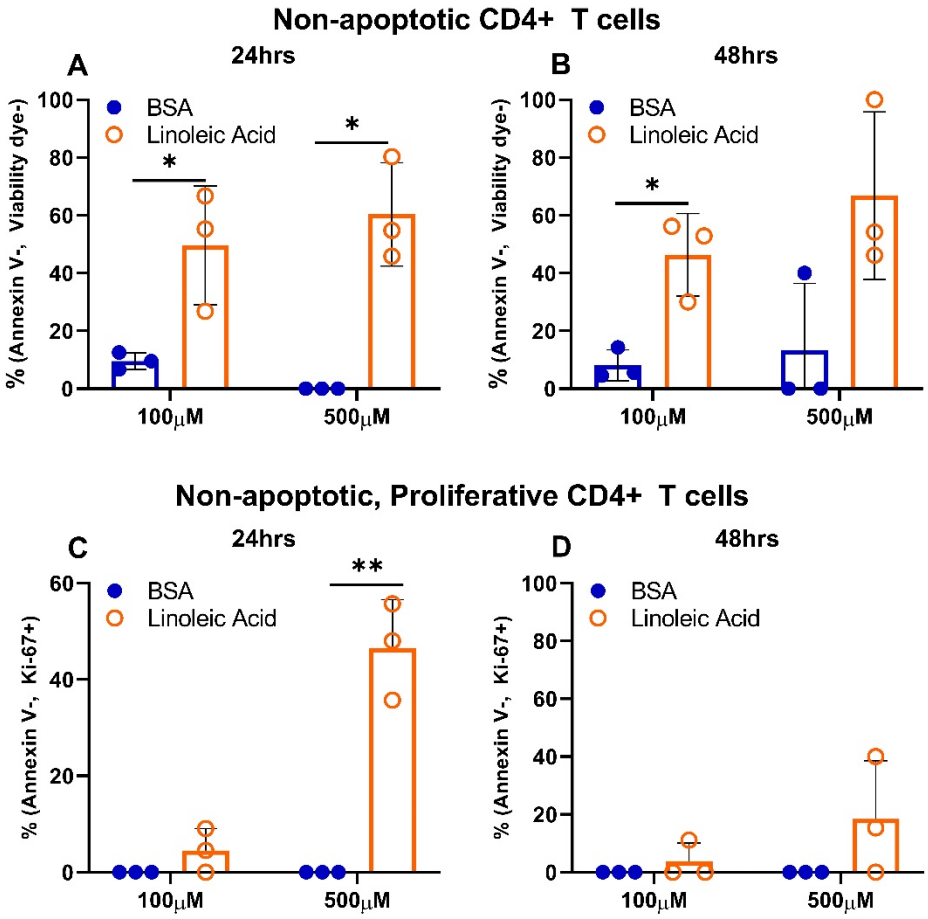

Figure 4. Frequencies of viable and proliferative, non-apoptotic T cells after LA splenocyte culture.

Graphs display an increased frequency of proliferation and survival in CD4+ T cells. T cells were exposed to LA or BSA control for 48 hours, followed by re-plating in fresh media, and samples were taken 24 hours and 48 hours post-re-plating for staining with Annexin V to measure apoptosis and viability dye to measure Live/Dead cells. **A)** Demonstrates that at 24 hours post-re-plating, there are more non-apoptotic CD4+ T cells in the LA-exposed group (high and low concentrations) than BSA controls. **B)** At 48 hours

post-re-plating, the trend stays the same where there are more non-apoptotic CD4+ T cells in the LA exposed group (high and low concentrations) compared to BSA controls. The graph in (**C)** demonstrates that 24 hours post-re-plating LA exposed cells at the high concentration are more proliferative than BSA control, while at 48 hours (D), there are no differences between the groups, and cells are not proliferating. Each symbol represents one mouse, n=3, and paired t-tests were used for statistics, graphs displayed as mean ± SD, **$p<0.002$ *$p<0.05$.

3.2 LA treatment of NOD mouse splenocytes expands IL-21-producing-CD4+ T cells and decreases IL-10-producing CD4+ T cells.

To characterize the phenotype and cytokine production of NOD T cells exposed to LA, NOD mouse splenocytes were cultured with LA at high and low concentrations for 48 hours. After 48 hours, splenocytes were re-stimulated with PMA and ionomycin and intracellular cytokine staining for flow cytometry performed. General gating for viability and surface markers was completed as described in **(Figure 1)**. After cytokine gating demonstrated by the representative plots in **(Figure 5A),** results show an increase in the frequency of CD4+/IL-21+ T cells at both high and low concentrations of LA **(Figure 5B)**. Also seen is a decrease in the frequency of CD4+/IL-10+ T cells **(Figure 5C)** at the higher LA concentration and no differences between groups for CD8+/IL-10+ T cells **(Figure 5E)**. There were no IFN-γ production changes by CD4+ or CD8+ T cells **(Figure 5D and 5F)**. Increases in IL-21 production by CD4+ T cells are of interest due to the role IL-21 has in T1D, where it is found elevated in T1D patient serum and is a requirement for pathogenesis in NOD mice (100,104,131,223). The exposure to LA may be promoting T cells to become more like a Th17 or T follicular helper (Tfh) phenotype which is characterized by the production of IL-21 (126). Both Th17 and Tfh T cell subsets have involvement in T1D disease development (99,128,129). Decreases in IL-10-producing CD4+ T cells by LA are of interest due to the ability of IL-10 to dampen effector T cell responses (132), which is needed to prevent autoimmunity in T1D (119,166). Thus, LA may promote the pathogenicity of CD4+ T cells by expanding IL-21 producers and

reducing IL-10 producers. These changes to anti-inflammatory and inflammatory cytokine ratios can significantly influence the development of diabetes.

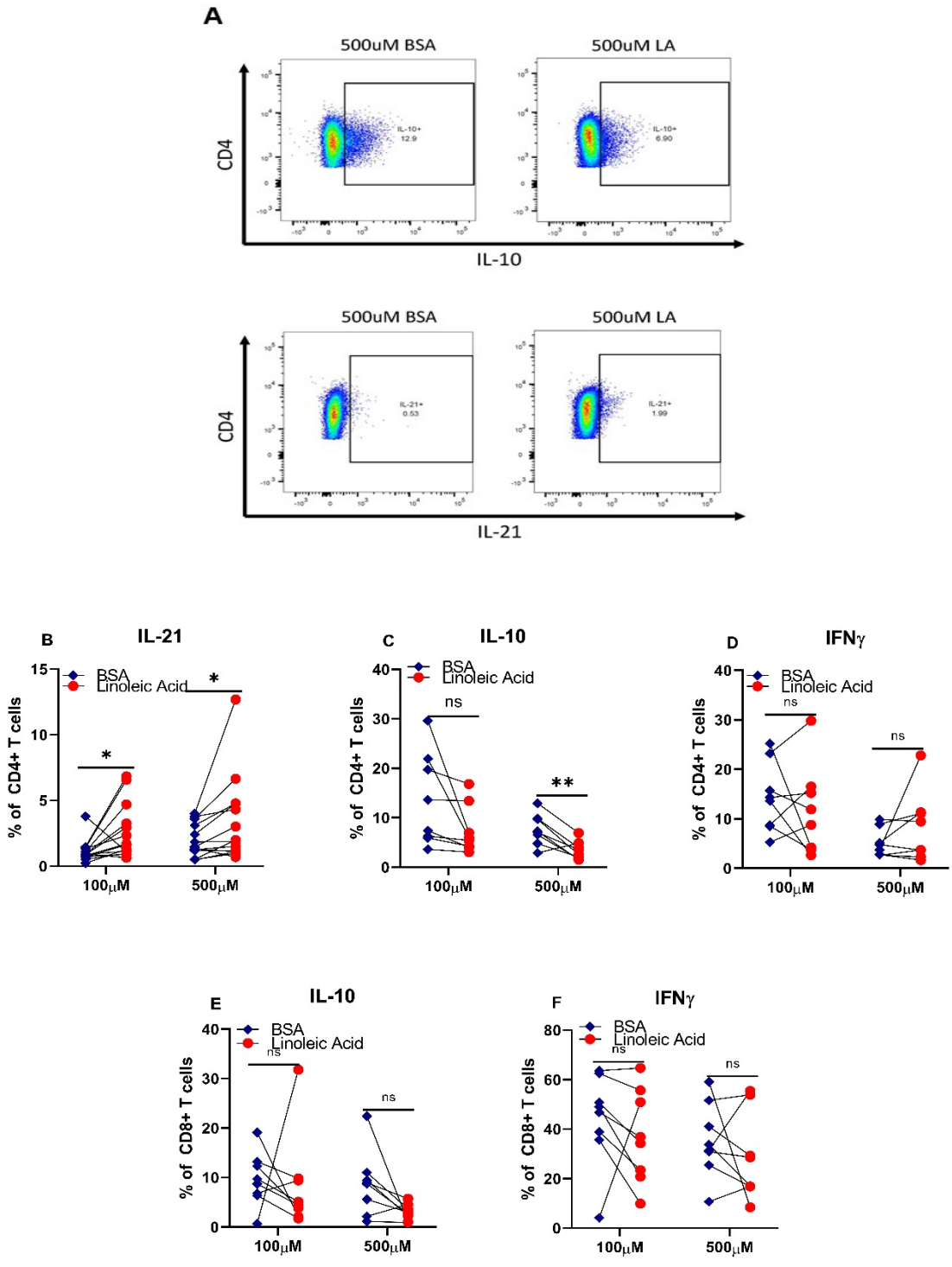
A
500uM BSA
500uM LA
CD4
IL-10
IL-10+
12.9
IL-10+
6.90
500uM BSA
500uM LA
CD4
IL-21
IL-21+
0.53
IL-21+
1.99
B
IL-21
% of CD4+ T cells
BSA
Linoleic Acid
*
*
100μM
500μM
C
IL-10
% of CD4+ T cells
BSA
Linoleic Acid
ns
**
100μM
500μM
D
IFNγ
% of CD4+ T cells
BSA
Linoleic Acid
ns
ns
100μM
500μM
E
IL-10
% of CD8+ T cells
BSA
Linoleic Acid
ns
ns
100μM
500μM
F
IFNγ
% of CD8+ T cells
BSA
Linoleic Acid
ns
ns
100μM
500μM

Figure 5. LA exposure expands IL-21-producing CD4+ T cells and decreases IL-10-producing CD4+ T cells.

Graphs demonstrate the frequency of cytokine production by CD4+ and CD8+ T cells. Splenocyte cultures exposed to LA or BSA controls were re-stimulated after 48 hours of incubation with PMA and Ionomycin for 4 hours for cytokine production. T cells and cytokines were gated according to the gating strategy described and shown in **Figure 1.** After gating for viability and surface markers CD4 and CD8, the representative flow plots in **(A)** display how cytokine gating was completed for each surface marker. Graphs display an increase in IL-21 production by CD4+ T cells **(B)** at both LA concentrations. Also, within the CD4+ T cell population, there is a decrease in IL-10 production **(C)** at the highest LA concentration. There are no differences in IFN-γ production in either condition or concentration **(D)**. There are no differences in CD8+ cytokine production between LA and controls seen in **(E)** for IL-10 production and **(F)** for IFN-γ production. Table 10 demonstrates T cell cytokine production descriptive statistics for the media control. Each symbol represents one mouse, n=8, paired t-tests were used for statistics (comparing LA and BSA) $**p<0.002$ $*p<0.05$.

Table 10: Descriptive Statistics for Media Control T cell Cytokine Frequencies

	Media Control T cell Cytokine Production Frequencies (%)				
	% CD4+: IFNγ+	% CD4+: IL-10+	%CD4+: IL-21+	%CD8+: IFNγ+	%CD8+: IL-10+
Minimum	6.190	1.910	0.1600	26.80	1.360
Maximum	23.20	16.60	2.110	64.00	30.80
Range	17.01	14.69	1.950	37.20	29.44
Mean	11.80	8.884	1.091	46.91	11.84
Standard Deviation	5.797	6.250	0.5851	11.18	11.12
Standard Error of Mean	2.050	2.210	0.1623	3.953	3.933

3.3 The ratio of inflammatory to anti-inflammatory cytokine-producing T cells is altered by LA.

To determine how LA alters the balance of pro- and anti-inflammatory cell types in T1D, frequencies, and counts of cytokine-producing T cell subsets were obtained by flow cytometry from cultures of NOD mouse splenocytes exposed to LA during activation with αCD3 and αCD28 mAbs as described above. The ratios of cell counts were obtained using counting beads, and the ratio of IL-21:IL-10 was calculated for CD4+ T cells and IFN-γ: IL-10 for CD8+ T cells. Since LA promoted the expansion of CD4+ IL-21-producing T cells, we proposed that the ratio of inflammatory to anti-inflammatory cells would be skewed towards a ratio consistent with pathogenicity. LA's low and high doses altered the ratio of IL-21 and IL-10 for CD4+ T cells **(Figure 6A**); the same trend was seen for the cell counts ratio in **(Figure 6B)**. The data of altered cell ratios suggests that survival and proliferation of IL-21-producing cells are preferentially promoted by LA relative to IL-10-producing T cells. Graphs display that LA exposed CD8+ T cell percentage ratios **(Figure 6C)** for IFN-γ and IL-10 are only increased at the higher dose of LA **.** The CD8+ T cell count ratios for IFN-γ and IL-10 **(Figure 6D)** are trending towards the same skewed composition at the higher dose of LA which is considered pathogenic. The increased ratios of pathogenic CD4+ and CD8+ T cells demonstrate LA's ability to influence the composition of T cells within an environment. In the context of T1D, these specific changes to T cell ratios are considered pathogenic mainly due to the changes in IL-10, which are vital for maintaining tolerance (119,166).

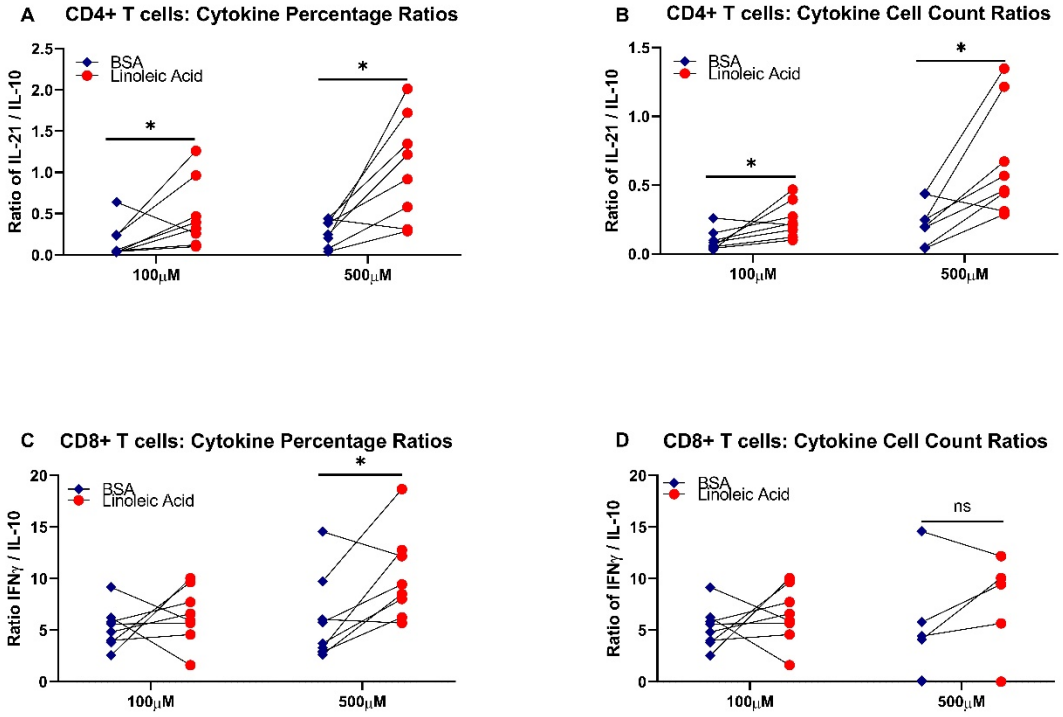

Figure 6. LA exposure alters the ratio of pro- and anti-inflammatory cytokines in CD4+ and CD8+ T cells.

Graphs display the ratios of cell frequencies and cell counts of cytokine-producing CD4+ and CD8+ T cells. Splenocyte cultures exposed to LA or BSA control were re-stimulated after 48 hours of incubation with PMA and Ionomycin for 4 hours for cytokine production. T cells expressing cytokines were gated according to the gating strategy describes in **Figure 1**. The ratios of cell counts were obtained using counting beads. The ratio of IL-21 and IL-10 producing CD4+ T cells was calculated, and IFN-γ and IL-10 for CD8+ T cells. The ratios were also calculated for the percentages of cytokine positive CD4+ and CD8+ T cells. Graphs demonstrate low and high LA doses altered the ratio of percentages of IL-21 and IL-10 for CD4+ T cells **(A)** and cell counts as seen in **(B).** For

LA-exposed CD8+ T cells, percentage **(C)** and cell count ratios **(D)** show that cells are trending towards pathogenic cytokine production at only the higher dose of LA. Each symbol represents one mouse, n=8, and paired t-tests were used for statistics. *$p<0.05$.

3.4 Glucose-free conditions have no impact on pathogenic cytokine production by LA-treated T cells.

T cell activation involves a metabolic switch to glycolysis, followed by glycolysis coupled with OXPHOS (189,267,268). The requirement for glucose is necessary if LA is being stored in lipid droplets as triacylglycerol, which requires a glycerol backbone produced from glucose (269,270). The glycolytic pathway is essential in T cell processes since this pathway is important early on in T cells' activation through CD3 and CD28 (185).

To understand the influence of glycolysis on IL-21 production by LA-treated T cells, splenocytes were cultured 48 hours with LA or BSA conjugated in glucose-free C10 media at high and low concentrations for 48 hours. After 48 hours, splenocytes were re-stimulated and stained for phenotype and cytokines and analyzed by flow cytometry. Results demonstrate that glycolysis is required for survival and production of IL-21 in LA-treated CD4+ T cells and controls. In glucose-free conditions, there were no changes in the number of live CD4+ T cells **(Figure 7A)** when compared to media and BSA controls**.** These data suggest that the enhanced survival seen in LA treatment requires the presence of glucose. There were also no changes in the number of IL-21-producing CD4+T cells **(Figure 7B)** in glucose-free conditions compared to controls. In glucose-

free and LA conditions, there are no changes in the number of IL-10 producing T cells **(Figure 7C)**, which is the opposite in conditions with normal glucose levels and where LA treatment reduces the number of IL-10 producing T cells **(Figure 5B)**.

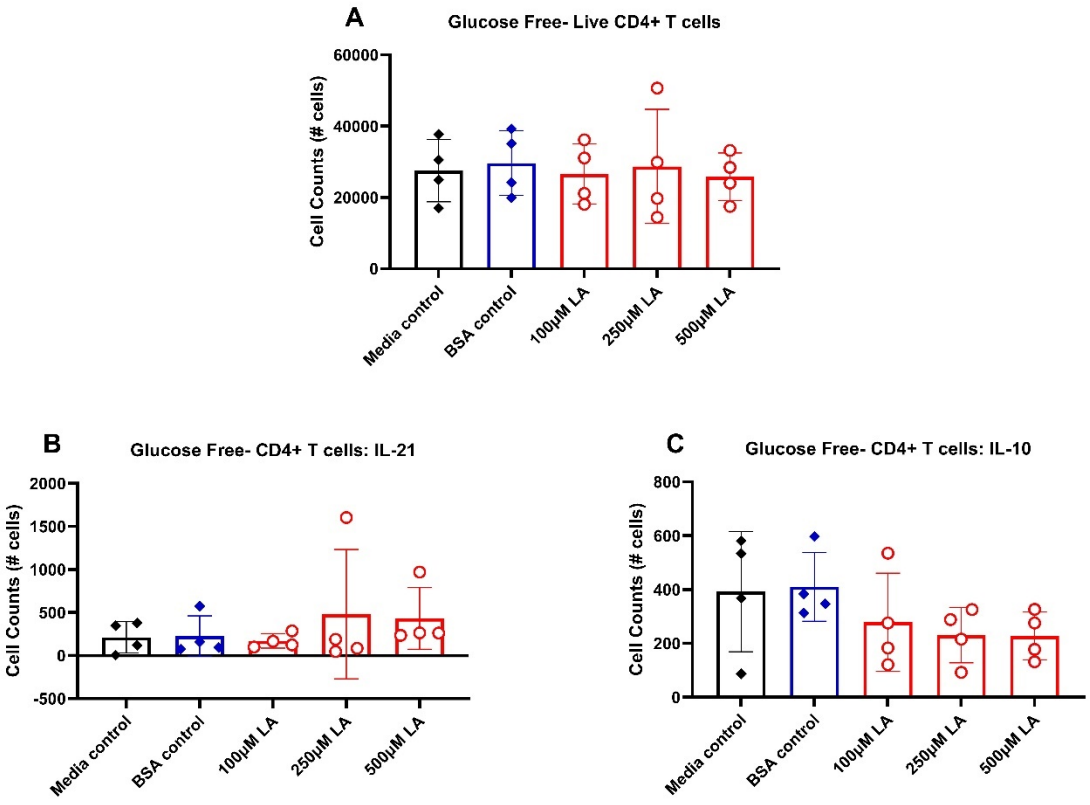

Figure 7. In glucose-free conditions, LA exposure does not alter CD4+ T cell cytokine production or viability.

Cell counts of viability and cytokine production by CD4+. Splenocyte cultures exposed to LA or controls in glucose-free conditions were re-stimulated after 48 hours of incubation with PMA and Ionomycin for 4 hours for cytokine production. Viable T cells

and cytokines were gated according to the gating strategy describes in **Figure 1 and Figure 5A.** Graphs display no differences in CD4+ T cell viability **(A)** between LA and BSA control-treated T cells. Also, there are no differences in CD4+ T cell production of IL-21 **(B)** and IL-10 **(C)** between LA-treated T cells and controls. Each symbol represents one mouse, n=4, graphs displayed as mean ± SD, Friedman test (non-parametric ANOVA) combined with Dunn's Multiple Comparisons Test were used for statistics.

3.5 Pathogenic cytokine production and viability of T cells in LA conditions require the presence of antigen-presenting cells.

3.5.1 Co-culture of BMDMs and BMDCs with T cells in LA conditions.

To determine if specific LA-treated APCs influence survival and cytokine production in T cells, BMDMs and BMDCs were generated using methods previously described (250,251). BMDMs and BMDCs were matured with LPS, and after incubation, adhered BMDMs were isolated from the cell culture dish. BMDCs were enriched using a dendritic cell enrichment kit as described in **Chapter 2** methods. BMDMs or BMDCs were co-cultured with isolated T cells at a 2:1 cell ratio (T cell: APC) in LA and control conditions. Results reveal that DCs co-cultured with T cells in LA conditions have a greater number of viable CD8+ T cells at high and low concentrations **(Figure 8E)** and viable CD4+ at high LA concentrations **(Figure 8A)** Co-cultures in LA conditions with macrophages did not display the same trend as DCs. Macrophages did not contribute to

more viable CD4+ **(Figure 8B)** or CD8+ T cells **(Figure 8E)** or differences in cytokine production between the LA and control groups **(data not shown)**. Also, culturing T cells alone with LA does not promote viability **(Figures 8C and 8G)**. These results demonstrate the requirement of DC contact with T cells or DC cytokine production in LA conditions that promotes viable T cells but does not impact T cell cytokine ratios. These data suggest that the altering of IL-10 and IL-21 cytokine levels in T cells in LA conditions could be a contribution to the influence of multiple APCs.

3.5.2 Co-culture of LA pre-treated APCs with T cells.

To determine whether changes in APCs caused by LA were sufficient to promote T cell survival and altered cytokine subset composition or whether both T cells and APCs required LA exposure, freshly isolated splenocytes were immunostained with an αCD3 fluorophore-conjugated antibody, and splenocytes were sorted via FACS keeping only cells that were CD3 negative (APCs) and disposing of CD3 positive cells (T cells). The FACS isolated APCs were cultured in fatty acid-free C10 media, BSA, and LA conditions at high and low doses (100μM and 500μM) for 24 hours. After 24 hours of APC pre-treatment with fatty acids, APCs were re-plated at a 3:1 ratio (APC: T cell) with enriched T cells from NOD mice, and the cells were co-cultured for 48 hours. The second set of APCs not exposed to LA were cultured with enriched T cells at a 3:1 ratio (APC: T cell), and LA (100μM and 500μM) added in at the time of co-culture. After 48 hours, cells were immunostained for viability, T cell phenotype, and cytokines. Results demonstrate no differences in viability **(Figure 9A and 9E)** and cytokine production in T

cells co-cultured along with APCs that were pre-treated with LA when compared to the media control. LA pre-treated APCs did not lead to differences in cytokine production by CD4+ **(Figures 9B-9D)** and CD8+ T cells **(Figures 9F and 9G).** These results suggest that LA directly affects T cells to influence T cells' viability and the altering of cytokines. A caveat in experimental design is that there could have been early cytokine production by LA pre-treated APCs that may have a role in the survival or cytokine production of T cells.

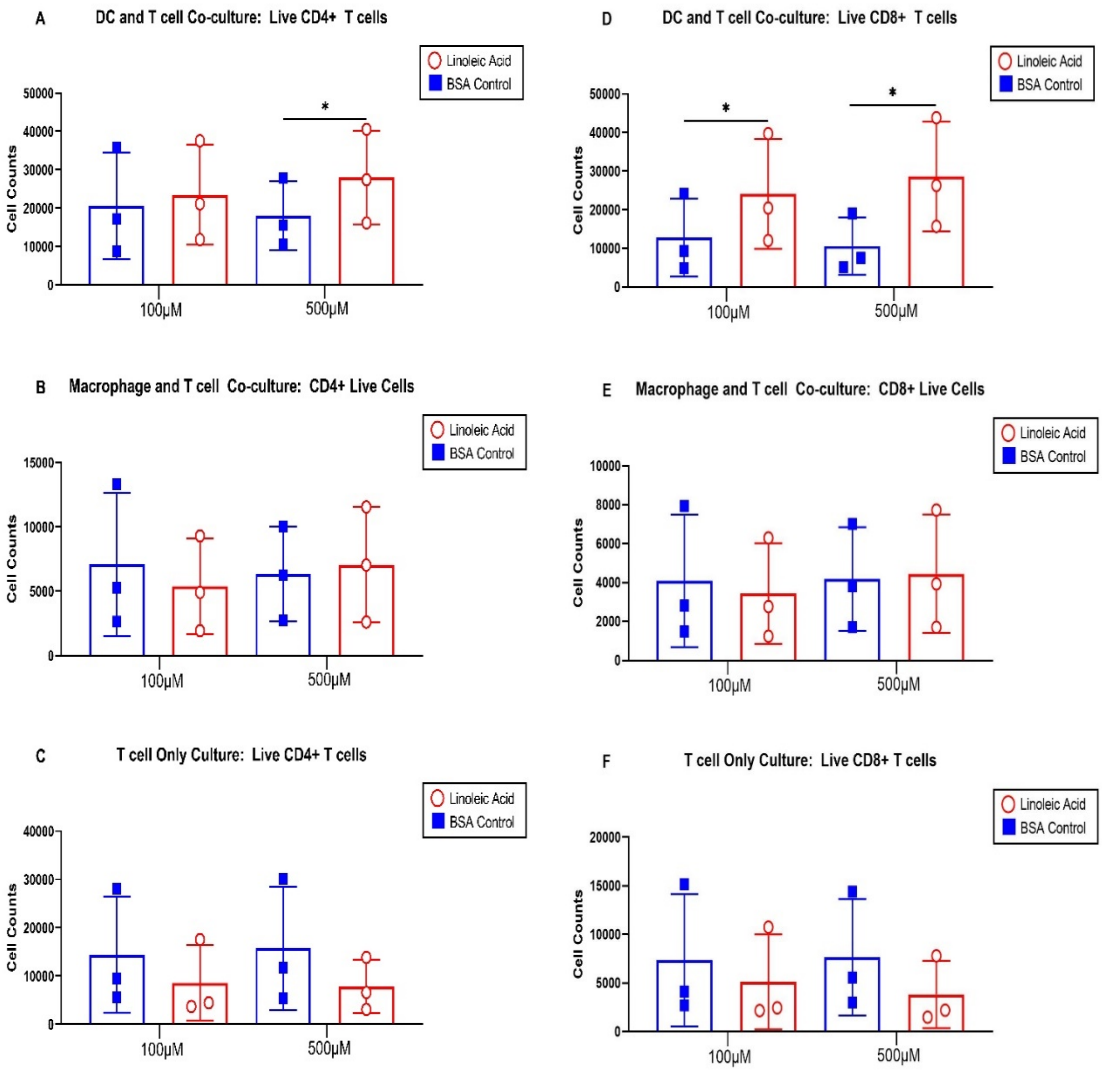

Figure 8. DCs exposed to LA enhance CD4+ and CD8+ T cell viability.

BMDMs and BMDCs were generated using methods previously described (250,251). BMDMs and BMDCs were matured with LPS, and after incubation, adhered BMDMs were isolated from the cell culture dish. BMDC populations were enriched using a dendritic cell enrichment kit as described in **Chapter 2** methods. BMDMs or BMDCs were co-cultured with T cells isolated by magnetic sorting at a 2:1 cell ratio (T cell: APC)

in LA and control conditions. Graphs reveal that DCs increase cell survival of CD4+ T cells but only at the high LA concentration **(A).** In the macrophage and T cell co-culture, there are no differences in viability between LA and BSA control **(B)**. Also, LA does not enhance the survival of T cells plated alone in culture **(C)**. At both high and low concentrations of LA, DCs enhanced the viability of CD8+ T cells **(D)**. In macrophage and DC co-cultures, no differences were demonstrated in the viability of CD8+ T cells **(E)**. The same trend is seen in CD8+ T cell-only cultures **(F),** where LA alone does not enhance viability. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, t-tests were used for statistics. *$p<0.05$.

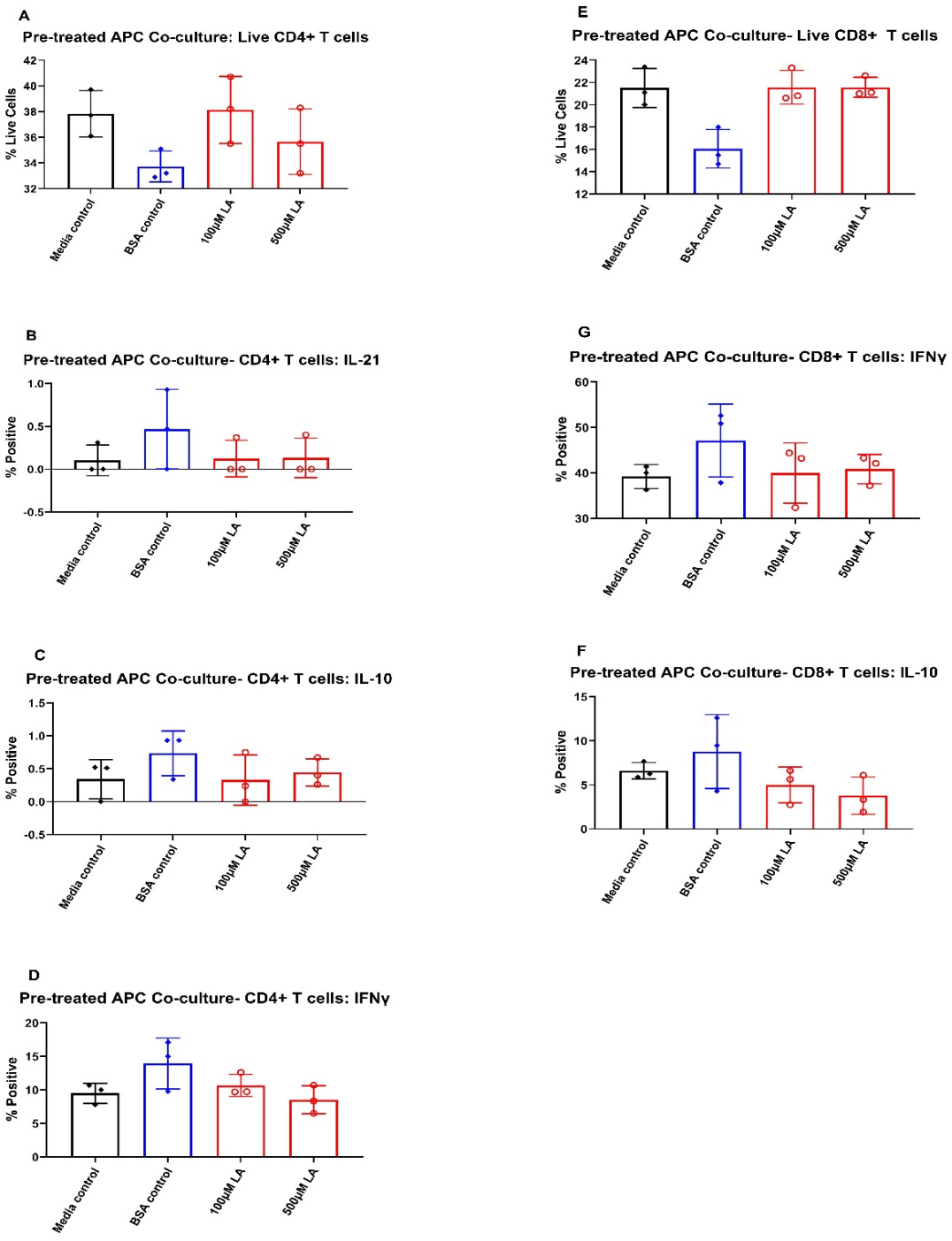

Figure 9. Pre-treatment of APCs with LA does not impact T cell cytokine production and T cell viability.

Isolated splenocytes were immunostained with an αCD3 fluorophore-conjugated antibody, and splenocytes were sorted via FACS keeping only cells that were CD3 negative (APCs) and disposing of CD3 positive cells (T cells). The FACS isolated APCs were cultured in fatty acid-free C10 media, BSA, and LA conditions at high and low doses (100μM and 500μM) for 24 hours. After 24 hours of APC pre-treatment with fatty acids, APCs were re-plated at a 3:1 ratio (APC: T cell) with enriched T cells from NOD mice, and the APCs were co-cultured for 48 hours with αCD3 and αCD28. After 48 hours, cells were immunostained for viability, T cell phenotype, and cytokines. Graphs display no differences in the number of CD4+ **(A)** and CD8+ **(E)** viable T cells. For CD4+ T cells co-cultured with LA pre-treated APCs, there are no differences in the production of IL-21 **(B)**, IL-10 **(C)**, and IFN-γ **(D)**. Within CD8+ T cells co-cultured with LA pre-treated APCs, there are no differences in IFN-γ (F) production and IL-10 **(G)**. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, Friedman test (non-parametric ANOVA) with Dunn's Multiple Comparisons Test were used for statistics,

3.6 LA-treated T cells do not have a Tfh or Th17 phenotype.

To verify that LA can skew T cells to a Tfh or Th17 phenotype, which typically produce IL-21 (126), splenocytes cultured 48 hours with LA or BSA were stained with T cell markers and transcription factors for these specific T cell subsets (Bcl-6, RORγt) (126) based on the panel in **Table 6**. Results demonstrate no significant changes to the mean fluorescence intensity of Bcl-6 or RORγt in LA-treated CD4+ T cells compared to controls (media and BSA). These data demonstrate that LA does not skew T cells to specific subsets known for IL-21 production (Tfh, Th17) **(Figures 10A and 10B)**. In a separate experiment where CD4+ T cells were polarized to Th17 with and without LA, there was a reduction of RORγt expression in T cells treated with 1mM LA **(Figure 11),** indicating that LA conditions skew cells away from Th17 phenotype in TH17 polarizing conditions. These data again confirm that Tfh and Th17 cell lineages are not involved in the production of IL-21 in LA conditions.

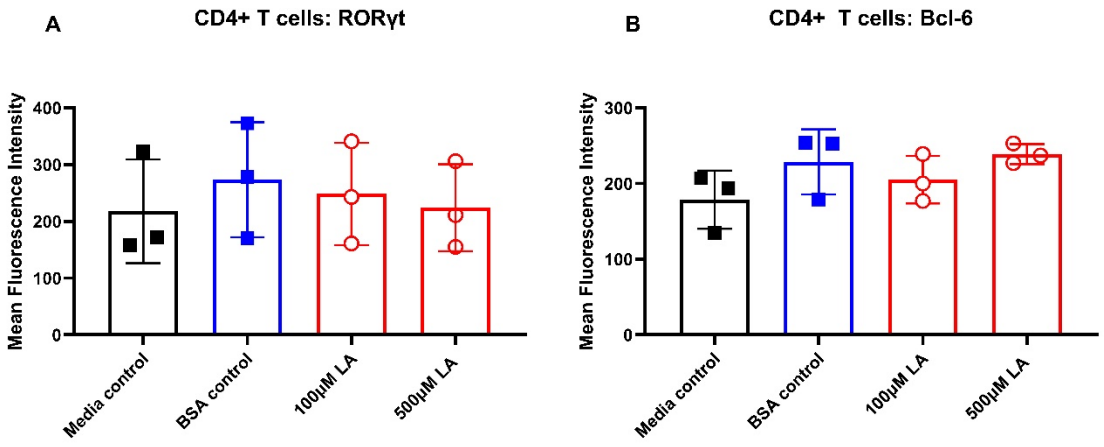

Figure 10. LA does not skew CD4+ T cell differentiation to Tfh or Th17 subsets. Splenocyte cultures exposed to LA or controls were re-stimulated after 48 hours of incubation with PMA and Ionomycin for 4 hours for cytokine production, cells were immunostained using the panel in **Table 6** using Tfh and Th17 lineage markers including transcription factors RORγt and Bcl-6 (126). Graphs display that LA has no influence on CD4+ T cell differentiation into Th17 cell subsets **(A)** as displayed by the MFI of RORγt, and LA did not promote differentiation of CD4+ T cells to a Tfh cell phenotype as displayed by the MFI of Bcl-6 **(B)**. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, one-way ANOVA with Tukey's Multiple Comparisons Test was used for statistics.

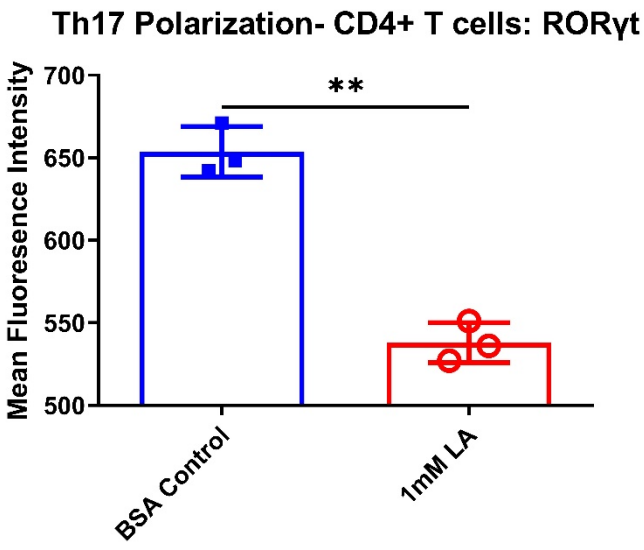

Figure 11. Th17 polarized cells exposed to LA have a reduction in the expression of lineage transcription factor RORγt.

Naïve CD4+ T cells were isolated and plated with αCD28, Th17-polarizing cytokines, and blocking antibodies using methods previously described (249). Th17-polarizing cytokines and blocking antibodies include αCD3, αCD28, IL-6 cytokine, IL-23 cytokine, human TGF-β, αIL-4, and anti-IFN-γ. CD4+ T cells in polarized conditions were incubated with LA or controls for 4 days, and afterward, T cells were re-stimulated with PMA and Ionomycin for 4 hours with BFA added an hour in and immunostained using the panel in **Table 9**. The graph above reveals that Th17-polarized cells cultured in LA have reduced expression of the Th17 lineage transcription RORγt indicating that LA does not skew cells to the Th17 subset but skews them away. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, t-test was used for statistics. **p<0.002

3.7 Accumulation of lipid droplets and changes to mitochondrial mass in T cells is promoted by LA exposure.

FAs can be used in many ways by the cell. Therefore, to understand if T cells are storing LA as TAGs for use in energy production, later on, the accumulation of lipids in T cells was measured by staining lipid droplets. Splenocytes cultured 48 hours with LA or controls were stained with Bodipy 493/503 and conjugated antibodies for T cell surface markers (CD4, CD8) and analyzed by flow cytometry. In CD4+ T cells **(Figure 12A),** there is an accumulation of lipids only at the higher concentration of LA (500μM). Results **(Figure 12B)** reveal an accumulation of lipids seen at 100μM and 500μM doses of LA in CD8+ T cells. These results suggest that CD8+ T cells store LA as neutral/non-polar lipids in lipid droplets (269), which is known to benefit memory CD8+ T cells that depend on fatty acid oxidation (271).

To measure if T cell mitochondria are undergoing biogenesis after LA treatment, which indicates FAO and OXPHOS, T cells were stained with Mitotracker dye which accumulates in active mitochondria and allows the quantification of mitochondrial mass, which is an indicator of mitochondrial biogenesis and mitochondrial content (173). Cells were also immunostained for surface markers CD4 and CD8. There are no differences in mitochondrial mass between LA and controls in CD4+ cells **(Figure 12C).** In contrast, CD8+ T cells **(Figure 12D)** exhibited an increase in mitochondrial mass at the high concentration of LA. There were no changes to mitochondrial mass in CD4+ T cells as measured by Mitotracker MFI. This data indicates that CD4+ T cells are not undergoing

mitochondrial biogenesis due to LA exposure. These results could indicate that LA is not being used as an energy source for FAO.

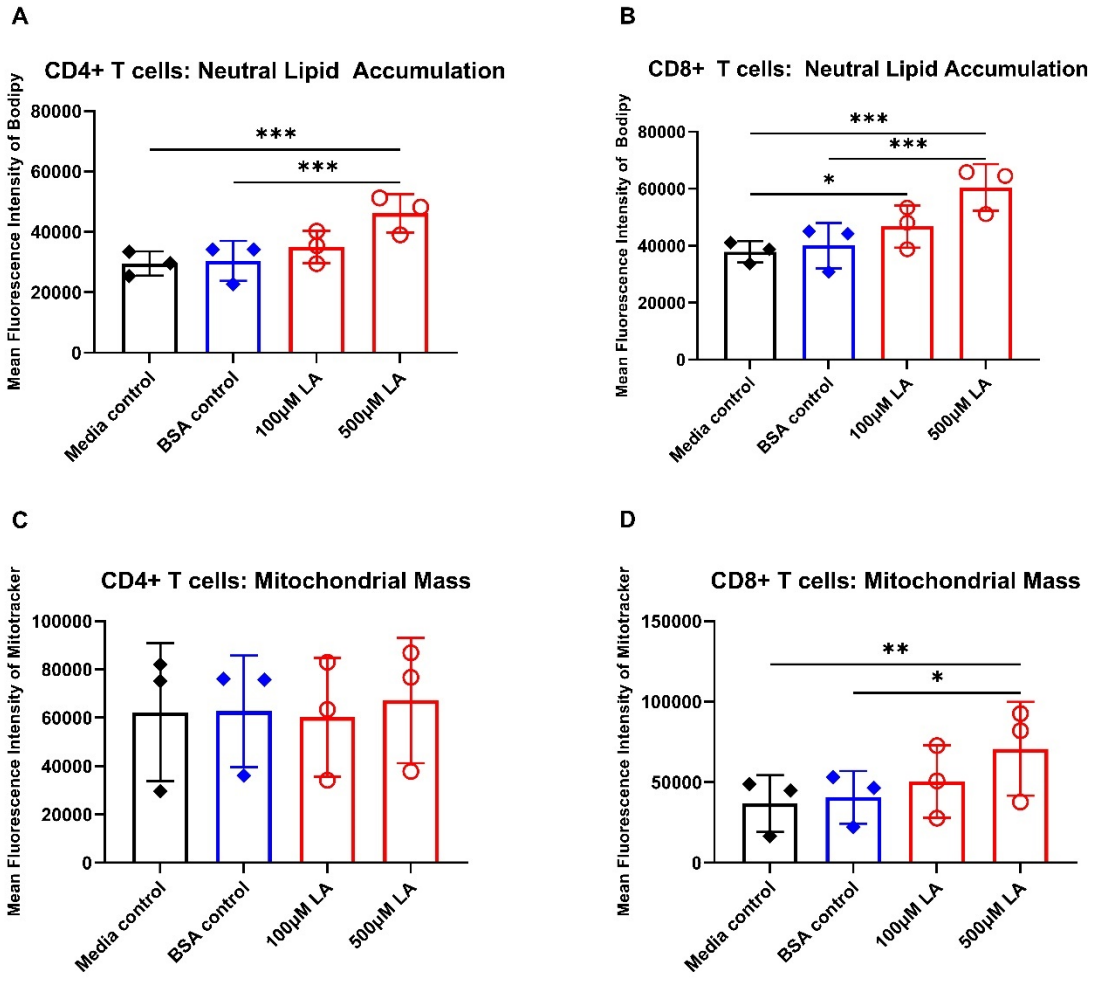

Figure 12. T cells exposed to LA have increased neutral lipid accumulation.

To understand how LA exposure affects lipid droplet accumulation and mitochondrial biogenesis, splenocytes cultured 48 hours with LA or controls were stained with Bodipy 493/503 and Mitotracker Green FM dye along with conjugated antibodies for T cell surface markers (CD4, CD8) and analyzed by flow cytometry using the panels in **Tables**

4 and 5. Graphs display that increased lipid accumulation is only seen in CD4+ T cells at the high concentration of LA **(A)** while there is increased neutral lipid accumulation in CD8+ T cells at both concentrations of LA **(B),** indicating that there are T cell-specific differences in how LA is handled by CD4+ and CD8+ T cells. When looking at mitochondrial mass as indicated by Mitotracker staining, it is revealed that CD4+ T cells in LA conditions do not increase the mitochondrial mass **(C)**, while CD8+ T cells display an increase in mitochondrial mass at the high concentration of LA **(D)**. CD8+ T cells are undergoing mitochondrial biogenesis in response to higher concentrations of LA and could be utilizing LA differently than CD4+ T cells. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, one-way ANOVA with Tukey's Multiple Comparisons Test was used for statistics, ***$p<0.001$ **$p<0.002$ *$p<0.05$.

3.8 LA shows specific and unique effects on T cell cytokine production compared to other FAs.

To quantify and determine cytokines exclusively produced by T cells in LA conditions, splenocytes were plated with 500μM of FAs, including LA, oleic acid, and α-linolenic acid, for 48 hours. After 48 hours, T cells were isolated from the splenocytes using a magnetic T cell isolation kit, and 1 x 10^5 cells re-plated in fresh media and re-stimulated using PMA and Ionomycin for 4 hours. After 4 hours, T cell supernatants were harvested and analyzed by multiplex ELISA for cytokines. It was revealed that LA treatment compared to controls and other FAs specifically led to increased levels of several cytokines and chemokines, including GM-CSF, IFN-γ, MIP-3α, IL-2, and IL-28B

(Figure 13). The increase in GM-CSF by LA **(Figure 13A)** is of interest since GM-CSF specifically plays a role in the pathogenesis of several autoimmune and chronic inflammatory diseases (272). GM-CSF-producing autoreactive CD4+ T cells have been found in T1D and are thought to be important in response to β-cell antigens (273). IFN-γ and IL-2 are critical cytokines in the pathogenesis of T1D and are shown to be increased by LA **(Figure 13B and 13E).** MIP-3α, also increased by LA (**Figure 13C),** is an important chemokine for attracting monocytes and memory lymphocytes to inflammation in autoimmune disorders like rheumatoid arthritis and other chronic inflammatory diseases (274). There are also increased levels of IL-28B in T cells treated with LA compared to other PUFAs **(Figure 13H),** IL-28B is a type III interferon that has a role in inflammatory processes and has been implicated in autoimmunity and cancer (275). There is a trend of decreased IL-10 production induced by oleic acid, linoleic acid, and α-linolenic acid **(Figure 13F)**. When looking at IL-21 production **(Figure 13G),** the trend of increased IL-21 by LA seen in intracellular cytokine staining was not seen in the supernatants analyzed by multiplex ELISA. This could be due to the normalization of the number of T cells per condition for re-stimulation in the multiplex ELISA. Also, the IL-21 measured is below the stated limit of detection, which could give rise to unreliable results **(Figure 13),** whereas flow cytometry data revealed more significant numbers of IL-21-producing T cells compared to other FAs and controls **(data not shown)**. There were no differences in the production of these specific cytokines in LA conditions compared to controls: IL-1β, IL-4, IL-6, IL-12, IL-13, IL-15, IL-17A, IL-17F, IL-23, IL-17, IL-31, IL-33, CD40L, and TNFβ **(data not shown)**. The multiplex ELISA data

suggests that LA impacts T cell cytokine production and promotes cytokine-based interactions with APCs.

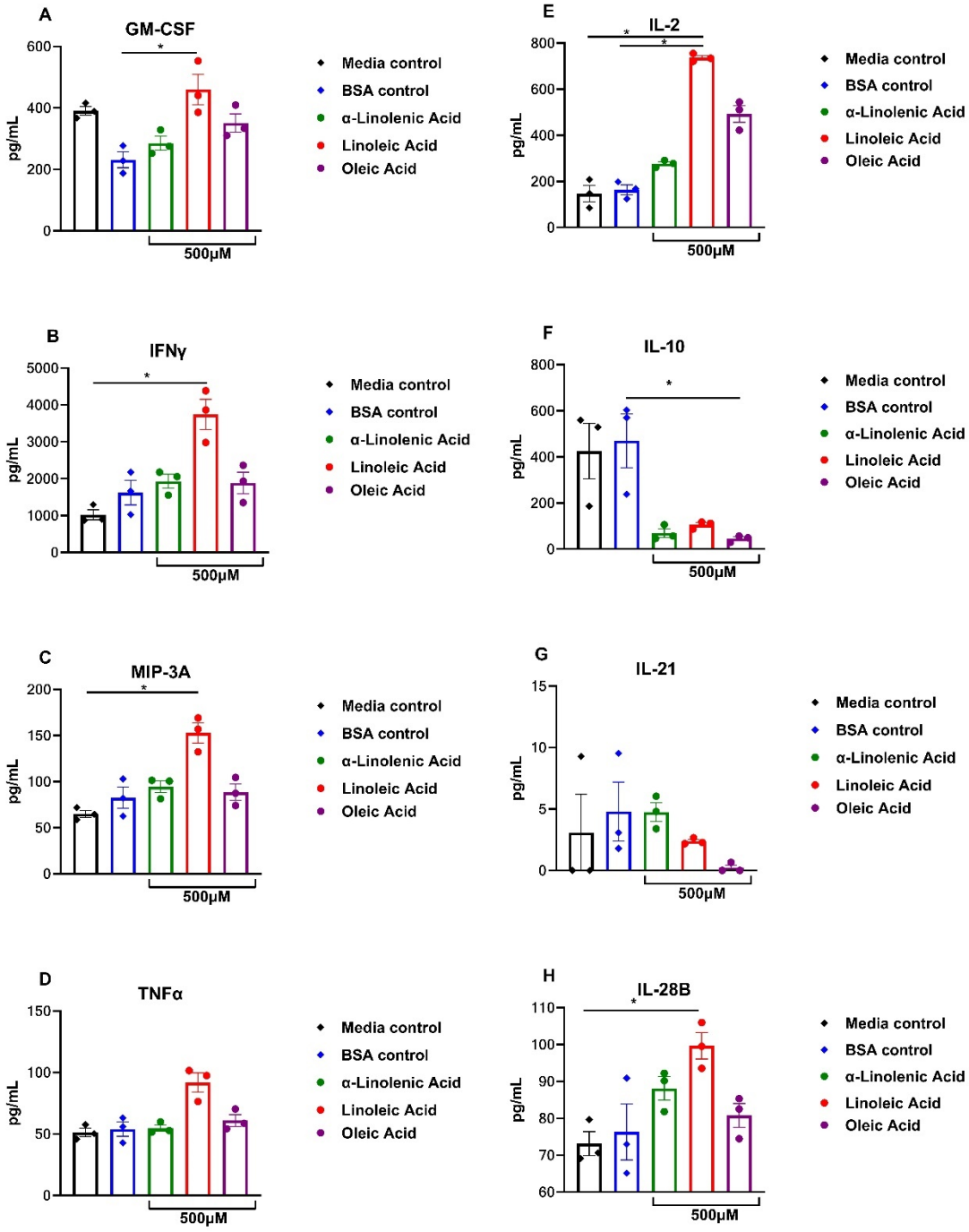

Figure 13. T cells in the cultured presence of LA have increased production of specific cytokines compared to other FAs and controls.

To understand how LA impacts cytokine production compared to other FAs, splenocytes were plated and activated in the presence of 500uM of FAs, including LA, oleic acid, α-linolenic acid, and controls for 48 hours. After 48 hours, T cells were isolated from splenocytes, re-plated in media, and re-stimulated using PMA and Ionomycin for 4 hours. After 4 hours, T cell supernatants were harvested and analyzed by multiplex ELISA for cytokines. Graphs reveal that LA led to significantly increased levels of GM-CSF **(A)**, IFN-γ **(B),** and MIP-3α **(C)**. While not significant, there is a suggested increase in levels of TNFα by LA **(D)**. The presence of LA increased the levels of IL-2 **(E),** which is essential for T cell proliferation (224). There is a trend that LA, oleic acid, and α-linolenic acid can suppress the production of IL-10 **(F)**. The production of IL-21 **(G)** is not enhanced by LA, but there is evidence that cell numbers may come into play since T cells were normalized to the same cell number before re-plating for PMA and ionomycin stimulation. Also seen is an increase in IL-28B **(H)** by LA compared to controls and other FAs. Each symbol represents pooled replicates of one mouse, n=3, graphs displayed as mean ± SD, Kruskal-Wallis test (non-parametric ANOVA) with Dunn's Multiple Comparisons Test were used for statistics, *$p<0.05$.

3.9 LA does not enhance IL-21 production in CD4+ T cells from a non-autoimmune mouse model.

To verify that the survival and IL-21 production influenced by LA treatment on NOD mouse T cells is specific to NOD mice, and therefore, T1D, C57BL/6J, and NOD mouse splenocytes were cultured with LA for 48 hours. After 48 hours, cells were re-stimulated and immunostained for viability, phenotype, and cytokine production. Results reveal an increase in IL-21 specific to NOD mouse CD4+ T cells at high and low LA concentrations compared to C57BL/6J mouse media only control **(Figure 14B).** Also revealed was that there are differences in the survival of splenocytes at lower concentrations of LA between NOD and C57BL/6J mice **(Figure 14A),** where NOD mouse cells have a greater percentage of viable CD4+ T cells**.** At concentrations greater than 100μM **(Figure 14A),** the differences in survival between T cells from the two mouse strains disappear, with higher concentrations of LA promoting comparable survival in C57BL/6J and NOD CD4+ T cells. These results indicate that while the LA promotion of viability in CD4+ T cells is not exclusive to NOD mice, the production of IL-21 in LA conditions is specific to NOD mouse CD4+ T cells.

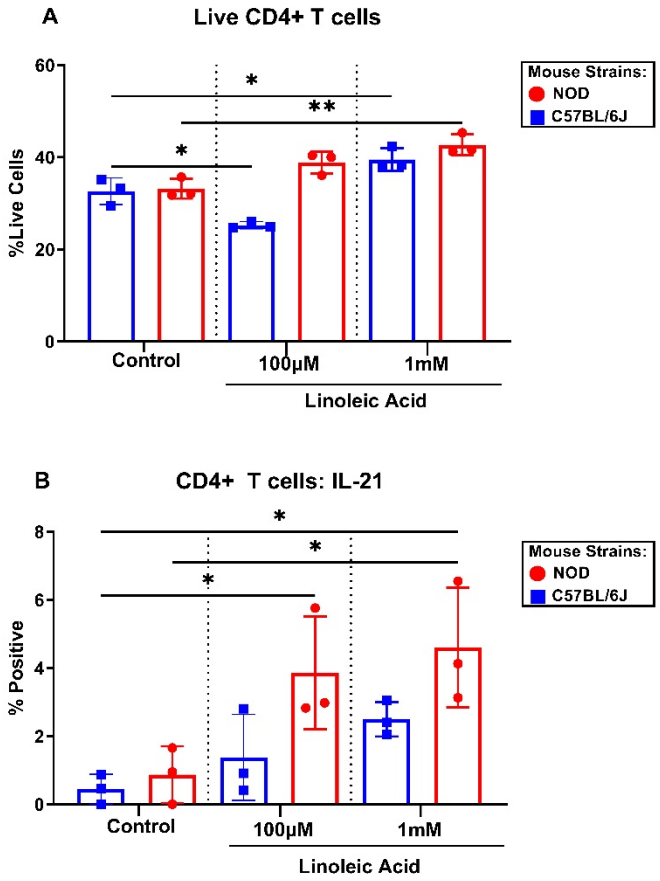

Figure 14. LA-induced IL-21 production by CD4+ T cells is limited to NOD mouse T cells.

C57BL/6J and NOD mouse splenocytes were cultured with LA or media control for 48 hours. After 48 hours, T cells were re-stimulated and immunostained for viability, phenotype, and cytokine production using the flow panel in **Table 3.** Graphs show that LA exposure enhances viability in CD4+ NOD mouse T cells at high and low concentrations while only enhancing viability in C57BL/6J mice at the higher concentration **(A)**. LAs' effects on viability are not specific to NOD mouse CD4+ T cells. There is increased IL-21 production induced by both LA concentrations in NOD CD4+ T cells but not in C57BL/6J mouse CD4+ T cells **(B)**. LA's effects on IL-21 production are

specific to NOD mice, and LA cannot increase IL-21 production in a non-autoimmune mouse model. Each symbol represents splenocytes from one mouse, n=3, graphs displayed as mean ± SD, one-way ANOVA test with Tukey's Multiple Comparisons Test were used for statistics, **$p<0.002$ *$p<0.05$.

CHAPTER FOUR: LINOLEIC ACID REDUCES THE EXPRESSION OF LIPID METABOLISM-REGULATING GENES IN T CELLS

Rationale

LA, an n-6 PUFA, can have one of many fates once inside of the cell. The fatty acid can be used for energetic processes, create lipid signaling molecules, be stored as triacylglycerols (TAGs), or influence gene expression through fatty acid-binding proteins or directly as ligands of specific transcriptional regulators (244,246,260). Adding more complexity, PUFAs such as LA can directly bind transcriptional regulators such as peroxisome proliferator-activated receptors (PPARs), which regulate a vast assortment of lipid metabolic and general metabolic processes (244–246). The PPARs are ligand-activated and also serve as sensors for lipids (276,277). PPARs specifically bind to certain response elements within promoters (PPREs) (245,246,276).

T cell subsets that depend on lipid metabolism include Tregs and memory T cells (110,254,271). How these T cell subsets use these pathways is vastly different from Tregs, which use lipids for FAO, and memory T cells are more prone to lipolysis and storing lipids as TAGs for long-term energy needs (110,176,254,271). It is not precisely known how in general, T cells use the lipids within their environments and which pathways specific lipids can influence. It has also not been established if there are genetic differences in T cells from autoimmunity-prone mouse strains or individuals that contribute to how they handle lipids from their environment since there is evidence that human and NOD mouse T1D progressors have altered bioenergetic needs before diabetes

development (64,77,91,92). This aim seeks to identify changes in gene expression underlying responses to LA in NOD mouse T cells. We hope to reveal how a LA environment influences the gene expression of NOD T cells.

Results

4.1 LA exposure alters the gene expression of NOD mouse T cells.

LA can bind and influence transcription factors, signaling proteins, or be used as an energy source (244,246). To determine how LA impacts T cells' gene expression, we measured differences in gene expression between LA- and BSA-treated T cells from NOD mice. Splenocytes were activated as described above and activated T cells were isolated after 48 hours using isolation with magnetic beads. RNA was extracted and converted to cDNA for Q-PCR analysis of genes related to the processing of lipids or metabolism found in **Table 1**. The data show reduced expression of *PPARα* in NOD mouse T cells **(Figure 15A)**, along with a reduction in expression of *SIRT4* **(Figure 15E),** a regulator of fatty acid oxidation and metabolism (278) at higher doses of LA treatment. There are no differences seen in the gene expression of *CD36* and *CPT1a* **(Figure 15C and 15D)** between LA and the control group, indicating that the presence of LA does not affect the translocation of fatty acids into the cell via CD36 (279) or into the mitochondria since CPT1a is an important enzyme involved in FAO metabolism and shuttles fatty acids from the cytosol into the mitochondrial matrix, the site of FAO (181,254,270). The results also show a decrease in expression of *PPARγ* at low and high doses of LA **(Figure 15B)**. These data suggest that there is gene repression by LA,

particularly in pathways involved in the regulation of lipid metabolism. A caveat of this specific aim is that it is unknown when after activation and exposure to LA, T cells reduce the expression of *PPARα* and *PPARγ* and how LA impacts these transcription factors' activation status and mechanisms. PPARγ functions as a nutrient sensor and regulates gene transcription to promote the storage of lipids like non-esterified LCFA (276,280,281). PPARα also functions as a nutrient sensor (282). It is the main regulator of lipid catabolism and increases the transcription of lipid catabolism genes (276,283). There is evidence that PPARα -/- male mice but not female mice with induced EAE developed more severe clinical signs of EAE due to enhanced Th1 autoimmunity (284). Since lipid metabolism is extensive and complex, broader gene expression studies must be completed to get a better idea of pathways and potential mechanisms where LA leads to the suppression of important lipid regulating transcription factor genes in T cells from an autoimmune mouse model.

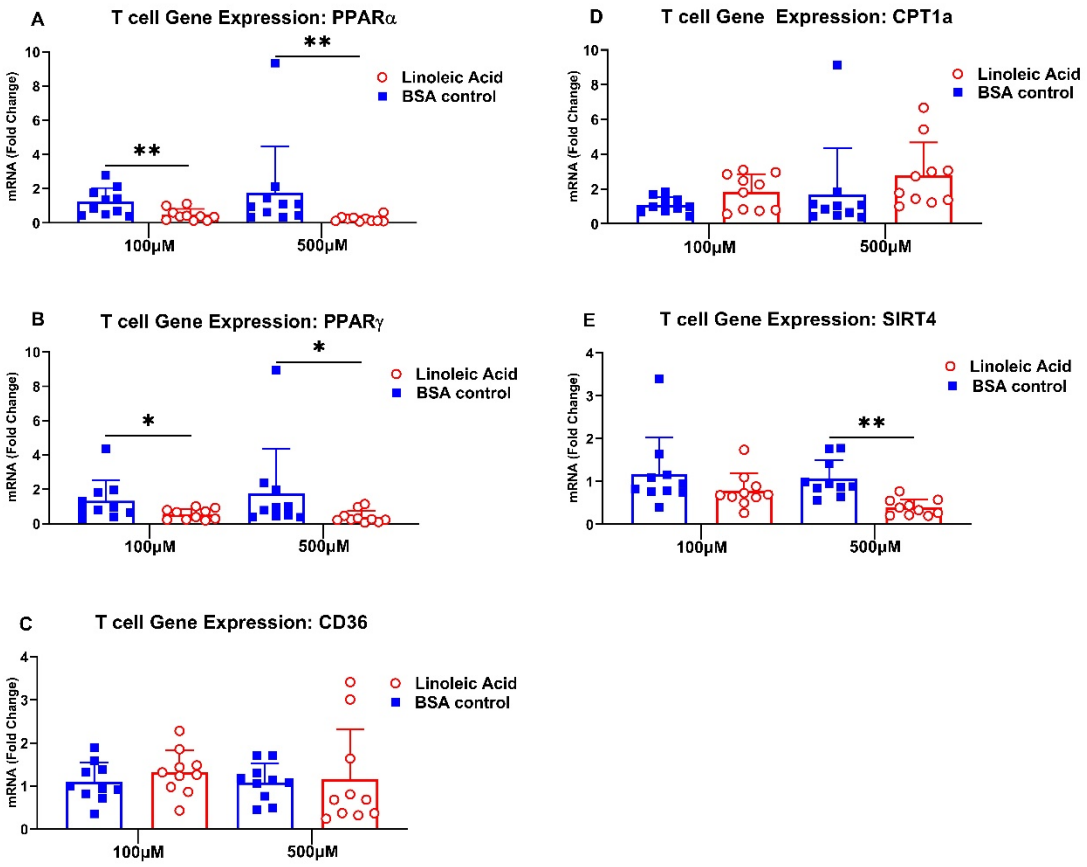

Figure 15. LA decreases T cell gene expression of lipid-regulating transcription factors PPARα and PPARγ.

T cells were isolated from LA and control splenocyte cultures using magnetic isolation, and RNA was extracted and converted to cDNA for Q-PCR analysis of genes related to the processing of lipids or regulation of lipid metabolism found in **Table 1.** Graphs reveal a decrease in gene expression of lipid-regulating transcription factors PPARα **(A)** and PPARγ **(B),** suggesting that LA regulates major transcription factors that further regulate cellular metabolic pathways mainly involved with lipid metabolism (276). There are no differences induced by LA in CD36 **(C)** expression or CPT1a **(D)** expression.

Mitochondria are not affected (173,254,279). Also seen is a reduction in SIRT4 (E) expression**,** a mitochondrial NAD+-dependent enzyme involved in regulating key metabolic processes, including mitochondrial energy transfer, cell survival, and FAO (285,286). Each symbol represents T cells from one mouse, n=10, graphs displayed as mean ± SD, Wilcoxon matched-pairs signed-rank test was used for statistics (non-parametric), **$p<0.002$ *$p<0.05$.

CHAPTER FIVE: THE IMPACT OF IL-7Rα BLOCKADE ON T CELL LIPID METABOLISM

Rationale

Elevated IL-7 levels or IL-7 signaling have been correlated with T1D and play a role in the expansion and function of autoreactive CD4+ and CD8+ T cells (168,241,287). Blocking the IL-7 pathway with anti-IL-7Rα mAbs has been shown to push memory CD4+ T cells into an inhibited state with reduced cytokine secretion and proliferation leading to prevention and reversal of T1D in NOD mice (15,16). T cell inhibition after anti-IL-7Ra treatment was mediated by enhanced expression of the co-inhibitory receptors (Co-IRs) PD-1, LAG-3, and TIM-3 (15,243). The role of co-inhibitory receptors, in general, is to regulate and inhibit effector T cell responses, especially in instances of inflammation and promoting self-tolerance (288). Co-IR expression on Tregs ensures their ability to control and suppress effector T cell responses (288). Expression of co-IRs also contributes to T cell exhaustion (289). T cell exhaustion is a differentiation state of T cells that develops due to persistent exposure to inflammatory signals or antigen and is characterized by T cells having loss of effector functions, up-regulation and sustained expression of co-IRs, and metabolic abnormalities (289). Evidence demonstrates anti-IL-7Ra treatment pushes T cells into an exhausted or inhibited state, which reduces the effector functions of autoreactive T cells (15,16,290).

It is known that IL-7 signaling is involved in promoting T cell homeostasis and TAG synthesis in memory T cells, giving the IL-7R signaling pathway a role in cell lipid metabolism (176,271,291). The synthesis of TAGs, which can subsequently be used for lipolysis and FAO, is necessary as a long-term energy source for memory T cell survival (176,196). Little is understood about how treatment with anti-IL-7Rα mAbs induces changes in metabolic pathways, including FAO, and how it impacts lipid metabolism in autoreactive T cells. Some changes noticed in Gene Set Enrichment Analysis (GSEA) analysis of CD4+ memory T cells isolated from NOD mice treated with anti-IL-7Ra mAbs versus rat IgG control Ab include downregulation of TCA cycle and oxidative phosphorylation pathways, and there is upregulation of the hypoxic response pathway, which may indicate metabolic dysregulation **(data not shown)**. These changes, however, do not fully describe the effects of IL-7R blockade on T cell lipid metabolism. Here, **we hypothesized that IL-7Rα blockade compromises lipid metabolic processes in autoreactive T cells and that in turn inhibits their function through co-IR expression and exhaustion to reduce the pathogenic functions of NOD mouse T cells.** Thus, we sought to determine whether IL-7 signaling plays a role in activated T cells' response to LA by measuring viability, cytokine production, and expression of the co-IRs PD-1, LAG-3, TIM-3, and TIGIT.

PD-1 expressed on T cells promotes negative regulation of signaling through the TCR and CD28 after binding with its ligands PD-L1 or PD-L2. This signaling inhibition can impede T cell activation, inhibit T cells from self-reactivity, and promote Treg

development and proliferation (288,292,293). It has been shown that PD-1 ligation results in metabolic changes to T cells where they transition from glycolysis during the effector response to FAO and OXPHOS, which is associated with cell survival and longevity (294).

LAG-3 is expressed on CD4+ T cells, CD8+ T cells, Tregs, Tr1 cells, and specific NK cell subsets (288). The primary role of LAG-3 is to inhibit T cell expansion after antigen exposure (288,295), but the exact signaling mechanisms for inhibition of T cell function and specific alterations to metabolism by LAG-3 engagement are still unknown.

Tim-3 is expressed on IFN-γ -producing Th1 cells and CD8+ T cells (296). Binding with its ligand galectin-9 can induce cell death in Tim3-expressing Th1 cells (297). The cytoplasmic tail of the Tim-3 receptor complex interacts with the TCR complex components to negatively regulate immune responses (298,299).

TIGIT is expressed on NK cells, T cells, Tregs, and Tfh cells. TIGIT works by blocking cell activation, proliferation, and effector functions by regulating components of the TCR complex (288,300,301). The ligands for TIGIT are CD155 and CD112, and these ligands can be expressed on APCs and T cells (288,300,302).

The Dooms lab previously showed that PD-1, TIM-3, and LAG-3 expression is increased after IL-7Ra blockade (15,243). Here, we wish to know if specific nutrient environments such as LA conditions influence cytokine production and the expression of co-IRs in T cells after anti-IL-7Rα treatment.

Results

5.1 IL-7Rα blockade does not promote differences in cytokines or exhaustion markers in an LA environment.

NOD mice were treated with anti-IL-7Rα or Rat IgG antibodies (0.5 mg IP). After three days, splenocytes were isolated and stimulated in vitro with αCD3 and αCD28 mAbs in the absence or presence of high and low doses of LA conjugated to FA-free bovine serum albumin at a 2:1 molar ratio. After culturing for 48 hours with and without LA, T cells were re-stimulated with PMA and ionomycin to induce cytokine production. Cytokines were analyzed by intracellular cytokine staining. Antibodies staining phenotypic and exhaustion markers (PD-1, LAG-3, TIM-3, TIGIT) were added to detect inhibited T cells, and a cell viability dye was added to assess live cell numbers (flow panel found in **Table 8)**. First, there were no differences in viability in CD4+ and CD8+ T cells in any treatment groups with and without LA **(Figure 16A and 16E)**. While this result does not recapitulate our data showing LA induces a survival benefit in activated T cells isolated from unmanipulated NOD mice, it is feasible that the in vivo treatment with a large dose of rat IgG or anti-IL-7Ra antibodies altered the T cell activation state in a way that muted their response to LA. Moreover, results demonstrate that IL-7R blockade did not alter cytokine production in response to LA **(Figure 16B-16D, 16F-16G)**. Results are comparable between IgG control also those exposed to LA **(Figure 16).** There were no differences in cytokine production between the T cells from mice treated with IgG and anti-IL-7Rα. Results also reveal no differences in co-inhibitory receptor expression (LAG3, TIGIT, PD-1, and Tim3), indicating that cells are not exhausted, and LA does

not influence the inhibition of cells by IL-7R blockade **(Figure 17)**. It is possible that the three-day treatment time of mice with anti-IL-7Rα was not enough to inhibit the cells to assess the expression of exhaustion markers and cytokine production. A more extended treatment regimen or extra dosage of anti-IL-7Rα needs to be explored. There is a trend of Tim-3 expression increased in CD8+ T cells regardless of IgG or anti-IL-7Rα treatment group **(Figure 17H);** this is an interesting observation considering Tim-3 has a role in suppressing Th1 cells and CD8+ T cells (288,296).

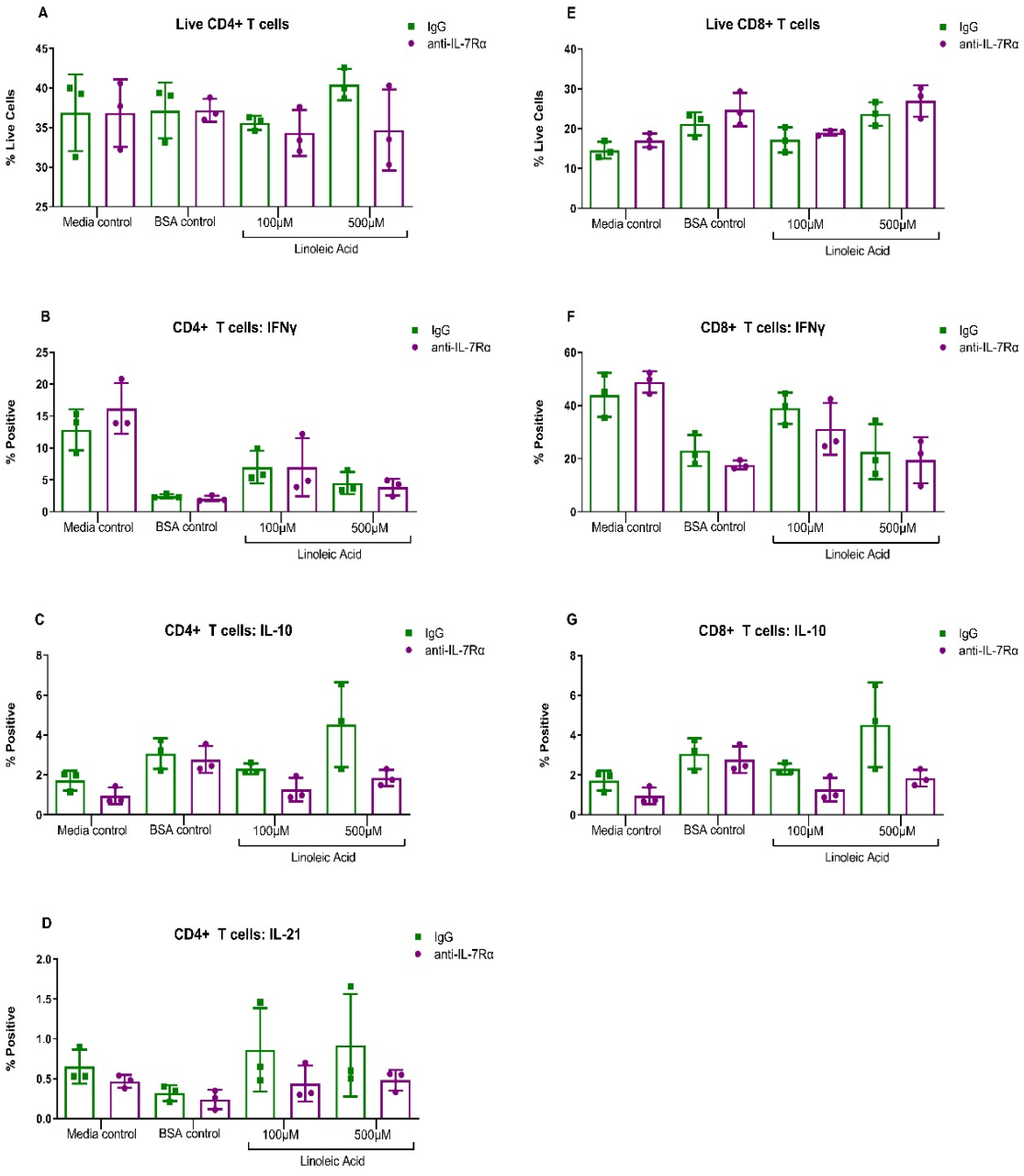

Figure 16. LA does not affect viability or cytokine production in T cells from anti-IL-7Rα treated mice.

Splenocytes from NOD mice treated with anti-IL-7Rα or Rat IgG antibodies were isolated and activated in vitro in the absence or presence of high and low doses of LA. After culturing for 48 hours with and without LA, T cells were re-stimulated. T cells were analyzed for markers of T cells and cytokines using the flow panel in **Table 3.** Graphs demonstrate no differences in cytokine production between anti-IL-7Rα and IgG-treated T cells cultured with and without LA. Within the CD4+ population, there were no differences in cell viability **(A)** and the production of cytokines IFN-γ **(B)**, IL-10 **(C)**, and IL-21 **(D)**. In the CD8+ T cell population, there were also no differences in viability **(E),** and there were no differences in the production of cytokines, including IFN-γ **(F)** and IL-10 **(G)**. This data indicates that LA does not impact viability or cytokine production in cells from anti-IL-7Rα treated NOD mice. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, Kruskal-Wallis test (non-parametric ANOVA) with Dunn's Multiple Comparisons Test were used for statistics.

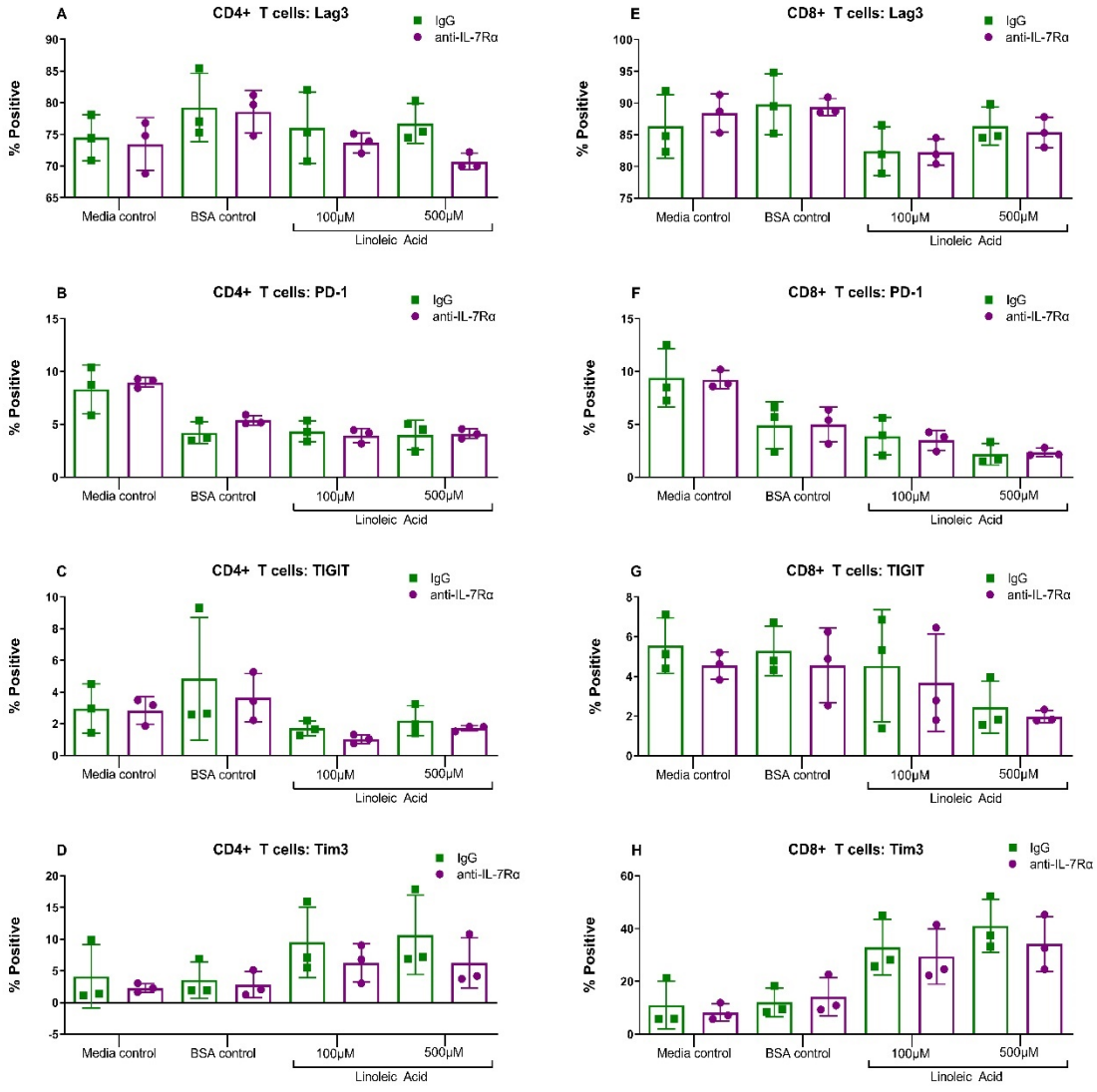

Figure 17 LA does not affect co-inhibitory receptor expression in T cells from anti-IL-7Rα treated mice.

Splenocytes from NOD mice treated with anti-IL-7Rα or Rat IgG antibodies were isolated and activated in vitro in the absence or presence of high and low doses of LA. After culturing for 48 hours with and without LA, T cells were re-stimulated. T cells were analyzed for markers of T cells and exhaustion (TIGIT, PD-1, LAG-3, TIM-3)

using the flow panel in **Table 8.** Graphs demonstrate no differences in co-inhibitory receptor expression between anti-IL-7Rα and IgG-treated T cells cultured with and without LA. Within the CD4+ population, there were no differences in the expression of Lag-3 **(A)**, PD-1 **(B)**, TIGIT **(C)**, and Tim-3 **(D)**. In the CD8+ T cell population, there were no differences in the expression of Lag-3 **(E)**, PD-1 **(F)**, TIGIT **(G)**, and Tim-3 **(H)**. However, there is a trend of Tim-3 expression being increased in CD8+ T cells regardless of IgG or anti-IL-7Rα treatment group **(H).** These data indicate that LA does not impact co-inhibitory receptor expression in CD4+ T cells from anti-IL-7Rα treated mice, which were expected to have changes in their metabolic state. Each symbol represents one mouse, n=3, graphs displayed as mean ± SD, Kruskal-Wallis test (non-parametric ANOVA) with Dunn's Multiple Comparisons Test were used for statistics.

5.2 IL-7Rα blockade of NOD T cells alters Aquaporin 9 expression but not other lipid metabolism-regulating genes.

To understand the role of the IL-7/IL-7Ra pathway on the expression of lipid metabolism-regulating genes, NOD mice were treated with anti-IL-7Rα or Rat IgG antibodies. After three days, CD4+ T cells were isolated from mouse splenocytes, and RNA was extracted for conversion to cDNA. Q-PCR analysis was completed to look at the expression of lipid metabolism-related genes found in **Table 1**. Although evidence exists that the IL-7 signaling pathway can regulate TAG formation (170,176), blocking the IL-7 signaling pathway did not change the expression of any genes that would regulate the processes of lipid metabolism, which include PPARα, PPARγ, and ACC1 (276,303) **(Figure 18A-18E).** Anti-IL-7Rα treatment reduced the expression of Aquaporin 9 **(Figure 18E)**, a channel protein used for importing glycerol into the cell (176). It is known that IL-7 signaling induces expression of Aquaporin 9 to import glycerol for TAG synthesis in CD8+ memory T cells; hence we expected blocking the IL-7R pathway may reduce Aquaporin 9 gene expression in CD4+ T cells (176). CPT1a is an important enzyme for FAO (173,254), and there are no differences in the expression of this gene between IgG and anti-IL-7Rα **(Figure 18D)**. The findings in **(Figure 18E)** need to be further characterized through studies of TAG accumulation after anti-IL-7Rα treatment, which could demonstrate that TAG synthesis is altered by anti-IL-7Rα and its implication on T cell survival. Once again, the dosing of anti-IL-7Rα could have implications on the results, and a more extended treatment regimen combined with

multiple dosing could allow for anti-IL-7Rα to exert its effects on T cell inhibition and lipid metabolic processes.

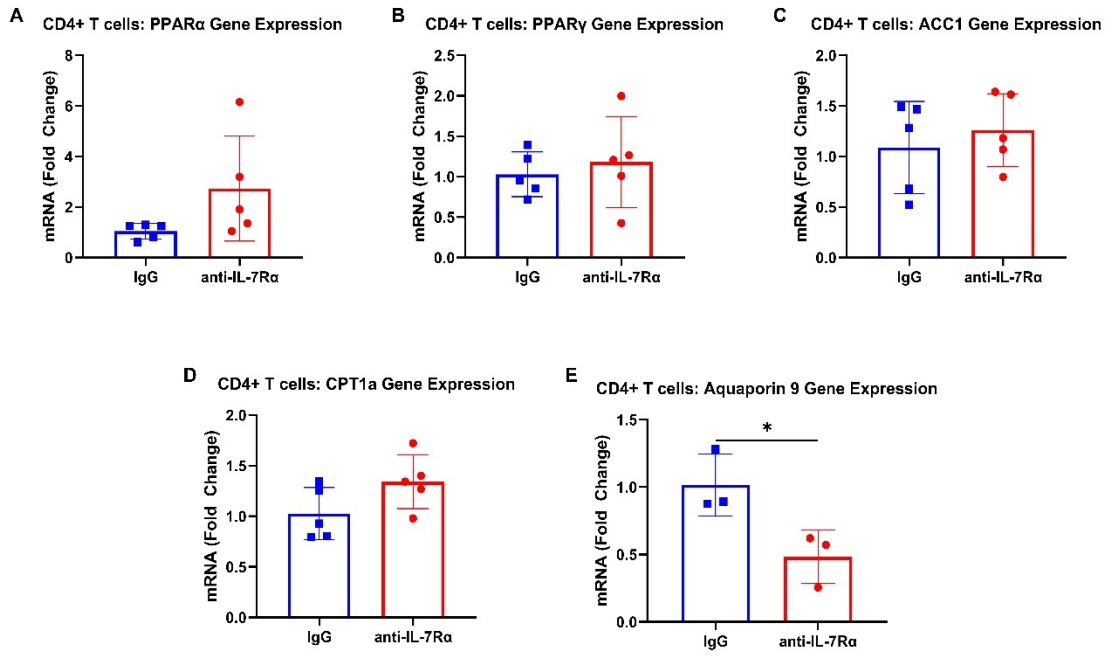

Figure 18. CD4+ T cells from anti-IL-7Rα treated mice have reduced Aquaporin 9 gene expression.

NOD mice were treated with anti-IL-7Rα or Rat IgG antibodies. After three days, splenocytes were isolated, and T cells were purified using a CD4+ magnetic negative selection kit. CD4+ T cell RNA was extracted and converted to cDNA for Q-PCR analysis of genes related to lipids' processing or lipid metabolism regulation found in **Table 1.** Graphs reveal no differences in the expression of lipid metabolism-related genes in CD4+ T cells from anti-IL-7Rα treated mice. There are no differences in the gene expression of lipid regulating transcription factors PPARα **(A)** and PPARγ **(B)** between

treatment groups. Also, there are no changes to expression seen in genes related to lipid handling, such as ACC1 **(C),** which is essential for fatty acid synthesis (303), and no changes in expression to CPT1a **(D),** which plays a role in translocating fatty acids into the mitochondrial matrix which is the site of FAO (173,254). Anti-IL-7Rα treatment reduces the expression of Aquaporin 9 **(E)**, a channel protein used for importing glycerol into the cell for TAG formation (176). Each symbol represents one mouse, n=5 or 3, graphs displayed as mean ± SD, unpaired t-tests were used for statistics. *$p<0.05$

CHAPTER SIX: DISCUSSION

6.1 Overall Summary of Results

In the studies described, we explored how exposure to LA influences the phenotype and cytokine production of TCR-activated T cells from the NOD mouse model of T1D. LA is a significant component of the Western diet and is known to contribute to inflammation and autoimmunity (257–259,263,304). Our studies revealed that in LA's presence, 48 hours after activation, there are a greater number of viable CD4+ and CD8+ T cells compared to controls **(Figure 2)**. To understand if LA enhanced viability due to cells being more proliferative or having enhanced survival, a proliferation/survival assay was completed and revealed that CD4+ T cells cultured in LA conditions had enhanced survival, indicated by the viability dye-/Annexin V- population, up to 48 hours after initial activation and re-plating compared to controls **(Figure 4A and 4B)**. CD4+ T cells exposed to higher concentrations of LA expressed higher levels of the proliferation marker Ki-67 compared to controls up to 24 hours after initial activation and re-plating **(Figure 3 and Figure 4C)**. These results suggest that high levels of LA promote T cell proliferation while low and higher levels can contribute to enhanced survival of T cells. This augmentation of T cell functions can be considered pathogenic in the context of T1D since it is unknown what supports initial autoreactive T cell activation, survival, and attack on pancreatic islets.

Our studies also demonstrate that LA directly alters cytokine subset composition in CD4+ T cells by promoting IL-21 production, a pathogenic cytokine involved in T1D, while reducing IL-10 **(Figure 5),** which is necessary for tolerance against autoimmunity

(119,166). In **Figure 6,** we revealed that the ratio of cytokines produced by CD4+ and CD8+ T cells is altered. The ratios of frequencies and cell counts of IL-21:IL-10 in CD4+ T cells **(Figure 6A and 6B)** are increased in LA conditions, indicating more IL-21-producing T cells than IL-10 producers. In CD8+ T cells, ratios of frequencies and cell counts of IFN-γ:IL-10 are trending towards altered subset composition at high concentrations of LA **(Figure 6C and 6D)**. These data suggest that LA conditions can alter the composition of cytokine-producing CD4+ and CD8+ T cells to be more pathogenic.

Furthermore, we have evidence that DCs contribute to enhanced survival **(Figure 8A and 8E)** either by cytokine production or cell contact with T cells, which we could not distinguish between in these studies. Also revealed was that LA does not change the differentiation state of CD4+ T cells into Tfh or Th17 as measured by expression of transcription factors for these subsets **(Figure 10A and 10B).** However, long-term studies have to be considered to understand the long-term outcomes of T cells exposed to LA.

LA in this study did not promote mitochondrial biogenesis in CD4+ T cells and increases in mitochondrial mass were only seen in CD8+ T cells, indicating that there is a difference in how LA is handled in CD4+ and CD8+ T cells **(Figure 12C and 12D)** and suggesting that CD8+ T cells could be utilizing LA as a fuel source which would promote the increase in mitochondrial mass. There were increases in lipid accumulation in LA-exposed CD8+ T cells **(Figure 12B)** and, at high LA concentrations, in CD4+ T cells **(Figure 12A)**. Surprisingly, LA exposure reduced expression of the lipid metabolism-

regulating transcription factors PPARα and PPARγ. However, there were no changes seen in the gene expression of CPT1a, an essential enzyme in the FAO pathway (173,254,270), or CD36, a fatty acid translocase (279) **(Figure 15)**. The gene expression analysis suggests a potential mechanism through which LA regulates cytokine production by reducing PPARα and PPARγ expression where evidence exists that reduction of the expression of these transcription factors can promote autoimmunity through many processes (246,260,276). Finally, we began to investigate the role of the IL-7 pathway in regulating the response to LA in NOD T cells. IL-7Ra blockade did not significantly alter co-IR expression **(Figure 17)** and the production of cytokines like IL-10, IL-21, and IFN-γ **(Figure 16)** in response to LA exposure. However, there are interesting data in **(Figure 17H)** that could suggest LA conditions increase Tim-3 expression on CD8+ T cells from both IgG and anti-IL-7Rα treated mice, but more studies must be completed to make a proper conclusion about this trend. We report that blocking the IL-7R signaling pathway did not change the expression of the lipid metabolism-regulating transcription factors PPARα and PPARγ in T cells **(Figure 18A and 18B)**. Interestingly, my data show that anti-IL-7Rα treatment reduces Aquaporin 9 gene expression on CD4+ T cells **(Figure18E)**. This could suggest that blocking the IL-7R pathway reduces TAG formation since Aquaporin 9 is involved in transporting glycerol into the cell for TAG formation (176). TAG formation is an essential mechanism for the long-term survival of T cells (176).

6.2 Significance of how an LA environment can modulate T cells and influence T1D autoimmunity.

In this study, LA was found to enhance NOD T cell survival and induce the production of IL-21 in CD4+ T cells. IL-21 has various effects on T cells, including proliferation and inhibiting the production of other cytokines (126). IL-21 plays a role in T1D development and is elevated in T1D patients (104,131). This cytokine is also vital for the differentiation of Th17 cells and is produced by Tfh cells important in the germinal center formation and have also been found elevated in T1D patients (104,126). This study also revealed that, along with increases in IL-21 production, there is a decrease in IL-10-producing T cells in both CD4+ and CD8+ T cells. This is significant since IL-10 is an anti-inflammatory cytokine important for regulating Th1 effector responses and IFN-γ secretion (132,305). IL-10 can be produced by most immune cells, but Tregs and Tr1 cells are responsible for secreting IL-10 to regulate effector responses, and Tr1 cells can be induced by IL-10 (132,305). It has been shown that a lack of IL-10 leads to autoimmune pathology in many experimental animal models of autoimmunity such as RA (306), SLE (307), and EAE (307,308). T1D Tregs are less functional and fail to induce tolerance and suppress autoreactive effector T cells (135) via cell to cell contact and production of cytokines such as IL-10 (132,135). The decrease in IL-10 induced by LA is critical to characterize in T1D since the standard mechanisms of tolerance induced by Tregs are unstable, and LA could promote pathogenicity by altering the composition of T cells producing pro-inflammatory and anti-inflammatory cytokines. In this study, it is still not clear if the promotion of IL-21 producers and decrease in IL-10 producing T

cells is due to the expansion of the pathogenic population via proliferation or if LA induces apoptosis of IL-10-producing T cells, or if LA prevents apoptosis in T cells producing pathogenic cytokines.

It is still unknown if chronic exposure to LA could promote plasticity in NOD mouse T cells long-term, a possibility that is not addressed in this study. This study specifically observed the short-term effects within 48 hours of activation. More research needs to be done to understand the long-term effects of chronic exposure to fatty acids like LA to understand its impact on T cells' differentiation state fully. Using the NOD mouse model, mice fed with diets supplemented with n-3 fatty acids EPA and DHA led to a reduction in the percentages of pro-inflammatory T cell subsets and reduced the incidence of diabetes (94). In the described study by Bi et al. (94), EPA and DHA were able to inhibit the activation of mTORC1 by arachidonic acid and had similar effects on mTORC1 as treatment with rapamycin which has been demonstrated to prevent diabetes in humans (94,309). This signifies that it is still worthwhile to investigate whether long-term consumption of an n-6 fatty acid or an LA-rich diet could influence individuals' risk of autoimmunity. This study characterizes how LA can influence the pathogenicity of autoreactive-prone T cells from NOD mice. Since high LA intake is a component of the Western diet (257,258,261,310), understanding the mechanisms by which dietary factors such as PUFA's impact on T cell differentiation states and cytokine production can help to gain an understanding of how T cells are modulated by diet-derived factors present in tissue environments. There have been many observations that the Western diet rich in the n-6 fatty acid LA has increased inflammation and autoimmunity in humans and animal

models (257,263,304,310,311). In the NZBWF1 mouse model of SLE, feeding NZBWF1 mice a corn oil-based Western-type diet rich in LA raised plasma autoantibodies, and mice had a higher incidence of glomerulonephritis compared to mice fed a diet rich in n-3 fatty acids (263). It is still unknown what the direct impact on immunity is or by which mechanisms fatty acids exert their effects on cells since nutrition-based research is very contradictory depending on what is being studied (259,312,313). This describes a need for more specific research to support studying how dietary factors impact specific cell types in inflammatory and autoimmune conditions.

The observation that LA induces CD4+ IL-21-producing T cells with a survival advantage and decreases IL-10-producing CD4+ T cells may point to how changes in the lipid environment in tissues and the periphery could influence the pathogenicity of cells in T1D. IL-21 is a crucial cytokine in the development of autoimmunity (99,121), including T1D, where autoreactive CD4+ T cells produce IL-21 to help activate autoreactive CD8+ T cells (223). IL-21 has also been shown to skew T cells towards FAO (236), which was not seen in my study through gene expression analysis of FAO-related genes like CPT1a and measures of mitochondrial mass by flow cytometry. Once again, this could be an issue of acute compared to chronic T cell exposure to LA. It is unlikely that these changes happened at earlier time points since IL-21 was not produced before 48 hours and IL-10 production was not reduced before 48 hours. Results demonstrate that the mitochondria are not undergoing biogenesis due to LA treatment in CD4+ T cells, as shown by no changes in mitochondrial mass, but increased mitochondrial mass is seen in CD8+ T cells at higher LA concentrations. These data give

evidence that CD4+ and CD8+ T cells may have different responses to LA. Also, the increase in lipid droplet formation in both CD4+ and CD8+ T cells indicates that cells are not using LA as a fuel source in FAO but may be converting LA into other lipid mediators such as Arachidonic acid, converting it for storage as TAGs, or LA can be incorporated into the membrane (257,258,261,310,314).

The data suggesting LA not being used as a fuel source is also confirmed by the decrease in expression of PPARα since PPARα is a positive regulator of FAO and promotes the expression of genes involved in the FAO pathway (276,315,316). The reduction in gene expression of the transcription factor PPARα may point to a possible mechanism of the decrease in expression of lipid metabolism pathway genes since many are regulated by PPARα (276,316). The abundance of lipid metabolic pathways regulated by PPARα and PPARγ adds to these studies' complexity and requires a broader analysis of gene expression to look for changes in other genes regulated by these transcription factors. Also, a decrease in expression could be regulated directly by overexposure to the ligand, which in this study is LA (282,316). Since PPARs have roles as nutrition sensors (276,316,317), it would be interesting to explore how chronic exposure to LA influences the activation state of the transcription factors PPARα and PPARγ. The decrease in PPARα specifically has connections to autoimmunity, where PPARα -/- male mice have a more severe clinical expression of EAE via Th1 responses when EAE is induced with MOG (284). Also, it was demonstrated that there is sexual dimorphism present in terms of how much PPARα is expressed between male and female mice where male mice have higher levels of PPARα expression, and this expression is sensitive to androgen levels

(284). This indicates that lower expression levels of PPARα influence autoimmunity and requires further investigation of how LA can decrease expression of PPARα and if that is connected to IL-21 production and reductions in IL-10 seen in NOD mouse T cells.

In this study, it was demonstrated that LA reduced the gene expression of PPARγ. When specifically looking at the role of PPARγ in autoimmunity, it has been shown that PPARγ activation negatively regulates Th17 differentiation but has no impact on Th1, Th2, or Treg subsets (318,319). Th17 cells have been implicated in the pathogenesis of many autoimmune diseases, including MS and T1D (126,281). Using T cells from both MS patients and the EAE mouse model, it has been demonstrated that activation of PPARγ using pharmacological agonist pioglitazone selectively suppresses Th17 differentiation (319). Also, 13s-HODE, an LA derivative serving as an agonist of PPARγ, has been demonstrated to have similar effects on Th17 suppression like pioglitazone (319,320). Further research is needed to specify how diet and fatty acids like LA are used by the cells to produce lipid mediators such as 13s-HODE compared to other lipid mediators that could have antagonistic effects on PPARγ. It is known that Δ-5-desaturase, Δ-6-desaturase, and elongase, along with specific LOX and COX enzymes further down the conversion pathways mediate the conversion of LA into inflammatory and anti-inflammatory lipid mediators (314,321,322). Exactly how cells like T cells choose to convert LA into either inflammatory or anti-inflammatory mediators has yet to be addressed and is a topic not covered in these studies. The data demonstrating reduced PPARγ gene expression points to another potentially complex mechanism by which IL-21 could have been induced in LA conditions and have contributions to autoimmune

pathogenesis. Further studies into the pathways that convert LA must be addressed in the future, especially since studies demonstrated that arachidonic acid consumption by NOD mice influenced T1D pathogenesis by elevating the percentages of Th17 cells (94). In Bi *et al*., mice were fed chow supplemented with arachidonic acid, a derivative of the essential fatty acid LA(94).

While the enhanced survival of T cells in LA conditions is not exclusive to NOD mouse T cells, it is still a significant finding since enhanced survival can promote greater numbers of pathogenic T cells and may be a factor that needs to be considered in autoimmunity. There have been findings supporting the roles of specific fatty acids influencing T cells and therefore inducing inflammation or autoimmunity (94,124,311). For example, in non-alcoholic fatty liver disease, LA induces selective loss of CD4+ T cells but not CD8+ T cells (304). In our studies, the opposite is seen where LA conditions enhanced CD4+ T cells survival and pathogenic cytokine production. The differences between our studies and previously published data on the effects of LA in CD4+ T cells in NAFLD are that the disease context is different; namely, T1D is autoimmune vs. NAFLD is inflammatory (304,311). The T cells studied are different, splenocytes compared to T cells from the liver (304). This highlights the importance of studying individual FA effects on T cells from different disease states since each FA has distinct effects on different cell types from the body dependent on the cell type-specific needs, environment, and genetic factors. This study presents data that suggest a constant high level of LA exposure in tissues could allow enhanced proliferation and survival of T cells

producing pathogenic cytokines. Decreasing the number of T cells producing anti-inflammatory cytokines which may influence the development of T1D.

6.3 Future Directions

This study demonstrated that the essential fatty acid LA, which is obtained through the diet, can modulate T cell survival, proliferation, and cytokine production in the NOD mouse model. This cytokine modulation involved increasing cytokines that are considered pathogenic in T1D and other autoimmune diseases while decreasing anti-inflammatory cytokines essential for establishing immune tolerance.

Future directions for this type of study involving dietary environmental factors and their impact on immune cells would need to involve methods for a broader analysis of cytokines and genes to get a complete picture of how altering nutritional status influences metabolic pathways that modulate immune status. Experiments in this study focused on specific cytokines involved in the pathogenesis of T1D and looking at specific genes involved in lipid metabolic processes. While we were able to obtain data supporting increases of IL-21, decreases in IL-10, and reduction of lipid metabolism-regulating genes PPARα and PPARγ, we could not obtain any information into the mechanisms or pathways that could have been involved in cytokine modulation. Using multiplex ELISA analysis of T cell cytokines, we were able to obtain information about more cytokines impacted by LA but do not fully understand how or why these cytokines increase in the presence of this fatty acid.

We did gain some knowledge of how APCs can impact this T cell cytokine modulation by LA, but broader studies of more specific DC and macrophage subsets need to be completed as there are studies supporting specific subsets that could be influenced by dietary fatty acids. There is also much literature supporting the direct effects of specific fatty acids on T cells (94,124,311), and the differences between direct effects on T cells and contributions by APCs also exposed to fatty acids have to be distinguished to fully understand the impact of fatty acids on specific cell types. Studies of lipid metabolism are complicated due to the many impacts of different fatty acids on many different cell types. Adding more complexity is that lipids can affect gene transcription, cell metabolism, have effects via specific pathways mediated by enzymes, or influence pathways via membrane and lipid raft changes affecting GPCRs or tyrosine kinase receptor signaling (246,260,261,276,323). The effects of fatty acids on each of these roles in each specific immune cell type need to be fully characterized to understand how fatty acids within specific diets can influence immune cell function and promote or reduce autoimmunity and inflammatory disorders.

Some future directions specific to this study would be to complete *in vivo* experiments through a feeding study in NOD mice with read-outs being kinetics of diabetes incidence, a more comprehensive analysis of multiple genes through microarray or RNAseq, large scale multicolor flow cytometry, and proteomic approaches like mass spectrometry. Utilizing these methods would allow more detailed knowledge of how specific dietary lipids alter different immune cells involved in autoimmunity. Also, by defining specific pathways fatty acids regulate in NOD mice, we can better

mechanistically understand how lipid metabolism regulates T cell cytokine production in T1D and other autoimmune diseases.

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