Metabolic Mechanisms of Nutritionally Regulated Hormone Signaling on T Helper Cell Function

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Nutritionally regulated hormones communicate nutritional status to cells and tissues throughout the body. In the case of overnutrition or obesity, levels of leptin, insulin, and insulin like growth factor 1 (IGF-1) are increased, whereas they are decreased in the setting of undernutrition. Either undernutrition or overnutrition results in a dysregulated immune response that contributes to increased susceptibility to infection. Immune cells, especially T cells, are responsive to nutritional hormone signals. Leptin has been shown to promote inflammatory $CD4^+$ T cell differentiation, while insulin and IGF-1 have been shown to promote increased metabolism and function of various $CD4^+$ T cell subsets. Here, we set out to understand how nutritionally regulated hormone signaling to T cells impacts T cell metabolism and function and thereby influences immunity.

In Chapter 2, we investigated the requirement for leptin receptor signaling on T cells in driving obesity-associated inflammation and glucose intolerance. Leptin signaling has been shown to directly promote inflammatory T helper 1 (Th1) and T helper 17 (Th17) cell differentiation and function. Since T cells have a critical role in driving inflammation and systemic glucose intolerance in obesity, we sought to determine the role of leptin signaling in this context. Male and female T cell-specific

leptin receptor knockout mice and littermate controls were placed on low-fat diet or high-fat diet to induce obesity for 18 weeks. Weight gain, serum glucose levels, systemic glucose tolerance, T cell metabolism, and T cell differentiation and cytokine production were examined. In both male and female mice, T cell-specific leptin receptor deficiency did not reverse impaired glucose tolerance in obesity, although it did prevent impaired fasting glucose levels in obese mice compared to littermate controls, in a sex dependent manner. Despite these minimal effects on systemic metabolism, T cell-specific leptin signaling was required for changes in T cell metabolism, differentiation, and cytokine production observed in mice fed high-fat diet compared to low-fat diet. Thus, T cell-specific deficiency of leptin signaling alters T cell metabolism and function in obesity but has minimal effects on obesity-associated systemic metabolism. These results suggest a redundancy in cytokine receptor signaling pathways in response to inflammatory signals in obesity.

In Chapter 3, we investigated the impact of insulin and IGF-1 signaling on $CD4^+$ T cell metabolism and function. Insulin receptor (IR) and IGF-1 receptor (IGF-1R) have signaling pathways closely related to the leptin receptor signaling pathways, and are known to regulate metabolism. Using extracellular flux analysis, we determined that both insulin and IGF-1 increase glycolytic and oxidative metabolism of $CD4^+$ T cells, but insulin has a more potent effect. However, IGF-1 acts specifically on Th17 cells to increase their IL-17 production and metabolism. Furthermore, IGF-1 treatment decreases mitochondrial membrane potential and mitochondrial ROS (mROS) production in Th17 cells, presumably to protect the cells from oxidative stress. Interestingly, both IR and IGF-1R appear to be required for this effect. This could indicate that the hybrid IR/IGF-1R is required for mediating the effect of IGF-1 on mitochondrial membrane potential and mROS production. Finally, we determined that the decrease in mitochondrial membrane potential and mROS caused by IGF-1 treatment is mediated by uncoupling protein 2 (UCP2) activity, and that the effects of IGF-1 treatment can be reversed by UCP2 inhibition.

Altogether, these studies implicate nutritionally regulated hormones in promoting $CD4^+$ T cell metabolism and function both in health and disease.

Dedication

To my parents, Christine and Mark Kiernan, for their enduring support and encouragement throughout my academic pursuits.

Contents

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1. Introduction

1.1 CD4$^+$ T cell immunity and metabolism

1.1.1 CD4$^+$ T cells in immunity

The immune system consists of two main branches, innate and adaptive (1). The innate immune system is characterized by rapid and non-specific responses to infection, driven by recognition of pathogen associated molecular patterns. The innate immune response activates within minutes to hours, controlling the infection until the adaptive immune response can activate and clear the infection. The adaptive immune response consists of many specialized cells, including T cells and B cells, which recognize specific molecular signatures of pathogens called antigens using highly specific antigen receptors. Thus, the response mounted by T cells and B cells is specific and potent for each pathogen encountered. Development and amplification of these specific responses takes about five days after initiation of the infection response.

CD4$^+$ T cells, also called T helper cells, are a subset of the T cell lineage and have a critical role in coordinating the immune response (1). CD4$^+$ T cells produce cytokines to direct other immune cells to perform their functions. For example, B cells are stimulated to produce antibodies, and macrophages are directed to kill engulfed pathogens. CD4$^+$ T cells are activated by recognition of antigen presented by antigen presenting cells such as dendritic cells. T cells require three signals to become fully activated: binding of the T cell receptor (TCR) to presented antigen, costimulation by binding of CD28, and cytokines from innate immune cells.

1.1.2 CD4$^+$ T cell subsets and their function

To address categories of pathogen threats, the immune system deploys effector programs with specific functional characteristics to deploy the appropriate response. Upon activation, CD4$^+$ T cells implement one of several functional programs that is determined by the cytokine environment in which they are activated (1). Specifically, innate immune cells produce cytokines that direct T cells to upregulate a cellular program that determines their differentiation into functional T cell subsets. In this way, the CD4$^+$ T cell coordination response is determined by the nature of the pathogen being targeted. Each subset of CD4$^+$ T cells is driven by a lineage-defining transcription factor and produces a defined set of cytokines.

T helper 1 (Th1) cells are formed when CD4$^+$ T cells are activated in the presence of IL-12 (1, 2). Th1 cells are characterized by expression of the transcription factor, T-box expressed in T cells (Tbet; TBX21), and production of IFN-γ (1, 3). Th1 cells primarily coordinate the response to intracellular bacteria and viral infection, and they activate macrophages to dispose of infected cells.

T helper 2 (Th2) cells are formed when CD4$^+$ T cells are activated in the presence of IL-4 (4). Th2 cells are characterized by expression of transcription factor Gata binding protein 3 (Gata3) and production of IL-4, IL-5, and IL-13 (5). The primary function of Th2 cells is to coordinate the immune response against parasites and helminths (1). This is accomplished by recruiting eosinophils, basophils, and mast cells.

T helper 17 (Th17) cells are formed in the presence of TGF-β and IL-6(6, 7, 8). Th17 cells are characterized by expression of transcription factor RAR-related orphan receptor gamma (RORγT) and production of IL-17 (1, 9). Th17 cells primarily respond to infections by extracellular bacteria and fungi and recruit neutrophil responses.

Unlike the other subsets of $CD4^+$ T cells, some T regulatory (Treg) cells are programmed during development and others differentiate in the periphery in response to TGF-β and IL-2 (1, 10). Treg cells are characterized by expression of forkhead box P3 (FoxP3) and activation marker CD25 (IL-2 receptor alpha chain) (11, 12). The main function of Treg cells is to suppress effector T cell responses by production of cytokines such as TGF-β and IL-10, or direct cytolysis of activated $CD4^+$ or $CD8^+$ T cells (1). Treg cells are critically important in controlling the infection response to protect against damage caused by the immune system.

1.1.3 $CD4^+$ T cell metabolism

1.1.3.1 Metabolism of activated $CD4^+$ T cells

To perform their functions, immune cells must dynamically regulate their metabolism. Quiescent, naïve T cells require very low metabolic activity. Naïve T cell metabolism is characterized by glucose and amino acid catabolism, and is maintained by IL-7 signaling (13). Upon activation, T cells need to dramatically upregulate biosynthetic pathways to grow, divide, migrate, and produce cytokines. In general, T cells do this by upregulating several anabolic pathways including: glucose uptake and aerobic glycolysis, glutamine uptake and glutaminolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and fatty acid synthesis (13). T cell activation activates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway which drives the shift to anabolic metabolism (13). Furthermore, several nutrients such as glutamine also contribute to mTOR complex 1 (mTORC1) activation, which results in a feed forward loop promoting the anabolic metabolism of activated T cells (13).

Costimulation via CD28 induces upregulation of glucose transporter 1 (Glut1) on the T cell surface, which induces increased glucose uptake (14). Glut1 is required for increased glucose uptake, glycolysis, growth, and proliferation of T cells (14). Loss of Glut1 results in impaired T cell activation, while overexpression of Glut1 increases T cell activation (15). Without Glut1, effector T cell subsets also had reduced expansion (15).

Once glucose has entered the cell, T cells metabolize glucose through aerobic glycolysis (Figure 1). Aerobic glycolysis, while a less efficient way to make ATP than oxidative phosphorylation (OXPHOS), generates important intermediates that feed into other biosynthetic pathways (13, 16). Glucose-derived pyruvate can have multiple fates.

Pyruvate can be converted to lactate and exported from the cell (13). Alternatively, it can be used to synthesize certain amino acids or converted to acetyl-CoA which feeds into the TCA cycle. Glycolytic intermediates such as phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3PG) can be used for amino acid synthesis and serine biosynthesis, while glucose-6-phosphate (G6P) can feed the pentose phosphate pathway to support nucleotide synthesis (17, 18).

Glutamine uptake is also increased upon T cell activation with the upregulation of its receptor, alanine-serine-cysteine transporter 2 (ASCT2) (19). Metabolism of glutamine through glutaminolysis contributes to several anabolic pathways (Figure 1) (13). Glutamine can be converted to glutamate, which is a precursor for the synthesis of several amino acids as well as nucleotides. Glutamine metabolism feeds into the TCA cycle with conversion of glutamate to α-ketoglutarate. Additionally, glutamine is a precursor to glutathione, which performs an important antioxidant function in the cell by reducing reactive oxygen species (ROS) (20).

Both glycolysis and glutaminolysis feed intermediates into the TCA cycle (13, 16, 18) (Figure 1). The TCA cycle provides reduced electron carriers such as NADH and FADH2 that donate electrons for OXPHOS, as well as intermediates for amino acid, fatty acid, and nucleotide synthesis. In the inner mitochondrial membrane, electrons are passed from carriers (NADH, NADPH, and FADH2) to the electron transport chain (ETC). While the electrons move through the ETC, protons are pumped across the inner mitochondrial membrane to generate a proton gradient which then drives ATP synthesis. While activated effector T cell subsets most dramatically upregulate and rely on glycolysis, OXPHOS is also upregulated to provide ATP.

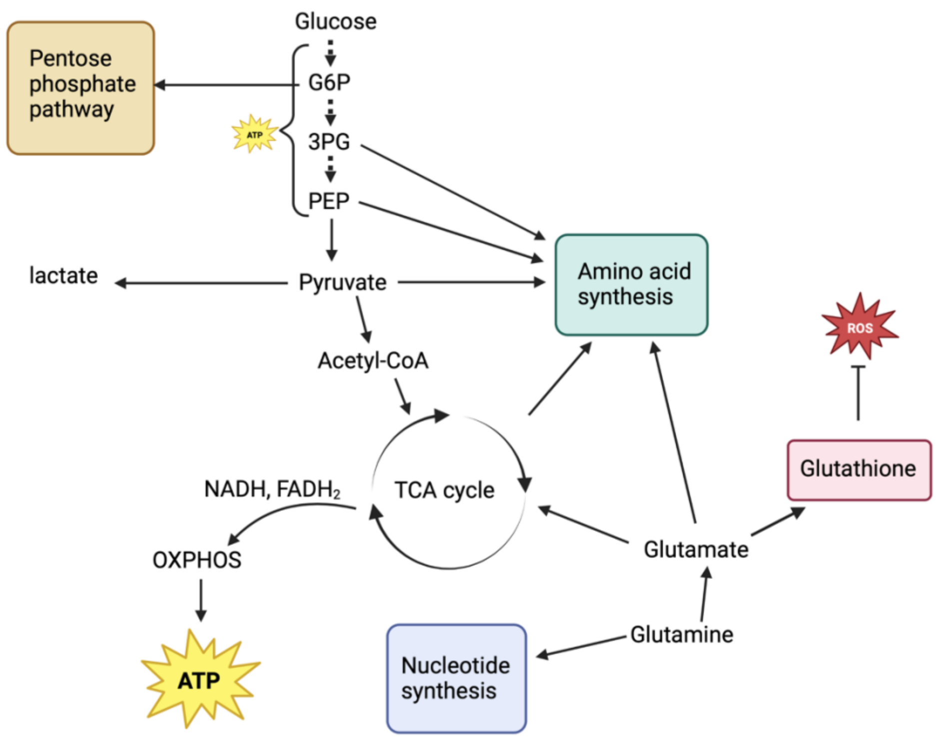

Figure 1: T cell activation promotes anabolic metabolic pathways.

Activation of T cells promotes increased cellular metabolism through pathways that support biosynthesis of macromolecules required for T cell function. Glycolytic intermediates support the pentose phosphate pathway and amino acid synthesis. Glutaminolysis supports nucleotide synthesis, amino acid synthesis and protects from ROS by increasing glutathione levels. Created with Biorender.

1.1.3.2 Th1 and Th2 cell metabolism

Specialized subsets of $CD4^+$ T cells have unique metabolic programs that correspond to their functions (Figure 2). In this way, T cell metabolism and function are intimately connected. In fact, modifying T cell metabolism has direct effects on the function and differentiation of T helper cell subsets.

Th1 and Th2 cells rely primarily on aerobic glycolysis and oxidative phosphorylation for energy. Both Th1 and Th2 cells are characterized by dramatically upregulated Glut1 expression compared to naïve $CD4^+$ T cells (21). Th1 cell fate is promoted by signaling through the PI3K/Akt/mTORC1 pathway(22, 23, 24). Specifically, mTORC1 activates Tbet, the Th1 lineage-defining transcription factor. Inhibiting mTORC1 results in decreased IFN-γ production by Th1 cells (25). Metabolism of Th2 cells differs from Th1 cells in that Th2 cells uniquely rely on mTORC2 signaling in addition to mTORC1 (26). Loss of mTORC2 only impacts Th2 differentiation, while mTORC1 deficiency results in loss of Th1, Th2, and Th17 cells (24, 26, 27).

1.1.3.3 Treg metabolism

Regulatory T cells have distinct metabolic needs as compared to effector T cell subsets (Figure 2). While Treg cells have greater glucose metabolism than naïve T cells, glycolysis is much lower in Treg cells than in activated effector T cell subsets(21, 28). Treg cells primarily rely on fatty acid oxidation and oxidative phosphorylation (29).

FoxP3 expression has been shown to inhibit PI3K/Akt/mTOR signaling, thus limiting glycolysis and the anabolic program induced in inflammatory T cell subsets (30). Overexpression of Glut1 in Tregs expands Treg cell numbers but reduces their suppressive function and FoxP3 expression, suggesting that Treg cells require reduced glycolytic metabolism (30). Furthermore, FoxP3 suppresses expression of myc, a transcriptional driver of glycolysis downstream of mTOR signaling (31). While Treg cells do not rely on mTOR, they seem to be driven by AMPK signaling, as shown by increased AMPK phosphorylation in Treg cells, and the promotion of Treg cell lineage by the AMPK activator, metformin (21, 28).

1.1.3.4 Th17 metabolism

Th17 cell metabolism is composed of a combination of aerobic glycolysis, glutaminolysis and fatty acid synthesis (Figure 2). Like Th1 and Th2 cells, Th17 cells rely on mTORC1 signaling and increased Glut1 expression (21, 24, 27). Th17 cells have a greater dependence on glutamine and glutaminolysis as compared to other T cell subsets. Glutaminolysis is required for Th17 differentiation, especially the conversion of glutamine to glutamate, which can feed the TCA cycle and other metabolic pathways (20). For example, glutamate supports glutathione production to protect against ROS which can be detrimental to Th17 cell function and differentiation (20). Furthermore, glutamine metabolism epigenetically inhibits FoxP3 expression in Th17 cells (32).

Interestingly, Th17 cells rely on fatty acid synthesis to produce the lipids they require (33). Deficiency in acetyl-CoA carboxylase (ACC1), an enzyme that drives the fatty acid synthesis pathway, results in diversion of Th17s to the Treg lineage (33).

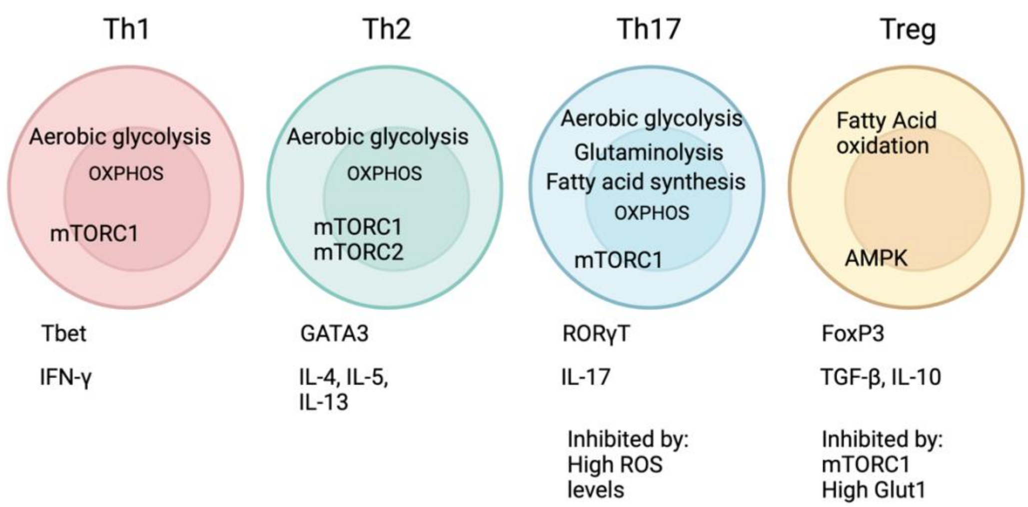

Figure 2: Metabolism of T helper cell subsets

CD4+ T cells differentiate into functional subsets with unique metabolic programs to support their functional requirements. Adapted from MacIver et al, Ann. Rev. Immunol, 2014. Created with Biorender.

1.1.3.6 Reactive oxygen species production and Th17 cells

The requirement and contribution of ROS to Th17 cell differentiation and function is debated. Several groups convincingly show that moderation or control of

ROS is required for optimal Th17 differentiation and cytokine production. Gerriets et al. show that inhibition of ROS with the antioxidant N-acetylcysteine (NAC) increases Th17 cell differentiation, suggesting that Th17 cells are sensitive to ROS and that ROS inhibits Th17 differentiation (34). This idea is further promoted by evidence showing Th17 cell cytokine production is decreased with pro-oxidant treatment, which induces ROS production (34, 35). Other groups showed similar results whereby NAC treatment increased Th17 cell differentiation and cytokine production (36, 37). These data suggest that abrogation of ROS levels by anti-oxidant treatment promotes Th17 cell differentiation.

In contrast, several studies demonstrated that Th17 cells require ROS for differentiation and function. Inhibiting mitochondrial ROS (mROS) production results in reduced Th17 differentiation in vitro (38). This suggests that Th17 cells require mROS during differentiation. Another study reported that increased Th17 differentiation coincided with increased ROS production in these cells (39). Treatment with the antioxidant NAC reduced Th17 differentiation.

Overall, the dependence of Th17 cells on ROS production is unclear. It seems that ROS signaling is important for T cell activation and to some extent for differentiation. However, there are divergent results suggesting that ROS is both required and inhibitory for Th17 differentiation. Further investigation of the role of ROS signaling in Th17 cells is needed.

1.2 The impact of nutritional status on immunity

Portions below are modified from the following publication: "The role of the adipokine leptin in immune cell function in health and disease" by Kaitlin Kiernan and Nancie J. MacIver, Frontiers in Immunology, 2021

Nutritional status and immune function are intimately connected (40). Overnutrition and undernutrition both confer susceptibility to infection. Obesity increases the risk of autoimmune disease while undernutrition protects against it. In these settings, the immune system is dysregulated, resulting in impaired or aberrant immune responses.

1.2.1 Undernutrition

Undernutrition is characterized by a state of immunodeficiency, which results in increased susceptibility to infection (40). Studies of undernourished children reported lymph node atrophy, reduced vaccine responses, and reduced effector T cell numbers (32). Interestingly, immunodeficiency associated with undernutrition confers some protection against autoimmune disease (40).

Undernutrition is associated with specific defects at the T cell level. First, T cell numbers in mice are decreased after a 48 hour fasting period or when fed a protein deficient diet (41, 42, 43, 44). Undernutrition results in T cell functional defects as well. Fasting during induction of a mouse model of multiple sclerosis protected against severe disease, suggesting that undernutrition reduces T cell function (45). Additionally, T cells from fasted mice produce less cytokine, and have reduced glucose metabolism (45).

1.2.2 Obesity

On the other hand, obesity is associated with a chronic, low-grade systemic inflammation that has been shown to promote the development of multiple disorders of health including type 2 diabetes, autoimmunity, nonalcoholic fatty liver disease, asthma, and cardiovascular disease (46, 47). This obesity-associated inflammation is characterized by increased circulating inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 6 (IL-6), as well as an increase in pro-inflammatory immune cells, particularly macrophages and lymphocytes (48, 49, 50, 51, 52, 53, 54).

While there is an increase in inflammatory immune cells in obesity, the response to infection is impaired (40). Obesity increases the risk of severe infection and death for several viral and bacterial illnesses. For example, patients with obesity are predisposed to increased severity of disease and mortality from influenza infection (55). Patients with obesity also had increased risk of severe outcomes from SARS-CoV-2 infection (55).

In the setting of obesity, both primary and secondary T cell responses to influenza infection have been shown to be impaired (56, 57, 58, 59, 60). Obese mice have increased levels of inflammatory T cells in the adipose tissue, particularly increased Th1, Th17, and $CD8^+$ T cells (60, 61). These cells contribute to the production of inflammatory cytokines IFN-γ and IL-17 that promote obesity-associated inflammation. Furthermore, T cells from obese mice have increased glucose uptake and oxidative metabolism (60). T cell metabolic dysfunction in obesity is rescued by administration of metabolic drugs such as metformin (60). Influenza outcomes were improved in obese mice treated with metformin, supporting the idea that restoration of "lean" T cell metabolism promotes improved T cell function (60). Thus, a dysregulated nutritional environment affects both immune cell function and metabolism.

1.3 Mediators of nutritional status on immunity

There are several hormones, adipokines and cytokines that are regulated by nutritional status. These include leptin, insulin, and insulin-like growth factor 1 (IGF-1), among others. These mediators act on many cells in the body to change cellular metabolism and function in response to the nutritional status of the organism. Thus, these hormones are primed to relay information about systemic nutritional status to immune cells, impacting immune cell function and metabolism.

1.3.1 Leptin

Portions below are modified from the following publication: “The role of the adipokine leptin in immune cell function in health and disease” by Kaitlin Kiernan and Nancie J. MacIver, Frontiers in Immunology, 2021

1.3.1.1 Leptin function

Leptin can be produced by multiple cells in the body, including immune cells, but is primarily produced by adipocytes in proportion to adipocyte mass, such that increasing adiposity leads to increased systemic concentrations of leptin (62, 63). Although leptin is produced in a diurnal manner (64), it is not a fast-acting signal or cytokine, but rather communicates stable nutritional status to the body as a whole. Leptin has a well-defined role as a metabolic mediator and communicator of nutritional status at the level of the hypothalamus where leptin receptors are highly expressed. Increased leptin signaling at the hypothalamus regulates appetite and leads to decreased nutrient intake and increased energy expenditure. Studies of leptin deficiency and fasting have demonstrated that leptin signaling is also required for normal reproductive hormone production, as well as thyroid hormone. Therefore, leptin plays a critical role in controlling energy homeostasis, metabolism, and neuroendocrine function (65, 66, 67).

Over the last two decades, it has become apparent that leptin also has a critical role as an immune modulator. This was initially observed in individuals with rare mutations in leptin or the leptin receptor, who are obese from lack of leptin signaling at the hypothalamus, but were also found to have an increased risk of intracellular infections secondary to immune cell deficiencies (68). Leptin has subsequently been shown to act on several different immune cell types and can affect both immune cell development and function. Through that mechanism, increased systemic leptin levels in diet-induced obesity directly promote obesity-associated inflammation.

1.3.1.2 Leptin effects on T cells

Leptin plays an important role in T cell development. Leptin deficiency has been shown to result in thymic atrophy and decreased circulating T cell numbers (69, 70, 71). Interestingly, leptin receptor has been found to be expressed on double negative, double positive and CD4 single positive thymocyte subsets, but not on CD8 single positive thymocytes (72). Moreover, leptin treatment rescued $CD4^+$ T cell development in leptin mutant (*ob*/*ob*) mice but did not rescue $CD8^+$ T cell development (72). Together this suggests that leptin is required for early T cell development and for later development of $CD4^+$ T cells, but not $CD8^+$ T cells.

$CD4^+$ T cells express high levels of the long isoform of the leptin receptor (Ob-Rb), which is significant because it is the only isoform that can signal through the Janus kinase (*JAK*)-signal transducer and activator of transcription (*STAT*) pathway (73**).** Leptin receptor signaling in T cells has been shown to promote survival, proliferation, cytokine production, and differentiation. *In vivo*, leptin treatment of wildtype (WT) mice was shown to inhibit steroid-induced apoptosis of lymphocytes (74). In response to leptin treatment, naïve $CD4^+$ T cells, but not memory T cells, showed an increase in proliferation in a mixed lymphocyte reaction (75). In an older study of human cells, monocyte-depleted peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA) and Concanavalin A (ConA) and treated with leptin had increased proliferation compared to untreated cells (76). More recent studies have demonstrated that $CD4^+$ T cells from leptin receptor mutant (*db/db*) mice have reduced proliferation when compared to WT $CD4^+$ T cells, suggesting that leptin signaling on $CD4^+$ T cells is required for proliferation (70).

JAK-STAT signaling is downstream of many lymphocyte receptors that promote the production of various cytokines. Thus, as one would predict, leptin treatment of bulk, non-differentiated T cells influenced cytokine production by these cells. Leptin treatment of $CD4^+$ T cells increased pro-inflammatory cytokine production, namely T helper 1 (Th1) cytokines interferon gamma (IFN-γ) and IL-2, while decreasing production of the T helper 2 (Th2) cytokine IL-4 (75). Moreover, activated $CD4^+$ T cells generated from T cell specific leptin receptor conditional knock out mice were found to produce less IFN-γ than WT $CD4^+$ T cells (70). Together, these data suggest that leptin promotes pro-inflammatory cytokine production in $CD4^+$ T cells.

Leptin has also been shown to play a role in the differentiation of T cells into functional subsets. Hypoleptinemia induced by fasting has been shown to suppress the number of effector T cells, but not regulatory T cells in mice. In fact, the same study found that while Treg cell proportions were increased in fasting, absolute numbers of Treg cells were unchanged, suggesting that leptin promotes the differentiation of effector T cells, but not Treg cells, and that any change in Treg cell proportions were indirect (71). In contrast, $CD4^+$ T cells isolated from fasted hypoleptinemic mice had decreased differentiation into T helper 17 (Th17) cells *in vitro* compared to $CD4^+$ T cells isolated from *ad lib* fed mice. When the fasted mice were given leptin injections twice daily, Th17 differentiation was restored, suggesting that leptin is critical for differentiation into Th17 cells (71). In support of this, Th17 differentiation *in vitro* was decreased in $CD4^+$ T cells isolated from mice with T cell specific knockout of leptin receptor compared to WT controls (71). Furthermore, T cell specific leptin receptor knock out mice had decreased frequency of Th17 cells and increased frequency of Treg cells in the lamina propria (77).

The mechanism by which leptin promotes Th17 differentiation has been investigated. Leptin signaling promotes transcription of RAR-related orphan receptor gamma (RORγt), which is the critical transcription factor for Th17 fate. When RORγt-deficient $CD4^+$ T cells were retrovirally transfected with a plasmid containing the *Rorc* gene, which encodes RORγt, leptin treatment was shown to increase transcription of

RORγt in these cells (78). This mechanism could also explain the inhibition of Treg differentiation by leptin, because Th17 and Treg cells have an antagonistic developmental program, where expression of the Th17 transcriptional program inhibits Treg development and vice versa, so that leptin promotion of Th17 fate by increasing RORγt transcription also directly inhibits Treg differentiation (79, 80). Given the pro-inflammatory effect of leptin on T cells, leptin is being investigated for use in cancer treatment to enhance the tumor-fighting action of T cells (81).

Interestingly, Treg cells express high amounts of leptin receptor, and have been shown to be capable of secreting leptin (82, 83). However, Treg cells are decreased in diet-induced obesity, which is consistent with the role of leptin in inhibiting Treg cell proportions, given that leptin levels are elevated in this setting (84). Treg cell proportions are also specifically decreased in the adipose tissue in diet-induced obesity, where leptin levels are expected to be highest (85). On the other hand, leptin mutant *ob/ob* mice were shown to have increased proportion of peripheral Foxp3+ $CD4^+$ Treg cells compared to WT mice, further supporting the role of leptin, and not obesity alone, in decreasing Treg cell proportions (83). Leptin has also been shown to inhibit Treg cell proliferation in primary human cells, and blockade of leptin binding to Treg cells using anti-leptin antibodies led to increased Treg cell proliferation (86).

Obesity-associated inflammation has been shown to drive insulin resistance, leading to type 2 diabetes (87). Methods that eliminate the inflammatory T cell response in obesity prevent insulin resistance and progression to type 2 diabetes. For example, several immunocompromised mouse models (NOD and SCID mice) have been found to be resistant to the development of obesity and insulin resistance when fed high fat diet (88). T cell-deficient TCR-knockout mice that lack $CD4^+$ and $CD8^+$ T cells had decreased obesity-induced macrophage infiltration into the adipose tissue and decreased insulin resistance on high fat diet compared to wildtype controls (89), and obese mice that lack IFN-γ had improved insulin sensitivity compared to obese wildtype controls (90). Similarly, knockout of the Th1-associated transcription factor T-bet improved insulin sensitivity in high-fat diet fed mice (91). Based on the pro-inflammatory effect of leptin on immune cells as described above, it is possible that obesity-associated hyperleptinemia is responsible, at least in part, for promoting the obesity-associated inflammation that leads to insulin resistance, glucose intolerance, and diabetes in obesity.

1.3.1.3 Leptin effects on T cell metabolism

It is now clear that leptin signaling through leptin receptor promotes a metabolic change in $CD4^+$ T cells. Since immune cell metabolism and function are intimately related, recent work has investigated if leptin-induced changes in $CD4^+$ T cell function are mediated by changes in T cell metabolism (16). This was first explored in a fasting model of hypoleptinemia. $CD4^+$ T cells isolated from fasted mice and activated *in vitro* showed decreased glucose uptake and decreased glycolytic rate compared to $CD4^+$ T cells isolated from *ad lib* fed control mice, suggesting that leptin signaling promotes glycolytic metabolism in $CD4^+$ T cells (70). As glycolytic metabolism is strongly associated with inflammatory function, this fits with the previously discussed role of leptin in promoting inflammatory cytokine production in $CD4^+$ T cells (70, 71). $CD4^+$ T cells isolated from leptin receptor mutant *db/db* mice also showed reduced glucose uptake, in part secondary to decreased glucose transporter Glut1 expression, and decreased glycolytic rate compared to WT $CD4^+$ T cells when activated *in vitro*. Additionally, $CD4^+$ T cells from *db/db* mice were less metabolically active with decreased extracellular acidification rate (ECAR), a measure of lactate production downstream of glycolysis, as well as decreased oxygen consumption rate (OCR), a measure of mitochondrial oxidation (70). These studies indicate that leptin receptor signaling in T cells leads to changes in cellular metabolism.

The functional subsets of $CD4^+$ T cells have distinct metabolic characteristics, and leptin influences the metabolism of these subsets in different ways. $CD4^+$ T cells were isolated from WT mice that were either fed *ad lib*, fasted for 48 hours to promote hypoleptinemia, or fasted while receiving twice daily intraperitoneal leptin injections, and differentiated *in vitro* into Th17 or Treg cells. Th17 cells generated from fasted mice showed decreased ECAR and OCR, but this was rescued when fasted mice received leptin injections (71). In contrast, Treg cell metabolism was not impacted by fasting (71).

To investigate the direct role of leptin signaling on T cell metabolism, $CD4^+$ T cells were isolated from T cell specific leptin receptor conditional knockout mice or controls and differentiated into Th17 or Treg cells *in vitro* (71). Th17 cells from leptin receptor knockout mice, but not Treg cells, showed decreased expression of key metabolic genes Glut1 and hexokinase 2 (HK2), which is a rate-limiting enzyme of glycolysis (71). Th17 cells from leptin receptor knockout mice also had decreased glucose uptake and lactate production compared to Th17 cells from controls, suggesting that leptin signaling promotes appropriate Th17 cells glycolytic signaling to fuel Th17 cell function (71). Combined, these data suggest that leptin has a T cell intrinsic effect on metabolism that promotes glycolytic and oxidative metabolism necessary for proper T cell function.

1.3.2 Insulin

1.3.2.1 Insulin function

Insulin is secreted by pancreatic beta cells in response to glucose levels in the blood, thus insulin levels are closely tied to nutritional status (92, 93). Insulin's primary systemic actions are directed toward three main cell types, although all cells are capable of responding. Insulin acts on skeletal muscle to increase glucose uptake and subsequent protein synthesis, glycogenesis, and lipogenesis. Insulin acts on hepatocytes to stimulate glycogenesis (store glucose in glycogen) while adipocytes are stimulated to increase lipogenesis. On the other hand, insulin signaling inhibits gluconeogenesis, lipolysis, and glycogenolysis. Overall, insulin's actions are anabolic, signaling to the body to store and use the available energy rather than depleting glycogen and lipid stores.

1.3.2.2 Insulin effects on T cells

Insulin receptor (IR) expression on T cells was first described many years ago. Antigen stimulation induced radioactive insulin binding to T cells, thus IR was explored as a marker of T cell activation (94, 95). Insulin was subsequently shown to act directly on T cells via IR (95). Upon stimulation, IR is internalized and signals through the PI3K and MAPK pathways (96, 97).

Insulin signaling has been shown to mediate a variety of functional effects on T cells. First, insulin treatment was shown to modulate T cell IL-2 production (98, 99). One group showed that insulin promotes the differentiation and function of T helper 2 cells, promoting the idea that insulin has an anti-inflammatory action (100). In contrast, however, insulin treatment was shown to increase T cell cytotoxicity in vitro (101).

Inducible knockdown of IR in rats and T cell specific deletion of IR in mice suggested that insulin signaling was required for optimal T cell cytokine production, proliferation, and migration (102, 103). Specifically, loss of IR on T cells has been shown to decrease IFN-γ, IL-4, and IL-10 production after in vitro stimulation (102, 103). IR knockdown T cells also show increased apoptosis in reduced serum conditions. T cells from IR knockdown mice had reduced inflammatory function, as demonstrated by reduced cytotoxic activity of $CD8^+$ T cells and improved clinical scores in a mouse model of GVHD (102). T cell specific deletion of IR resulted in impaired response to influenza characterized by increased weight loss and decreased antigen specific $CD4^+$ and $CD8^+$ T cells (103).

Recent literature has explored the role of insulin on Treg function and differentiation, especially in the context of a high fat diet mouse model. Insulin treatment decreased IL-10 production by Treg cells in vitro downstream of increased Akt activation (104). Studies using a Treg specific insulin receptor knockout mouse ($FoxP3Cre^+$ $IR^{fl/fl}$) confirmed that loss of insulin receptor on Treg cells increased IL-10 production by Treg cells (105). Furthermore, loss of IR on Treg cells improved glucose and insulin tolerance in mice fed high fat diet (HFD) (105). Signaling through insulin receptor substrate (IRS), a downstream modulator of insulin receptor signaling, was shown to inhibit FoxP3 expression and Treg function via mTORC1 (106). Furthermore, overexpression of IRS1 in Treg cells reduced their suppressive capacity such that adoptive transfer of these cells could not control colitis (106). Conversely, knockdown of IRS1 promotes Treg cell differentiation (106). Taken together, these data suggest an inhibitory effect of insulin signaling via IR/IRS1/Akt/mTORC1 on Treg function and differentiation.

1.3.2.3 Insulin effects on T cell metabolism

Given the role of insulin in modulating systemic metabolism, there has been some effort to understand the effect of insulin on metabolism of immune cells, and T cells in particular. Early studies demonstrated that insulin increased T cell glucose uptake, lactate production and cellular glycogen content, suggesting that insulin acts on T cells in much the same way as it acts on other cells such as adipocytes and myocytes (107). More recently, insulin was shown to increase the expression of glucose transporters Glut1, Glut3, and Glut4 on T cells from the blood (108). IR knockdown was shown to decrease Glut 3 and Glut4 mRNA expression on activated T cells (102). Furthermore, IR knockdown T cells had decreased glucose metabolism as demonstrated by decreased glucose uptake, decreased lactate production, and decreased lactate dehydrogenase expression (102). Insulin treatment of $CD4^+$ T cells was demonstrated to increase both glycolytic and oxidative metabolism as demonstrated by extracellular flux analysis (103).

1.3.3 Insulin-like growth factor 1

1.3.3.1 Insulin-like growth factor 1 hormone function

Insulin-like growth factor 1 (IGF-1) is closely related to insulin and is also regulated by nutritional status. Whereas insulin is primarily regulated by carbohydrate metabolism, IGF-1 is regulated by total energy intake and protein intake, such that the reduction of protein intake corresponds to the reduction in serum IGF-1 levels (109). IGF-1 is also regulated by insulin, such that increased insulin levels increase IGF-1, so IGF-1 is indirectly regulated by carbohydrate intake. IGF-1 is primarily produced in the liver and has endocrine effects, but it can also be produced locally and exhibit paracrine/autocrine action. It has been shown that T cells are capable of producing IGF-1 in some contexts, particularly in the skin, but the physiological relevance of this is unclear (110). While the primary role of insulin is stimulating glucose uptake from the blood, IGF-1 stimulates protein synthesis, cell growth, and prevents apoptosis for many cells throughout the body (109).

1.3.3.2 Insulin-like growth factor 1 effects on T cells

There is a long line of evidence demonstrating a role for IGF-1 on T cells. In historical studies, IGF-1 receptor (IGF-1R) was shown to be expressed on the surface of both $CD4^+$ and $CD8^+$ T cells, as well as other immune cells. $CD4^+$ T cells expressed more IGF-1R than $CD8^+$ T cells, with the $CD8^+$ T cells expressing lower affinity receptors. IGF-1R is upregulated on T cells upon activation, and particularly upon CD28 stimulation/engagement (111, 112, 113). IGF-1 treatment causes downregulation of IGF-1R on the T cell surface, which is the result of internalization of the activated receptor (114, 115).

IGF-1 treatment was shown to promote survival, proliferation, growth, and cytokine production of T cells in vitro. Activation of IGF-1R by IGF-1 binding induces tyrosine kinase activity of IGF-1R. Downstream, PI3K activates Akt, c-Jun N-terminal kinase (JNK), and c-Jun phosphorylation as well as mTOR activation in activated T cells (97, 116, 117, 118). Inhibiting PI3K activity or blocking IGF-1R removes protection from Fas-mediated apoptosis (111). Furthermore, IGF-1 treatment also protects T cells from Fas-mediated apoptosis, suggesting that IGF-1 signaling through IGF-1R promotes T cell survival (111, 118, 119). IGF-1 has also been shown to promote cell growth and proliferation of T cells (113, 117, 118, 120). Several studies demonstrated that IGF-1 treatment of T cells increased IL-2 production (115, 121, 122). In vitro differentiated Th2 cells treated with IGF-1 increased IL-10 and IL-4 production, but not IL-2. In vitro differentiated Th1 cells did not show differential cytokine production upon IGF-1 treatment (122). Taken together these data suggest that IGF-1 plays a role in promoting T cell activation and proliferation, while also protecting from apoptosis.

A recent study explored the role for IGF-1 signaling in Th17 cells, specifically(123). RNA sequencing data demonstrated that Th17 cells had upregulated levels of IGF-1R pathway genes, indicating a potential role for this pathway in Th17 cell differentiation and function. Indeed, DiToro et al demonstrated that loss of IGF-1R on T cells resulted in reduced differentiation of Th17 cells and reduced cytokine production. These results were validated in vivo when T cell specific IGF-1R knockout mice showed reduced disease in a mouse model of multiple sclerosis that is known to be mediated in part by Th17 cells. In addition to the functional differences observed, IGF-1 treatment was shown to promote increased glycolytic metabolism of Th17 cells. Together, these data present a compelling case that IGF-1 signaling through IGF-1R is required for optimal Th17 differentiation and function, and its action is in part mediated by impacting cellular metabolism.

1.3.3.3 Insulin-like growth factor 1 impacts on cellular metabolism

IGF-1 mediates many of its effects by influencing cellular metabolism, and metabolism of the organism. IGF-1 coordinates the metabolic response by increasing glucose uptake similarly to insulin (124, 125), increasing free fatty acid uptake (126), and increasing amino acid transport(127, 128, 129).

IGF-1 has been shown to act on mitochondria, specifically by promoting mitochondrial biogenesis, mitophagy, and mitochondrial function. In cancer cells, IGF-1 promotes mitochondrial biogenesis by increasing transcription of Peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1β) and PGC-1-related coactivator (PRC), transcriptional mediators of mitochondrial biogenesis (130). IGF-1 treatment preserves mitochondrial membrane potential as measured by tetramethylrhodamine, methyl ester (TMRM) staining(131). IGF-1 increases expression of Bnip3, which initiates the mitophagy program (130).

Interestingly, IGF-1 seems to have a protective effect against oxidative damage. After pretreating a myoblast cell line with hydrogen peroxide, IGF-1R phosphorylation increased in response to IGF-1 treatment (132). That is to say, oxidative stress sensitized these cells to IGF-1. Additionally, pretreatment with antioxidants or various mitochondrial inhibitors did not affect IGF-1R phosphorylation downstream of IGF-1(132). These data are confirmed by a study of human umbilical vein endothelial cells where IGF-1R activation by IGF-1 induced protection from oxidative stress caused by hydrogen peroxide administration (131). These data suggest that IGF-1R signaling increases in response to oxidative stress in cancer cells, possibly to initiate an anti-oxidant program. Furthermore, protection from oxidative stress and initiation of programs such as mitophagy are protective against apoptosis.

1.3.4 Hormone receptors and signaling

1.3.4.1 Leptin receptor

Portions below are modified from the following publication: "The role of the adipokine leptin in immune cell function in health and disease" by Kaitlin Kiernan and Nancie J. MacIver, Frontiers in Immunology, 2021

The mechanism of leptin's actions on immune cells is complex, in part because leptin receptor has several isoforms generated though alternative splicing, which each have differing signaling capacities (133). For example, T cells express the long form of the leptin receptor, particularly after activation, while neutrophils only express the short form, and natural killer cells express both the short and long form receptors (134). These isoforms differ primarily in the intracellular domain responsible for downstream signaling. While both the short and long receptor isoforms are capable of transmitting some signals inside the cell, it is believed that only the long form has complete signaling capabilities.

The long form of the receptor contains fully functional JAK2 binding sites, and upon leptin binding, the leptin receptor has been shown to homodimerize, bind to, and phosphorylate JAK2 (133). STAT3 proteins are then recruited to the receptor complex and phosphorylated, which leads to STAT3 dimerization, translocation to the nucleus, and binding to promoter sites. The system is highly regulated, as this signaling also leads to transcription of suppressor of cytokine signaling 3 (SOCS3), which is a negative regulator of the JAK/STAT signaling cascade. Leptin receptor can also signal through the PI3K/Akt and MAPK pathways through IRS-1/2 and SHP-2 recruitment, respectively (135).

1.3.4.2 Insulin and IGF-1 receptor

The insulin receptor (IR) and IGF-1 receptor (IGF-1R) are highly related dimeric transmembrane receptor tyrosine kinases. IR and IGF-1R share 48% amino acid sequence homology (136). While IR and IGF-1R have distinct downstream effects in the cell, their signaling pathways are closely related. IR is mainly known for generating metabolic effects downstream, while IGF-1R signaling generates mitogenic and proliferative effects. IR and IGF-1R are so closely related that IR and IGF-1R chains are capable of heterodimerizing to form an IR/IGF-1R hybrid receptor consisting of one IR chain and one IGF-1R chain. This hybrid receptor has further distinct signaling from IR and IGF-1R homodimers. IR and IGF-1R have distinct ligand binding capabilities (137). IGF-1R can bind both insulin and IGF-1 but has much greater affinity for IGF-1. Likewise, IR can bind both insulin and IGF-1 with greater affinity for insulin. IR/IGF-1R hybrid receptors have greater affinity for IGF-1 than insulin (138).

IR and IGF-1R signaling pathways have considerable overlap given their sequence homology. There are two primary arms of IR and IGF-1R signaling: the PI3K/Akt pathway and the Ras-MAPK pathway(97, 139). Binding of insulin or IGF-1 to the receptor results in a conformational change and phosphorylation of tyrosine residues in the intracellular domain (140, 141). IGF-1R has been shown to be internalized upon stimulation with IGF-1 (114, 142). The phosphorylation of IRS, Shc and other docking proteins such as Grb2 potentiate the downstream signaling cascades (143). Specifically, Grb2 activates Ras and the subsequent MAPK cascade which is responsible for promoting cell survival and DNA synthesis. The PI3K pathway is initiated by the phosphorylation of PIP2 to PIP3, which then activates Akt. Downstream of Akt are mTOR and GSK3b which promote protein synthesis and glucose metabolism, respectively. Phosphoproteomic analyses revealed that IR prefers signaling through

PI3K/mTORC, while IGF-1R preferentially phosphorylates Ras/Rho GTPases (144). In addition to these signaling cascades, SUMOylation of IGF-1 causes nuclear translocation where IGF-1R can phosphorylate transcription factors such as c-Jun N-terminal kinase (JNK), Jak, and SOCS transcription factors (145). Thus, IR and IGF-1R signaling potentiate complex pleiotropic effects on many cell types.

Despite the overlap in their signaling cascades, distinct programs of transcriptional regulation are apparent downstream of receptor activation (146, 147). Specifically, IGF-1R signaling promoted growth factor receptor bound protein 2 (Grb2) and Shc phosphorylation, while IR signaling promoted phosphorylation of insulin receptor substrate 1 (IRS-1). These events correspond to a bias toward cell cycle/proliferation and metabolic pathway regulation, respectively.

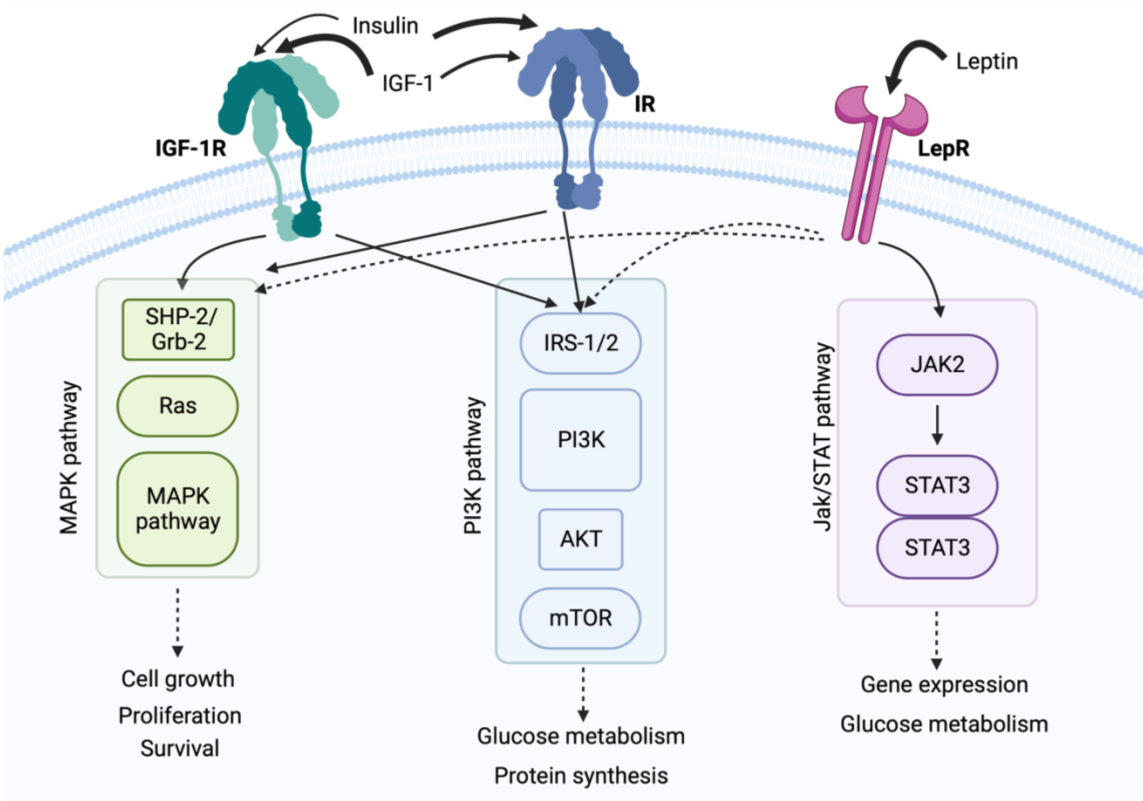

Figure 3: Signaling pathways downstream of nutritionally regulated hormone receptors

IGF-1R, IR, and LepR signal through overlapping pathways. IR and IGF-1R signal through the PI3K/Akt/mTOR and MAPK pathways to promote growth, survival and metabolic remodeling. LepR primarily exerts metabolic and functional effects through the Jak2/STAT3 pathway, but there is significant crosstalk with the PI3K and MAPK pathways. There is evidence that LepR and IR/IGF-1R signaling at the cell membrane such that insulin or IGF-1 treatment can induce phosphorylation of the leptin receptor and vice versa(148). Created with Biorender.

1.4 Dissertation Goals

To initiate the appropriate T cell response, three signals are canonically required. Signal 1 is TCR stimulation, which must be accompanied by signal 2, costimulation. Signal 3 is cytokine stimulation, which will provide information about the nature of the infection and push the T cell toward the appropriate subset. It is increasingly recognized that there is a fourth factor integrated into the decision matrix of T cell activation: nutritional signals. Protein hormones such as insulin and IGF-1 and adipokines such as leptin provide information about nutritional availability. Insulin and IGF-1 receptors signal through some of the same pathways that are required for T cell activation, which suggests that nutritional signals could augment T cell activation. Alternatively, nutritional signals act as a decision-making node whereby adequate nutrient availability licenses the activation, proliferation, and metabolic upregulation of the T cell. We set out to understand the requirement for and contribution of nutritionally regulated hormone signaling in the regulation of $CD4^{+}$ T cell metabolism and function.

1.4.1 Determine if leptin receptor signaling in T cells is critical for the development of T cell inflammation that drives glucose intolerance in obese mice.

Systemic metabolic dysfunction, especially glucose metabolism, is downstream of inflammatory cytokine production by immune cells in the adipose tissue. Systemic glucose intolerance in obesity has been shown to be driven by inflammatory immune cells, including T cells. Since leptin is an adipokine that increases in obesity, and functions to promote inflammatory cytokine production by T cells, leptin is poised to influence the inflammatory T cell environment in obesity. We set out to understand whether leptin signaling to T cells drives dysfunctional glucose metabolism in obesity.

1.4.2 Identify metabolic mechanisms by which insulin/IGF-1 signaling influence T cell differentiation and cytokine production.

While insulin and IGF-1 signaling to T cells has been explored in recent years, there are several unanswered questions remaining. Given the homology of insulin and IGF-1 receptors and their similar signaling pathways, the impact of insulin and IGF-1 to the metabolic and functional downstream consequences must be elucidated. Additionally, the mitochondrial consequences of insulin and IGF-1 signaling on $CD4^+$ T cells remain unknown. Since IGF-1 is known to promote mitochondrial biogenesis and function on other cells, we anticipate that IGF-1 will regulate mitochondrial function and metabolism in $CD4^+$ T cells.

2. Effects of T cell leptin signaling on systemic glucose tolerance and T cell responses in obesity

Portions of the following section were modified from the following manuscript: “Effects of T cell leptin signaling on systemic glucose tolerance and T cell responses in obesity.” Kaitlin Kiernan, Amanda G. Nichols, Yazan Alwarawrah, and Nancie J. MacIver, 2023 (in revision)

2.1 Introduction

Obesity, which is increasing at an alarming rate in the United States and other developed countries (149), is associated with a wide range of comorbidities, including type 2 diabetes, hyperlipidemia, hypertension, heart disease, ischemic stroke and several types of cancer (150, 151, 152, 153, 154, 155). Numerous studies have demonstrated that the development of many of these obesity-associated comorbidities is promoted by a chronic, low grade inflammatory state (156, 157, 158, 159, 160). In mouse studies of visceral adipose tissue, pro-inflammatory immune cells are preferentially increased in the adipose tissue of obese mice compared to lean mice. In particular, increases in M1-like pro-inflammatory macrophages, $CD8^+$ T cells, T helper 1 (Th1) cells, and B cells are observed (161). In contrast, there are decreased percentages of T regulatory (Treg) cells, T helper 2 (Th2) cells, natural killer T (NKT) cells, eosinophils, and type 2 innate lymphoid (ILC2) cells. This influx of inflammatory immune cells into obese adipose tissue promotes the production of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), and interferon gamma (IFN-γ), which contribute to the chronic inflammatory state (metaflammation) observed in obesity (161, 162, 163, 164, 165, 166, 167). Moreover, inflammatory cytokine production by adipose tissue immune cells has been shown to induce insulin resistance in obesity.

Although pro-inflammatory macrophages are the most predominant immune cell population in obese adipose tissue (168), multiple studies have now shown that inflammatory T cells also play a key role in driving obesity-associated inflammation. In a study of recombinase activating gene 1 knockout (Rag1$^{-/-}$) mice, in which T and B cells are not present, but the macrophage compartment remains intact, the Rag1$^{-/-}$ mice fed high-fat diet showed increased adiposity and weight gain, but had decreased inflammation in the adipose tissue compared to wildtype mice (169). In particular, there were decreased inflammatory innate immune cells present in the adipose tissue, and IFN-γ levels were decreased (169). These data suggest that adaptive immune cells, likely T cells, significantly contribute to the inflammatory environment of the adipose tissue in obesity either by recruitment of inflammatory innate immune cells or maintenance of the inflammatory environment by cytokine production. Moreover, mouse models that knockout the T cell receptor (TCR$\beta^{-/-}$), the Th1-associated transcription factor T-bet, or the Th1-associated cytokine IFN-γ have been shown to be protected against insulin resistance and diabetes when placed on high-fat diet (91, 170). This suggests that not only are T cells required for the inflammatory environment in obesity, but they are also required for the development of the systemic metabolic phenotype associated with obesity.

Adipose-derived cytokines, called adipokines, can contribute to the inflammatory environment of the adipose tissue in obesity. One such adipokine is leptin, which is secreted in proportion to adipose tissue mass and is therefore increased in the setting of obesity and decreased in undernutrition (171). Leptin is best known for its role in the hypothalamus where leptin receptors are highly expressed and leptin signaling leads to decreased appetite and increased energy expenditure. The roles of leptin in neuroendocrine function, energy homeostasis and metabolism have been thoroughly reviewed (65, 66, 67). Interestingly, leptin also has a critical role as an immune modulator. Leptin has been shown to affect multiple immune cell types and mediate varying effects depending on the cell type or activation status of the cell, with particularly significant and striking effects on T cell development and function (171).

To start, leptin is required for early T cell development in the thymus. Double negative, double positive and CD4 single positive, but not CD8 single positive, thymocytes were shown to express leptin receptor (72). Furthermore, leptin administration to leptin deficient mice rescued $CD4^+$ T cell development, but not $CD8^+$ T cell development (72). In addition to the developmental requirement, leptin has also been shown to influence $CD4^+$ T cell function and differentiation. In particular, leptin promotes $CD4^+$ T cell differentiation into Th1 and T helper 17 (Th17) functional subsets and increases their IFN-γ and IL-17 production, respectively (71). One mechanism by which leptin may influence $CD4^+$ T cell function and differentiation is through cellular metabolism (70, 71). Specifically, leptin has been shown to increase expression of the glucose transporter Glut1 and increase glucose uptake and glycolytic metabolism, thereby promoting inflammatory cytokine production (70, 71). Thus, leptin modulates metabolism systemically at the level of the hypothalamus, but also at the level of the immune cell, where it can influence immune cell function.

Given the role of T cells in driving obesity-associated inflammation and insulin resistance and the role of leptin in promoting $CD4^+$ T cell inflammatory function, we asked: what is the role of leptin signaling in driving T cell inflammation in the context of obesity-associated changes in systemic metabolism? To answer this question, we set up the following studies (Figure 4). T cell-specific leptin receptor conditional knockout mice and littermate controls were either fed high-fat diet (60% kcal from fat) or low-fat diet for 18 weeks, starting at weaning. Body weights and blood glucose levels were monitored throughout the study. At the completion of the dietary intervention, a glucose tolerance test was performed, and $CD4^+$ T cells were isolated from spleens to measure cellular metabolism and function.

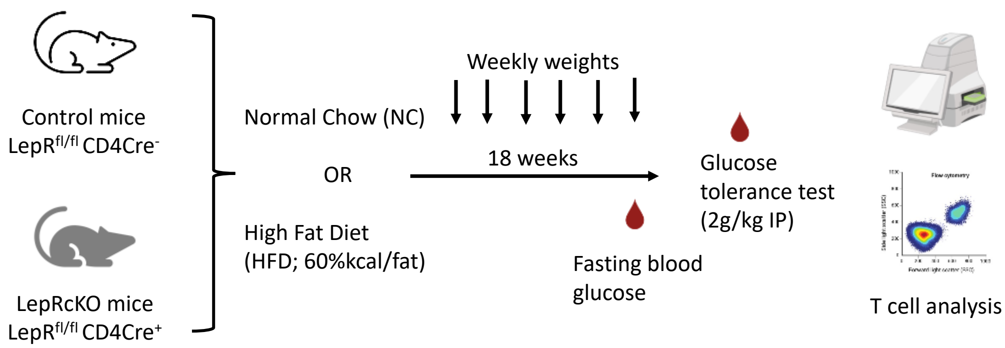

Figure 4: Experimental setup exploring requirement for leptin receptor signaling in T cells in diet induced obesity

2.2 Results

To determine the role of leptin signaling in driving T cell inflammation in the context of obesity-associated changes in systemic metabolism, we generated T cell-specific leptin receptor knockout mice by crossing leptin receptor floxed (*LepR$^{fl/fl}$*) animals with mice expressing a Cre recombinase transgene under the control of the CD4$^+$ promoter (*CD4Cre*) on the C57BL/6 background, as we have previously described (70, 71). These mice have a selective deletion of leptin receptor in both CD8$^+$ and CD4$^+$ T cells. Given our prior work demonstrating the effect of leptin on CD4$^+$ T cells, particularly Th1 and Th17 cells, as well as the relatively low expression of leptin receptor on $CD8^+$ T cells compared to $CD4^+$ T cells, we anticipated that this deletion would affect the metabolism and function of $CD4^+$ T cells more so than $CD8^+$ T cells (70, 71).

2.2.1 Leptin receptor deficiency on T cells does not confer protection from systemic glucose intolerance in diet-induced obesity

T cell-specific leptin receptor knockout mice ($CD4Cre^+LepR^{fl/fl}$) and littermate controls expressing leptin receptor ($CD4Cre^-LepR^{fl/fl}$) were placed on high-fat diet (HFD; 60% kcal from fat) or low-fat, normal chow (NC; 10% kcal from fat) for 18 weeks, starting at weaning at 3 weeks of age. This study design allowed us to collect longitudinal data during the development of obesity. The mice on HFD gained significantly more weight than the mice on NC, but there was no difference in weight gain between T cell-specific leptin receptor knockout mice and littermate controls on the same diet (Figure 5A). These findings were consistent in both male and female mice; however, male mice on HFD showed increased weight gain at an earlier age than female mice, as well as greater weight gain overall (Figure 5B-C).

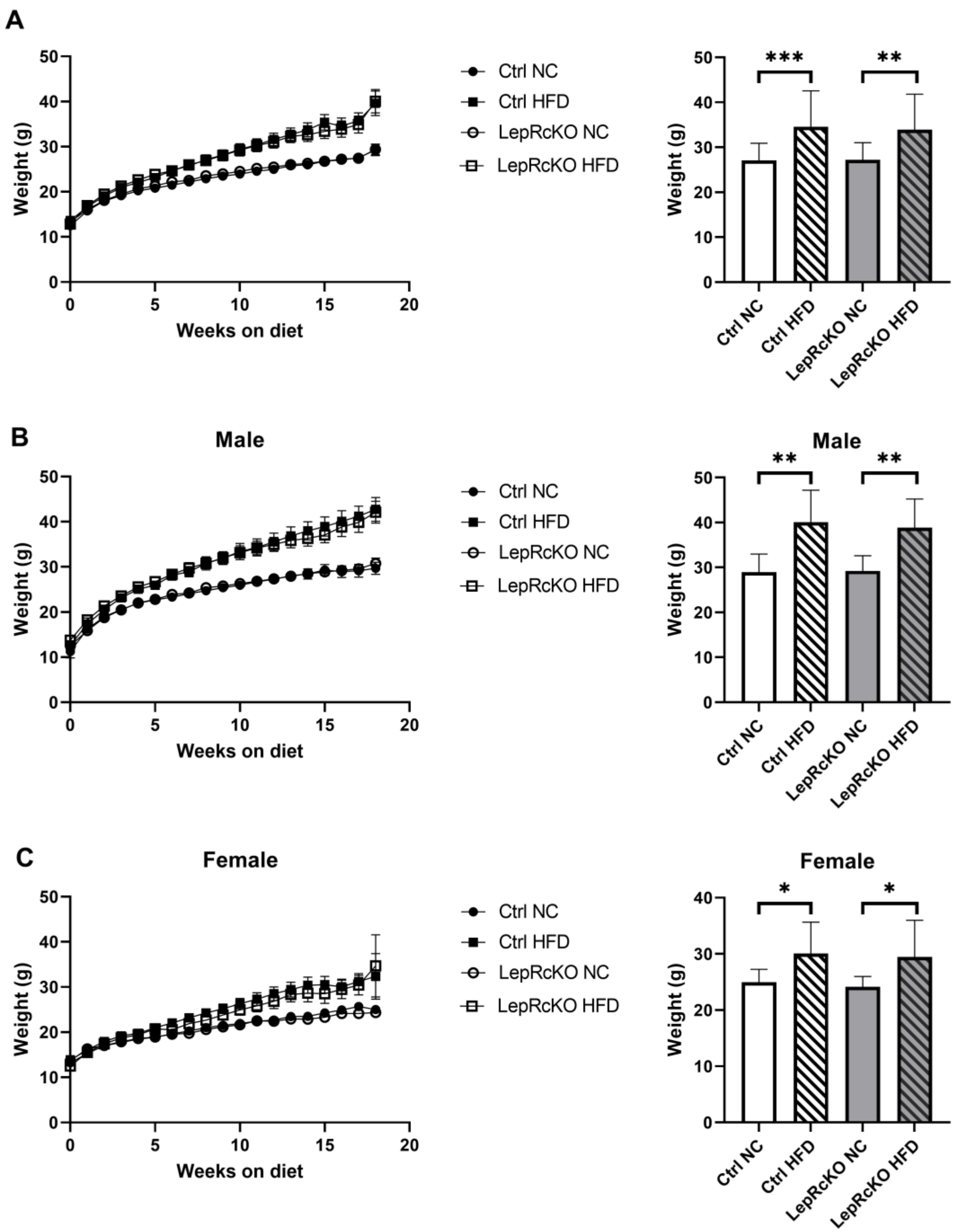

Figure 5: T cell-specific leptin receptor knockout mice and littermate controls have equivalent weight gain following high-fat diet.

T cell-specific leptin receptor knockout mice (LepR cKO) and littermate controls (Ctrl) were fed low-fat, normal chow (NC) or high-fat diet (HFD) for 18 weeks. Body weights were measured weekly and graphed versus time. Data plotted as mean ± standard error. Bar graphs show weights at 16 weeks on diet. Data shown as mean ± standard deviation. All experimental mice (n=18-20/group) are shown in (a), male mice (n=9-14/group) are shown in (b), female mice (n=9-11/group) are shown in (c) over the course of the experiment and then at 16 weeks (bar graphs). Data analyzed using student's t test with Welch's correction (*p<0.05; **p<0.01)

Following 18 weeks on diet, mice were fasted for 4 hours to allow for measurement of fasting blood glucoses. Fasting blood glucose levels of control mice on HFD were significantly elevated compared to control mice fed NC; however, these differences were not observed between leptin receptor conditional knockout mice on HFD compared to NC (Figure 6A). When these data were separated by sex, we found that control male, but not female, mice on HFD had significantly higher fasting blood glucose levels than their NC fed counterparts (Figure 6B-C). Again, this significant difference in weight gain between NC and HFD-fed male control mice was not observed in male mice with T cell-specific leptin receptor deficiency (Figure 6B).

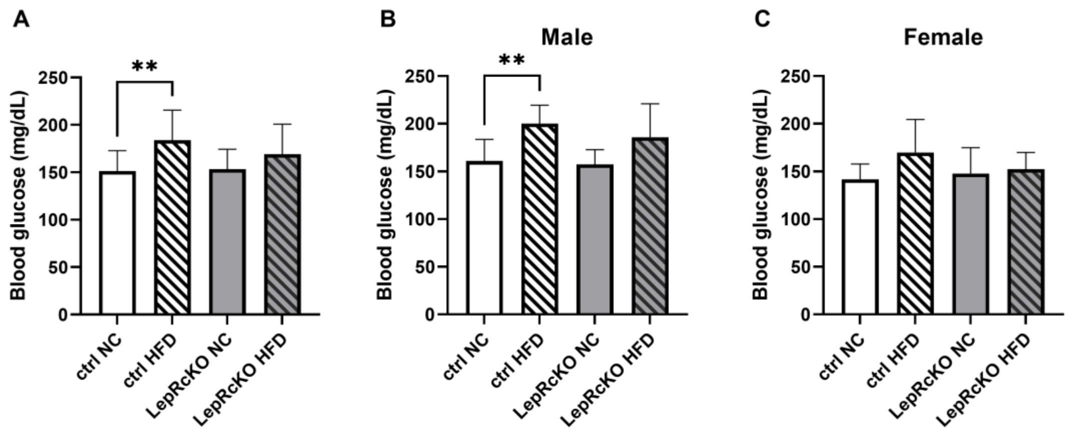

Figure 6: Leptin receptor knockout prevents impaired fasting glucose levels in male, but not female, mice fed high-fat diet.

T cell-specific leptin receptor knockout mice (LepR cKO) and littermate controls (Ctrl) were fed low-fat, normal chow (NC) or high-fat diet (HFD) for 18 weeks. Fasting blood glucose readings were taken after a 4 hour fast. Readings from all experimental mice (n=12-16/group) are shown in (a), fasting blood glucose readings from male mice (n=6-9/group) shown in (b), fasting blood glucose readings from female mice (n=6-7/group) are shown in (c); analyzed using student's t test with Welch's correction (**p<0.01)

Mice from each experimental group were then subjected to a glucose tolerance test. As shown in Figure 7A and B, mice fed HFD had significantly impaired glucose tolerance compared to mice fed NC, but there were no significant differences in glucose tolerance between T cell-specific leptin receptor knockout mice or control mice on either NC or HFD. These findings were maintained when mice were separated into male or female groups; however, male mice on HFD had more impaired glucose tolerance than female mice on HFD (Figure 7C-D), consistent with previous observations in the literature (172, 173, 174, 175).

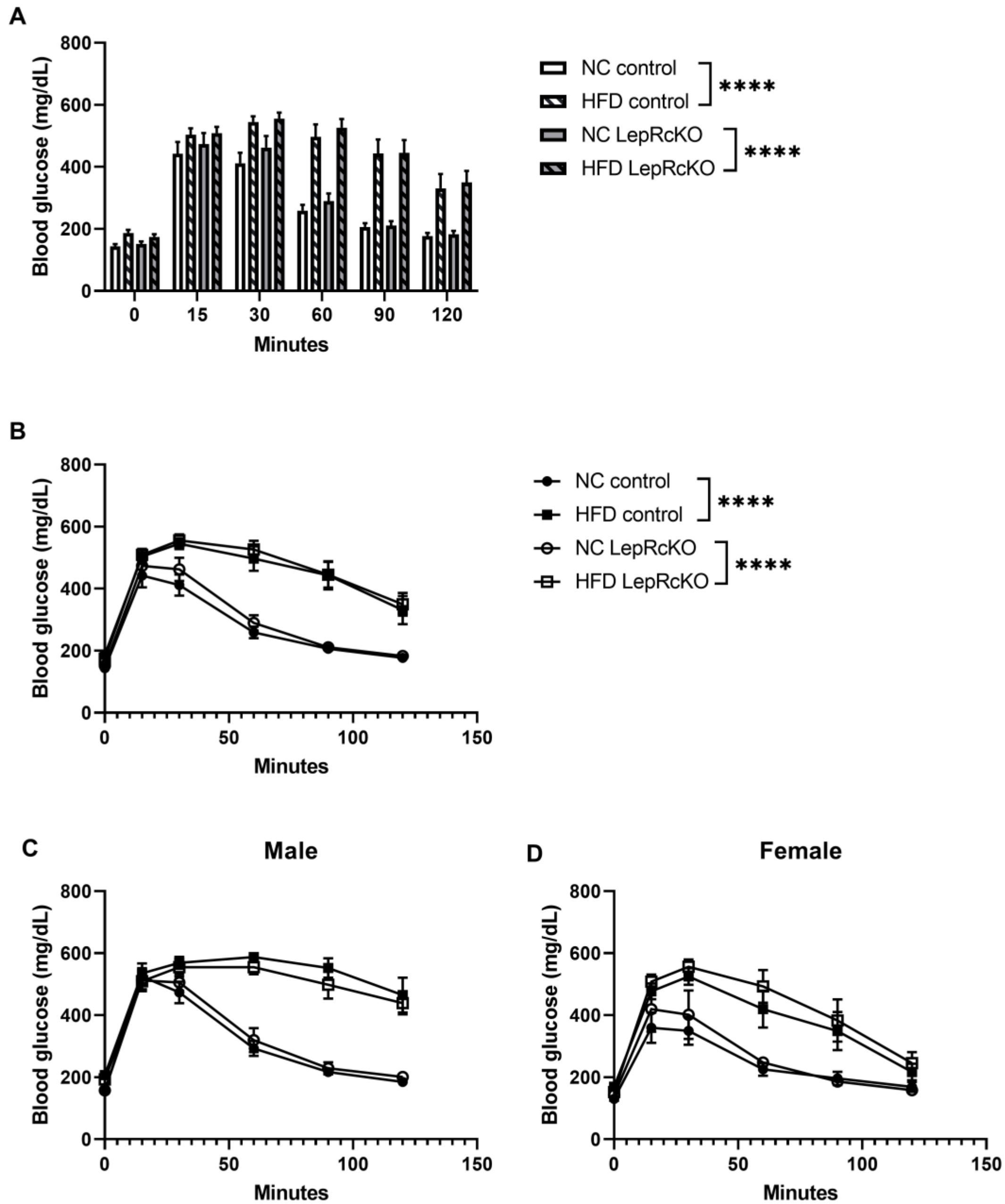

Figure 7: T cell-specific leptin receptor knockout mice have similar glucose tolerance to littermate control mice following high-fat diet.

Glucose tolerance test (GTT) was conducted after 18 weeks on low-fat, normal chow (NC) or high-fat diet (HFD). Mice were fasted for 6 hours; baseline blood glucose measured by tail vein bleed (0-minute timepoint). 2 g/kg glucose was injected intraperitoneally, and blood glucose (mg/dL) measured at 15, 30, 60, 90, 120 minutes post-injection. Bar graph shows all experimental mice (n=10-13/group) mean ± standard error (a), Line graph shows all experimental mice (n=10-13/group) mean ± standard error (b), male mice (n=5-7/group) mean ± standard error shown in (c), female mice (n=5-7/group) mean ± standard error shown in (d); analyzed using generalized estimating equations (GEE) model (****p<0.0001)

2.2.2 Metabolic effects of obesity on CD4$^+$ T cells depend on leptin receptor expression

Two days following glucose tolerance testing, mice were euthanized, and T cell metabolism and function were evaluated. Since CD4$^+$ T cell metabolism has been previously shown to be altered in obesity (176), we sought to characterize the metabolic phenotype of CD4$^+$ T cells from T cell-specific leptin receptor knockout mice versus littermate control mice on either NC or HFD. To that end, CD4$^+$ T cells were isolated from spleens and analyzed using extracellular flux analysis. CD4$^+$ T cells isolated from control mice fed HFD, but not from T cell-specific leptin receptor knockout mice fed HFD, showed significantly increased basal oxygen consumption rate (OCR, a surrogate marker for mitochondrial oxidation) compared to mice fed NC (Figure 8A). In contrast, CD4$^+$ T cells from T cell-specific leptin receptor knockout mice or littermate control mice on either NC or HFD did not show any difference in basal extracellular acidification rate (ECAR, a surrogate marker for glycolytic metabolism) (Figure 8B). The OCR/ECAR ratio is a useful measure to determine what proportion of the energy being produced in the cells is coming from mitochondrial oxidation versus glycolytic metabolism. In our study, control mice on HFD had a significantly increased OCR/ECAR ratio compared to control mice on NC (Figure 8C). However, there was no difference in OCR/ECAR ratio observed between T cell-specific leptin receptor knockout mice on NC or HFD (Figure 8C). These results suggest that leptin signaling is required, at least in part, for changes seen in T cell metabolism in obesity.

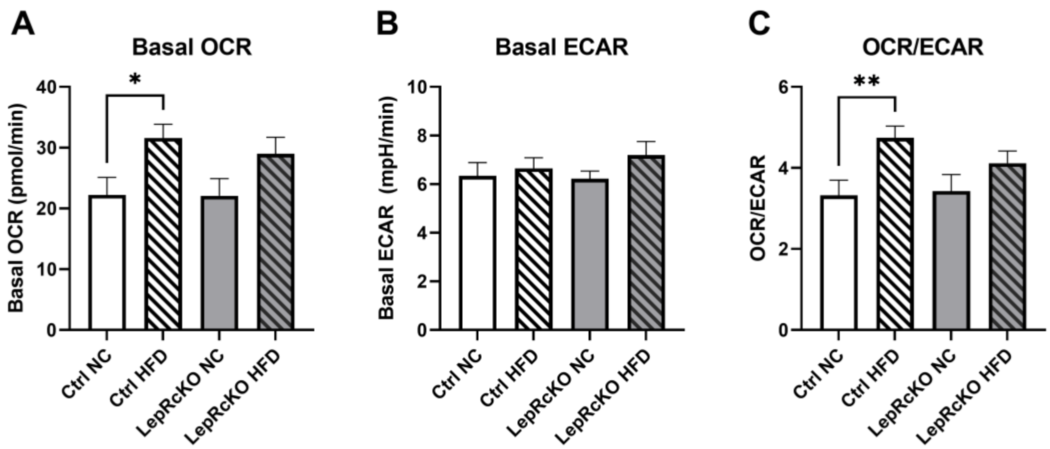

Figure 8: T cell-specific leptin signaling is required for changes in CD4+ T cell metabolism observed in mice fed high-fat diet compared to low fat diet.

T cell-specific leptin receptor knockout mice (LepR cKO) and littermate controls (Ctrl) were fed low-fat, normal chow (NC) or high-fat diet (HFD) for 18 weeks. CD4+ T cells were isolated from spleens and extracellular flux analysis was performed. Basal oxygen consumption rate (OCR) (mean ± standard error) shown in (a), basal extracellular acidification rate (ECAR) (mean ± standard error) shown in (b), OCR/ECAR ratio (mean ±standard error) shown in (c). Data analyzed using student's t test with Welch's correction (*$p<0.05$; **$p<0.01$; n=15-19/experimental group)

2.2.3 Functional changes in obesity require leptin receptor in $CD4^+$ T cells, but not $CD8^+$ T cells

To investigate T cell function in the experimental groups, splenocytes were analyzed by intracellular flow cytometry to determine the proportion of cells producing the pro-inflammatory cytokines IFN-γ and IL-17 or expressing the Treg-associated transcription factor Foxp3. Overall, control mice fed HFD had significantly increased proportion of IFN-γ producing $CD4^+$ T cells than control mice on NC (Figure 9A). However, no difference was observed in IFN-γ producing $CD4^+$ T cells between T cell-specific leptin receptor conditional knockout mice on NC versus HFD (Figure 9A). Interestingly, both T cell-specific leptin receptor conditional knockout and littermate control mice on HFD had significantly increased proportions of IFN-γ producing $CD8^+$ T cells when compared to mice on NC (Figure 9B). One possible explanation for the difference seen in IFN-γ production by $CD4^+$ versus $CD8^+$ T cells in the knockout mouse may be that $CD8^+$ T cells are less leptin responsive than $CD4^+$ T cells.

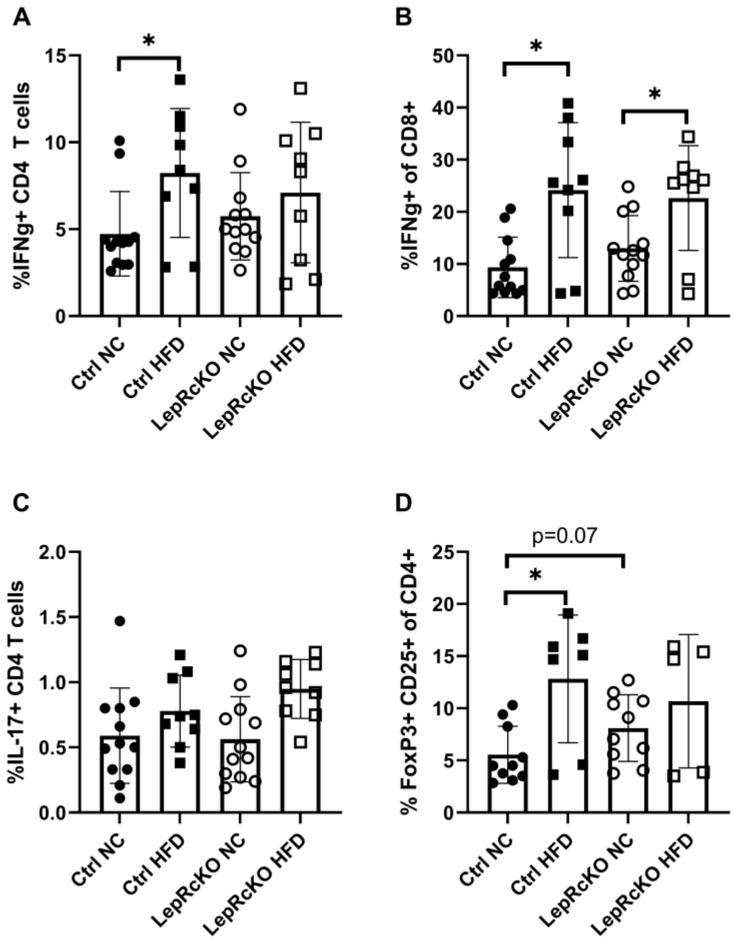

Figure 9: Splenic CD4+ T cells isolated from leptin receptor knockout mice are altered following high-fat diet compared to CD4+ T cells isolated from littermate controls.

T cell-specific leptin receptor knockout mice (LepR cKO) and littermate controls (Ctrl) were fed low-fat, normal chow (NC) or high-fat diet (HFD) for 18 weeks. CD4+ T cells were isolated from spleens and cytokine production or Foxp3 transcription factor was measured by intracellular flow cytometry. Proportion of splenic CD4+ T cells that produce IFN-γ (a), Proportion of splenic CD8+ T cells that produce IFN-γ (b), Proportion of splenic CD4+ T cells that produce IL-17 (c), Proportion of splenic CD4+ T cells that produce CD25 and Foxp3 (Treg cells) (d); analyzed using student's t test (*p<0.05) with Welch's correction. Each plotted point represents one mouse; n=9-12/experimental group.

We found no statistically significant difference in the proportion of $CD4^+$ T cells producing IL-17 in mice fed HFD compared to mice fed NC in both T cell-specific leptin receptor knockout and littermate control mice (Figure 9C). Lastly, we examined changes in Treg cell numbers, as defined by the proportion of $CD4^+$ T cells expressing the transcription factor Foxp3 and the activation marker CD25. The proportion of Treg cells was increased in control mice, but not T cell-specific leptin receptor knockout mice, fed HFD compared to mice fed NC (Figure 9D). Altogether, these findings demonstrate that T cell-specific leptin signaling is required for some of the changes in $CD4^+$ T cell metabolism and function observed in obesity but has minimal effects on obesity-associated systemic metabolism.

2.3 Discussion and Conclusions

As an increasing proportion of the United States population is diagnosed with obesity, it is becoming critically important to understand the implications of obesity for the health of those individuals. Studying the immune response is an important aspect of this, as obesity-associated changes in immune cells have been shown to drive inflammation which promotes systemic metabolic disease (157). Specifically, it has been demonstrated that T cells have a critical role in driving obesity-associated inflammation leading to systemic insulin resistance (169). We have previously shown that leptin is a key regulator of both $CD4^+$ T cell metabolism and effector cell differentiation and function (70, 71). Given the role of T cells in driving obesity-associated inflammation and insulin resistance as well as the role of leptin in promoting $CD4^+$ T cell inflammatory function, we set out to determine whether leptin signaling in T cells was required for the development of glucose intolerance and insulin resistance in obesity.

Our conditional leptin receptor knockout mouse, which has leptin receptor selectively deleted in T cells, allowed us to interrogate whether leptin signaling to T cells could be promoting T cell metabolic changes, increased inflammatory cytokine production, and subsequent systemic metabolic disease in the context of obesity. We have previously reported metabolic differences between T cells from C57BL/6 mice fed NC versus HFD (176). The data shown here support our previous work showing that $CD4^+$ T cells from diet-induced obese control mice have increased oxidative metabolism (OCR), as well as an increased ratio of oxidative to glycolytic metabolism (OCR:ECAR) compared to $CD4^+$ T cells from lean control mice. However, $CD4^+$ T cells from HFD versus NC-fed T cell-specific leptin receptor knockout mice did not show the same metabolic changes. These results suggest that direct leptin signaling mediates metabolic remodeling of $CD4^+$ T cells in obesity resulting in significantly increased basal oxidative metabolism and an increase in the ratio of oxidative to glycolytic metabolism characteristic of T cells from obese mice.

We also found differences when we interrogated the proportions of splenic T cells from our experimental groups. An increase in the proportion of IFN-γ producing

$CD4^+$ T cells was observed in control mice fed HFD compared to NC, which was not observed in $CD4^+$ T cells from T cell-specific leptin receptor knockout mice. On the other hand, the proportion of IFN-γ producing $CD8^+$ T cells was increased following HFD compared to NC in both T cell-specific leptin receptor knockout mice and littermate controls. This differing result in $CD4^+$ versus $CD8^+$ T cells is consistent with the fact that leptin receptor is expressed at lower levels on $CD8^+$ T cells than $CD4^+$ T cells. Therefore, $CD8^+$ T cells may retain their IFN-γ producing phenotype in the context of HFD-induced obesity, even when leptin receptor is deleted.

To our surprise, deletion of leptin signaling in T cells had a mild influence on fasting blood glucose in obesity but did not significantly affect systemic metabolic disease. Glucose tolerance tests between HFD-induced obese T cell-specific leptin receptor knockout mice and littermate controls did not show any significant differences. One possible explanation for these negative results is a redundancy in signaling pathways downstream of leptin as well as other nutritionally regulated hormones that are increased in the setting of obesity. For example, IL-6 is known to be increased in obesity, shares signaling pathways with leptin, and can have synergistic effects on T cells. In the absence of leptin signaling in obesity, other cytokine signals may be compensatory.

One limitation of our study is that we only investigated the role of leptin receptor signaling directly on T cells. While T cells have been shown to be important for obesity-associated inflammation, there are other immune cells that have been shown to have a role in this process. In particular, macrophages are known to be important drivers of inflammation in the adipose tissue in the setting of obesity. To this point, several studies have shown that reducing macrophage recruitment to or eliminating macrophages from the adipose tissue improves insulin sensitivity (177, 178, 179, 180, 181). Furthermore, IL-1 receptor I deficient mice have improved glucose tolerance and reduced inflammation in obesity, suggesting that macrophage production of IL-1β is a critical inflammatory driver in obesity (180). A study investigating the role of leptin signaling in myeloid cells found that specific deletion of leptin receptor in myeloid cells did not significantly affect glycemic control (182). However, selective reconstitution of leptin receptor in myeloid cells in mice that were otherwise leptin receptor deficient resulted in improved glucose tolerance. This combination of results suggests that there is a complex signaling network involving immune cells, adipose tissue, and soluble mediators such as leptin and other nutritionally regulated cytokines. Future studies should investigate the roles of other immune cells, endocrine signals, and inflammatory cytokines in driving obesity-associated inflammation and glucose intolerance.

In this study, we set out to understand whether the leptin signal to T cells in obesity is sufficient to drive systemic inflammation and metabolic disease. Using our T cell-specific leptin receptor conditional knockout mouse, we interrogated the role of leptin signaling on the pathogenesis of obesity as driven by the T cell. We found that loss of leptin signaling to T cells was insufficient to influence glucose tolerance in obesity, although leptin deficiency did prevent impaired fasting glucose levels compared to control mice in the HFD setting, in a sex dependent manner. Despite these minimal effects on systemic metabolism, T cells isolated from leptin receptor knockout mice on HFD lacked some of the metabolic and functional changes observed in T cells from control mice on HFD. These results contribute to a body of work that aims to understand the signals influencing immune cell function and inflammation in obesity. Understanding immune cells in obesity is critical for the development of therapeutics to ameliorate the consequences of obesity and subsequent metabolic disease. With an increasing proportion of individuals with obesity in both the United States and around the world, treating the inflammation and downstream consequences of obesity will become an increasing priority.

2.4 Materials and Methods

2.4.1 Animals

T cell-specific leptin receptor (LepR) knockout mice were generated by crossing CD4Cre transgenic mice with LepR-floxed mice (Jackson Laboratory). The following genotypes were studied: *CD4Cre⁺LepR* $^{fl/fl}$ (leptin receptor knockout mice; LepRcKO) and *CD4Cre⁻LepR* $^{fl/fl}$ (controls; Ctrl). Mice were weaned at 3 weeks of age onto either low-fat, normal chow (NC, 10-kcal% fat, LabDiet, St. Louis, MO) or high-fat diet (HFD, 60-kcal%

fat, Research Diets, New Brunswick, NJ) and remained on this diet for 18 weeks. Mice were group housed (up to 5 per cage), maintained at ambient temperature, and given ad libitum access to food and water. Mouse weights were collected weekly. All animal protocols were approved by the Institutional Animal Care and Use Committees at Duke University or the University of North Carolina at Chapel Hill.

2.4.2 Blood glucose readings and glucose tolerance tests

Blood glucose readings were taken using a One Touch Verio glucometer and strips. Mice were fasted for 4 hours prior to measuring blood glucose. Tails were snipped and one drop of blood deposited onto glucometer strip. To measure glucose tolerance, mice were fasted for 6 hours prior to glucose tolerance test. A baseline blood glucose reading was taken from each mouse. Mice were then injected intraperitoneally with 2 g/kg body weight of glucose. Blood glucose readings were taken at 15 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes following injection.

2.4.3 Tissue collection and processing

Mice were euthanized using CO_2 inhalation. Spleens were mashed and strained in PBS, washed, and resuspended. A portion of splenocytes were set aside for flow cytometry and the rest were used to isolate $CD4^+$ T cells using the StemCell $CD4^+$ T cell isolation kit (StemCell technologies, Vancouver, BC, Canada). Isolated $CD4^+$ T cells were analyzed by extracellular flux analysis.

2.4.4 Flow cytometry

Two million splenocytes were fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer kit (eBioscience) and stained for Foxp3 following the manufacturer instructions. For the identification of Treg cells, the following antibodies were used: BV421 Armenian Hamster anti-mouse CD3e (Biolegend, San Diego, CA), BV605 rat anti-mouse CD4 (Biolegend), PeCy7 rat anti-mouse CD25 (Biolegend), AF488 rat anti-mouse Foxp3 (Biolegend). For the identification of effector T cells (Th1 and Th17) and evaluation of their function, the following antibodies were used: BV421 Armenian Hamster anti-mouse CD3e (BD BioSciences), BV605 rat anti-mouse CD4 (Biolegend), AF488 rat anti-mouse CD8a (Biolegend), APC rat anti-mouse IL-17A (Biolegend), and PE/Cy7 rat anti-mouse IFNγ (Biolegend). Five million splenocytes were stimulated for 4.5 h in complete media containing Golgi Plug (2 μg/ml) (BD Biosciences), PMA (50 ng/ml) (Sigma-Aldrich, St. Louis, MO), and ionomycin (1 μg/ml) (Sigma-Aldrich), then permeabilized and fixed with Cytofix/Cytoperm kit (BD Biosciences) and stained for IFN-γ and IL-17A following the manufacturer's protocol. Samples were acquired on a ThermoFisher Attune NxT flow cytometer, and data were analyzed using FlowJo (Treestar, Ashland, OR).

2.4.5 Metabolic flux assays

CD4+ T cells were washed with Seahorse XF RPMI 1640 media (Agilent, Santa Clara, CA) and plated at a density of 250,000 cells/well (50 μL) in a Seahorse XFe96 plate (Agilent) pre-coated with Cell-Tak (Corning, Corning, NY). After spinning down the plate at 200 rpm for 1 min, the plate was incubated for 30 min in a humidified 37°C incubator in the absence of CO_2. Seahorse XF RPMI 1640 media (130 μL) was added, and the plate was incubated for an additional 20 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe96 Analyzer (Agilent).

2.4.6 Statistical analysis

Comparisons between groups were analyzed using t-test with Welch's correction assuming Gaussian distribution. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA). All data was determined as significant by $p<0.05$. Glucose tolerance test (GTT) analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC) at a two-tailed significance level of 0.05. All available GTT measurements within the study were included in the analysis. Marginal models using generalized estimating equations (GEE) were implemented to account for the correlation between repeated measurements of GTT levels in each mouse. A GEE-type model with a normal distribution and identity link was implemented to test the association between sex and GTT levels as well as diet and GTT levels. An exchangeable working correlation structure was used along with the robust variance estimator.

3. Metabolic mechanisms of insulin and IGF-1 signaling on T cell function and differentiation

3.1 Introduction

Insulin and IGF-1 are nutritionally regulated hormones, the levels of which are altered by blood glucose levels and nutritional status. Insulin is secreted from pancreatic beta cells in proportion to blood glucose levels and is best known for promoting glucose uptake in insulin responsive metabolic cells and tissues. In the case of obesity, insulin levels are increased at baseline due to peripheral insulin resistance. IGF-1, on the other hand, is regulated by protein levels and is growth hormone responsive. The diverse actions of IGF-1 include stimulating growth, inhibiting apoptosis, and promoting glucose uptake, similar to insulin. IGF-1 levels are low in states of protein malnutrition, but free IGF-1 is increased in obesity (183).

Insulin and IGF-1 have been shown to have effects on $CD4^+$ T cells. Insulin has been shown to promote select T cell functions. Whole body knockdown of insulin receptor in Wistar rats demonstrated that $CD4^+$ T cells without insulin receptor exhibited reduced cytokine production and proliferation, as well as increased apoptosis (184). In mouse studies, insulin was found to promote $CD4^+$ T cell production of IFN-γ, as well as both glycolytic and oxidative metabolism (103). Moreover, loss of insulin signaling to T cells caused reduced antigen-specific influenza responses in mice (103). These data suggest that insulin promotes $CD4^+$ T cell metabolism and function.

IGF-1 has also been shown to have various effects on T cells. One group recently demonstrated that IGF-1 signaling to Th17 cells increased both metabolism and IL-17 production, as measured by extracellular flux analysis and flow cytometry, respectively (123). Specifically, IGF-1 treatment of Th17 cells increased glycolytic and oxidative metabolism, and loss of IGF-1R on T cells was protective against EAE (123). Together, these data suggest a role of IGF-1 and IGF-1R signaling in regulating Th17 fate, function, and metabolism. Additionally, IGF-1 is known to promote mitochondrial function and biogenesis, but whether this is the case in T cells remains unknown (185).

Here we identify the specific effects of insulin and IGF-1 on $CD4^+$ T function and metabolism, with an emphasis on mitochondrial responses, including mitochondrial membrane potential. Our results suggest a cytoprotective function of IGF-1 in $CD4^+$ T cells, and especially in skewed Th17 cells. We also report the novel finding that IR levels are not changed in $CD4^+$ T cells following activation, but IGF-1R levels are, and we demonstrate a requirement for both IR and IGF-1R to mediate IGF-1 effects on Th17 cell metabolism. These results suggest that IGF-1 is signaling through the hybrid receptor, which has not been described on $CD4^+$ T cells to date.

3.2 Results

3.2.1 T cell activation is accompanied by increased IGF-1R expression

Previous publications demonstrating that $CD4^+$ T cells have increased binding of fluorescently labeled insulin following activation have led to the assumption that T cell activation increases IR expression (94, 103, 186). However, insulin can bind IGF-1R as well as IR, albeit at a lower affinity (187). Thus, we examined both IR and IGF-1R expression following $CD4^+$ T cell activation. Interestingly, we observed that IR gene expression is only mildly changed over the course of activation; however, IGF-1R gene expression was dynamically upregulated, with mRNA expression peaking at 6 hours post activation (Figure 10A-B). To support these results, we also examined protein expression of IR and IGF-1R following $CD4^+$ T cell activation using immunoblot, and we found that IGF-1R protein expression was indeed upregulated following activation and accumulated over time (Figure 10C). Conversely, IR protein expression was unchanged following $CD4^+$ T cell activation (Figure 10C). We observed no difference in activation marker expression or proliferation when activated $CD4^+$ T cells were treated with insulin or IGF-1 in serum free conditions (Figure 11).

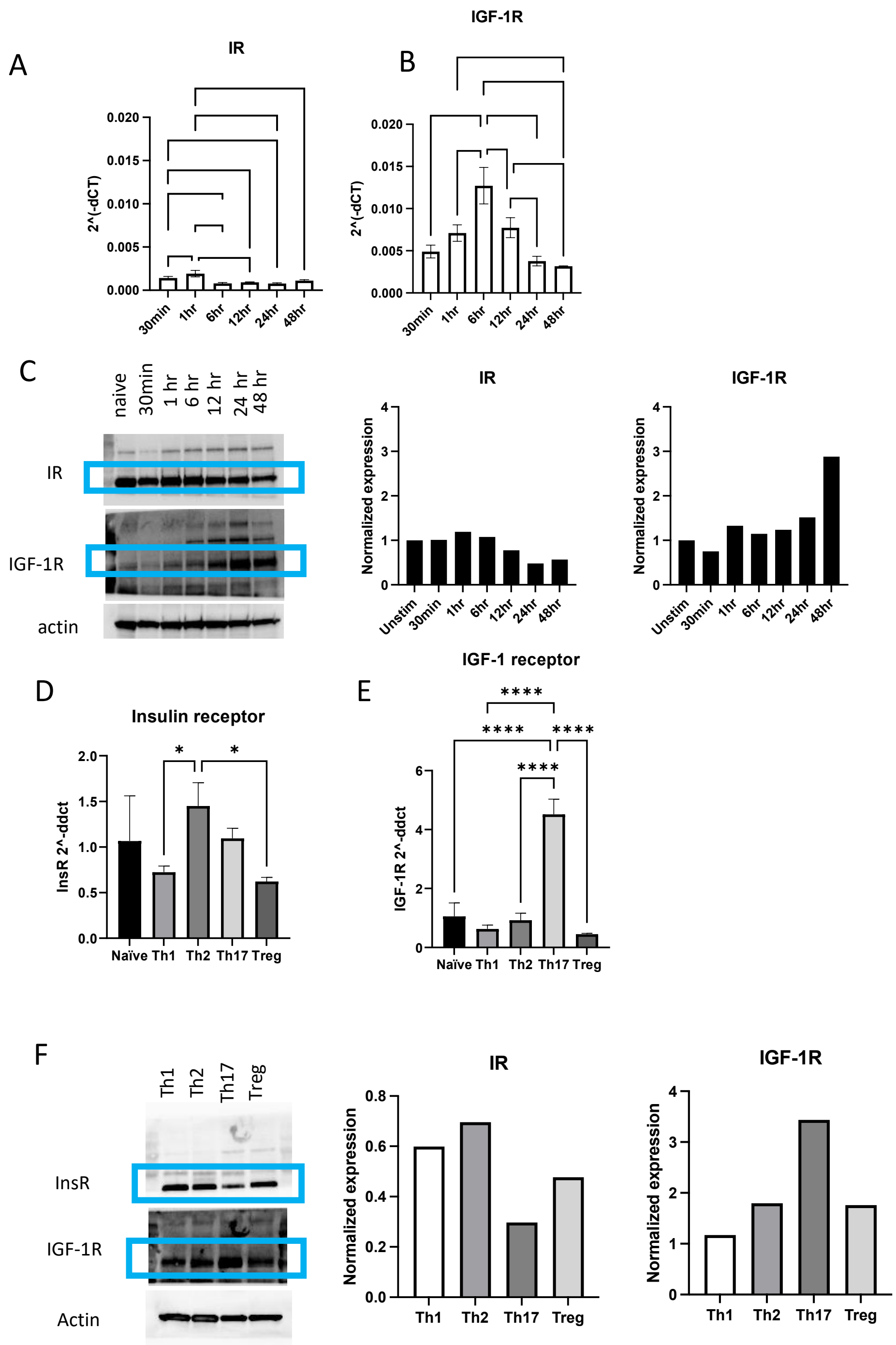
A
IR
2^(-dCT)
30min 1hr 6hr 12hr 24hr 48hr
B
IGF-1R
2^(-dCT)
30min 1hr 6hr 12hr 24hr 48hr
C
naive 30min 1 hr 6 hr 12 hr 24 hr 48 hr
IR
IGF-1R
actin
IR
Normalized expression
Unstim 30min 1hr 6hr 12hr 24hr 48hr
IGF-1R
Normalized expression
Unstim 30min 1hr 6hr 12hr 24hr 48hr
D
Insulin receptor
InsR 2^-ddct
Naïve Th1 Th2 Th17 Treg
E
IGF-1 receptor
IGF-1R 2^-ddct
Naïve Th1 Th2 Th17 Treg
F
Th1 Th2 Th17 Treg
InsR
IGF-1R
Actin
IR
Normalized expression
Th1 Th2 Th17 Treg
IGF-1R
Normalized expression
Th1 Th2 Th17 Treg

Figure 10: IGF-1R is dynamically regulated by $CD4^+$ T cell activation and differentiation

(A-C) $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 antibodies for 0hr (naïve), 30min, 1hr, 6hr, 12hr, 24hr, 48hr. Expression of IR *(A)* and IGF-1R *(B)* mRNA was analyzed by qRT-PCR. Separate samples were analyzed for IR and IGF-1R protein expression by western blot *(C)*. **(D-F)** $CD4^+$ T cells were differentiated *in vitro* to functional T helper subsets. IR *(D)* and IGF-1R *(E)* mRNA expression was analyzed by qRT-PCR. Separate samples were analyzed for IR and IGF-1R protein expression by western blot *(F)*. Results representative of three independent experiments. (*$p<0.05$; **$p<0.01$, ***$p<0.001$ ****$p<0.0001$)

We next interrogated the gene expression levels of IR and IGF-1R on $CD4^+$ T helper cell subsets (Th1, Th2, Th17, Treg), which we generated via *in vitro* differentiation, as described in the Methods. Each functional subset showed unique expression of IR and IGF-1R (Figure 10D-E). Most striking was a dramatic increase in IGF-1R expression on Th17 cells compared to the other $CD4^+$ T cell subsets. These results were consistent with protein expression, as determined by immunoblot (Figure 10F), which confirmed that Th17 cells have the highest expression of IGF-1R protein among the helper $CD4^+$ T cell subsets.

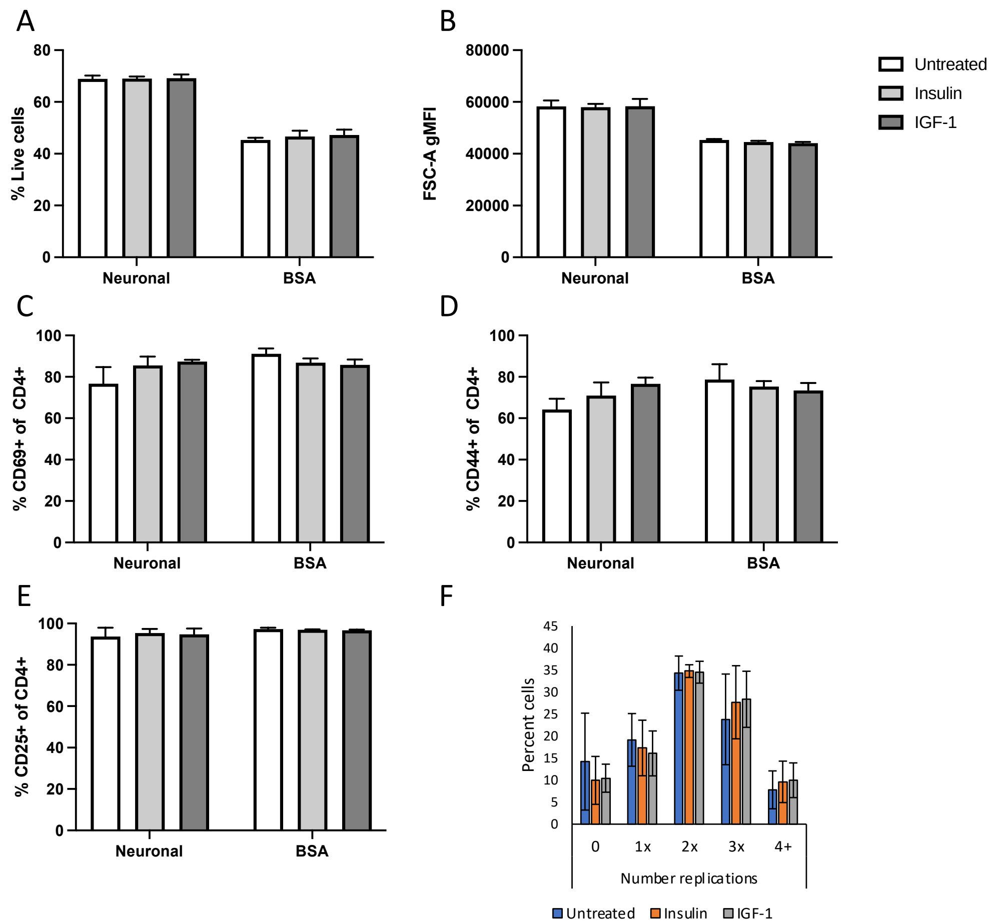

Figure 11: Insulin and IGF-1 treatment do not impact CD4+ T cell viability or activation marker expression

(A-E) $CD4^+$ T cells were activated for 48 hours on anti-CD3/CD28 coated plates in serum free media supplemented with either Neuronal serum or 0.35% BSA and treated with insulin or IGF-1 for the last 24 hours of activation. (A)Viability assessed by flow cytometry viability dye staining. (B) Cell size determined by FSC-A geometric mean fluorescent intensity (C-E) Activation marker expression measured by flow cytometry for CD69 (C), CD44 (D), and CD25 (E), (F) $CD4^+$ T cells stained with cell trace violet and activated for 72 hours on anti-CD3/CD28 coated plates in serum free media supplemented with either Neuronal serum or 0.35% BSA and treated with insulin or IGF-1. Proliferation assessed by flow cytometry. Data representative of at least 2 independent experiments; n=3 per experiment. Data analyzed using students t-test, no significant differences found.

3.2.2 IGF-1 treatment promotes cytokine production of activated $CD4^+$ T cells

Given the increased expression of IGF-1R on Th17 cells, we next asked if IGF-1 treatment of activated $CD4^+$ T cells increased IL-17 production *in vitro*. To test this, $CD4^+$ T cells were activated with plate-bound antibodies to CD3 and CD28 in neuronal serum (insulin/IGF-1 free) or serum free conditions to prevent any confounding effect of growth factors or hormones present in serum/media. Following 24 hours of activation, cells were treated with or without 50 ng/mL IGF-1, and cytokine production was examined at 48 hours. IGF-1 increased IL-17 production in activated $CD4^+$ T cells, as measured by both intracellular flow cytometry and ELISA (Figure 12A-C). However, insulin treatment did not significantly increase IL-17 production (Figure 12B-C). Furthermore, neither insulin nor IGF-1 treatment affected IFN-γ production of activated $CD4^+$ T cells (Figure 12D). To understand whether this effect of IGF-1 on IL-17 production is dependent on IR or IGF-1R signaling, we activated $CD4^+$ T cells from IR cKO (*CD4-Cre*$^+$ *IR*$^{fl/fl}$) and IGF-1R cKO (*CD4-Cre*$^+$ *IGF1R*$^{fl/fl}$) mice and littermate controls in full serum conditions and measured IL-17 production. Both IR cKO and IGF-1R cKO $CD4^+$ T cells showed reduced IL-17 production as measured by ELISA, suggesting that both IR and IGF-1R signaling are required for increased IL-17 production in activated $CD4^+$ T cells (Figure 12E).

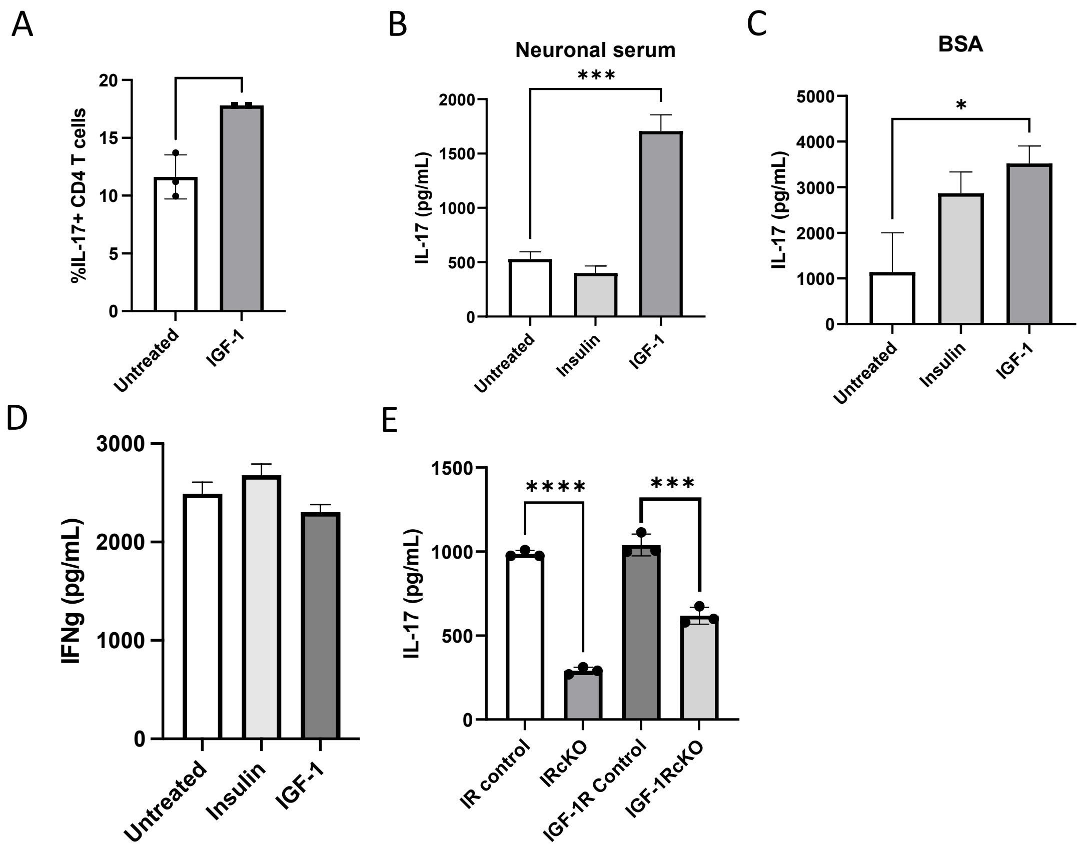

Figure 12: IGF-1 treatment increases IL-17 production by CD4+ T cells.

(A-D) CD4+ T cells were activated for 48 hours on anti-CD3/CD28 coated plates in serum free conditions and treated with IGF-1 for the last 24 hours of activation. IL-17 expression was analyzed by flow cytometry *(A)*. Serum free media was either supplemented with Neuronal serum *(B)* or 0.35% BSA *(C)*, and IL-17 production was measured by ELISA. (D) Serum free media was supplemented with Neuronal serum and IFN-γ production was measured by ELISA. *(E)* CD4+ T cells from spleens of IRcKO and IR cKO mice along with littermate controls were activated for 48 hours in full serum conditions. Serum samples were collected and analyzed for IL-17 production by ELISA. Data representative of at least 2 independent experiments; n=3 per experiment. Data analyzed using students t-test (*p<0.05; **p<0.01, ***p<0.001 ****p<0.0001)

3.2.3 Insulin and IGF-1 promote increased metabolism of $CD4^+$ T cells

Since both insulin and IGF-1 exert effects on metabolic cells and tissues, with insulin particularly well known for increasing glucose uptake, we examined the effects of insulin and IGF-1 on $CD4^+$ T cell metabolism. Treatment of activated $CD4^+$ T cells with IGF-1 (50 ng/mL) increased glucose uptake, as measured by uptake of tritiated 2-deoxyglucose, while treatment with insulin (10 ng/mL) did not (Figure 13A). Insulin treatment significantly increased basal oxidative consumption rate (OCR; a measure of mitochondrial respiration and a surrogate for oxidative phosphorylation), basal extracellular acidification rate (ECAR; a measure of lactate production, and a surrogate for glycolytic metabolism), the ratio of oxidative to glycolytic metabolism (OCR/ECAR), maximal respiration, spare respiratory capacity, ATP production, and proton leak in activated $CD4^+$ T cells, as measured using extracellular flux analysis (Figure 13B-H). Similar to insulin treatment, IGF-1 treatment of activated T cells significantly increased basal OCR, basal ECAR, the OCR/ECAR ratio, maximal respiration, ATP production, and proton leak, but not spare respiratory capacity. (Figure 13B-H). Overall, however, the metabolic changes in activated $CD4^+$ T cells following IGF-1 treatment were less striking than the metabolic changes seen in activated $CD4^+$ T cells treated with insulin. These data indicate that activated $CD4^+$ T cells become more metabolically active in response to either insulin or IGF-1 treatment, though the insulin effect is more potent.

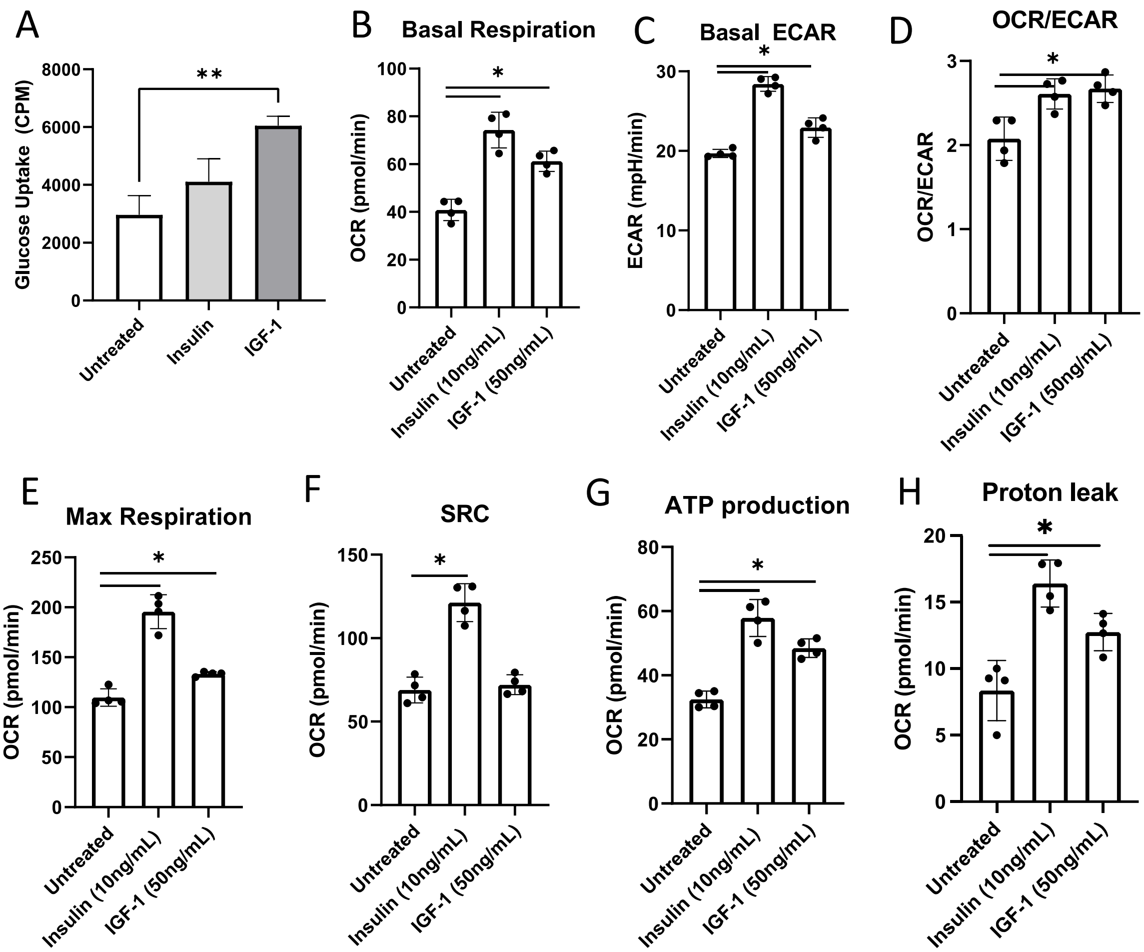

Figure 13: Insulin and IGF-1 modulate CD4+ T cell metabolism

CD4+ T cells from C57BL/6 mice were activated with anti-CD3 and anti-CD28 antibodies for 48 hours in neuronal serum supplemented media and treated with or without insulin (10ng/mL) or IGF-1 (50ng/mL) for the last 48 hours of activation. (A) Glucose uptake was measured by uptake of tritiated 2-deoxyglucose. (B-H) extracellular flux analysis was used to measure basal OCR, basal ECAR, OCR/ECAR ratio, max OCR, SRC, ATP production, and protein leak.

3.2.4 IGF-1 uniquely impacts mitochondrial membrane potential of activated CD4$^+$ T cells

IGF-1 has been implicated in several mitochondrial processes such as mitochondrial biogenesis, mitophagy, and mitochondrial function (185). We therefore measured the mitochondrial mass and mitochondrial membrane potential of activated CD4$^+$ T cells treated with insulin or IGF-1. To measure mitochondrial mass, we stained activated CD4$^+$ T cells with Mitotracker Green (MTG). Neither insulin nor IGF-1 caused any significant difference in mitochondrial mass compared to untreated cells (Figure 14A-B). In order to generate ATP in the mitochondria, mitochondrial membrane potential is generated by pumping protons into the intermembrane space which creates a gradient and polarizes the inner mitochondrial membrane. This proton gradient ($\Delta\Psi$) is then used to generate ATP via ATP synthase. To measure the degree of inner mitochondrial membrane polarization, we stained cells with tetramethylrhodamine (TMRE), which is a cell permeant positively charged dye that accumulates in active mitochondria due to their relative negative charge. Inactive or depolarized mitochondria with decreased membrane potential fail to sequester TMRE. IGF-1 treatment of activated CD4$^+$ T cells significantly decreased TMRE staining, while insulin treatment had no significant effect (Figure 14C-D), suggesting that IGF-1, but not insulin, decreases mitochondrial membrane potential (MMP) in activated CD4$^+$ T cells.

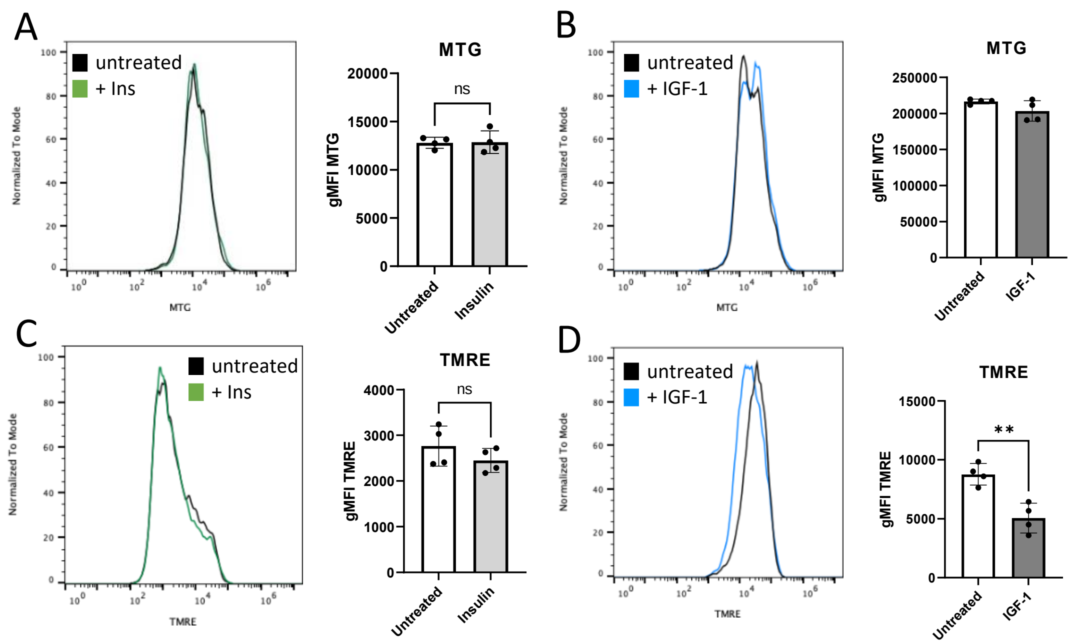

Figure 14: IGF-1 treatment decreases mitochondrial membrane potential in activated CD4+ T cells

CD4+ T cells from C57BL/6 mice were activated with anti-CD3 and anti-CD28 antibodies for 48 hours in neuronal serum supplemented media and treated with or without insulin (10ng/mL) or IGF-1 (50ng/mL) for the last 48 hours of activation. (A and B) Mitochondrial mass was measured by mitotracker green gMFI (geometric mean fluorescence intensity) (C and D) Mitochondrial membrane potential measured by TMRE gMFI by flow cytometry. Data representative of at least 2 independent experiments. Representative histograms shown; n=3-4 per experiment. Data analyzed using students t-test (**p<0.01)

3.2.5 IGF-1 treatment of Th17 promotes increased mitochondrial metabolism

Given that IGF-1R is significantly upregulated on Th17 cells compared to other CD4+ T cell subsets and causes an increase in IL-17 production by activated bulk CD4+ T cells, we investigated the effect of IGF-1 treatment on the function and metabolism of Th17 cells. Th17 cells were differentiated from $CD4^+$ T cells *in vitro*, and then treated with or without IGF-1 for 48 hours. IGF-1 treatment of differentiated Th17 cells did not increase the percentage of IL-17 positive cells but did increase IL-17 production as measured by mean fluorescent intensity, (Figure 15A-B). IGF-1 also altered the metabolism of differentiated Th17 cells, with increased basal OCR, basal ECAR, max OCR, spare respiratory capacity, ATP production, and proton leak, mirroring the results seen in bulk activated $CD4^+$ T cells treated with IGF-1 (Figure 15C-H). In contrast, Th1 and Treg cells treated with IGF-1 did not show any change in IFN-γ or Foxp3 expression or increased cellular metabolism (Figure 16).

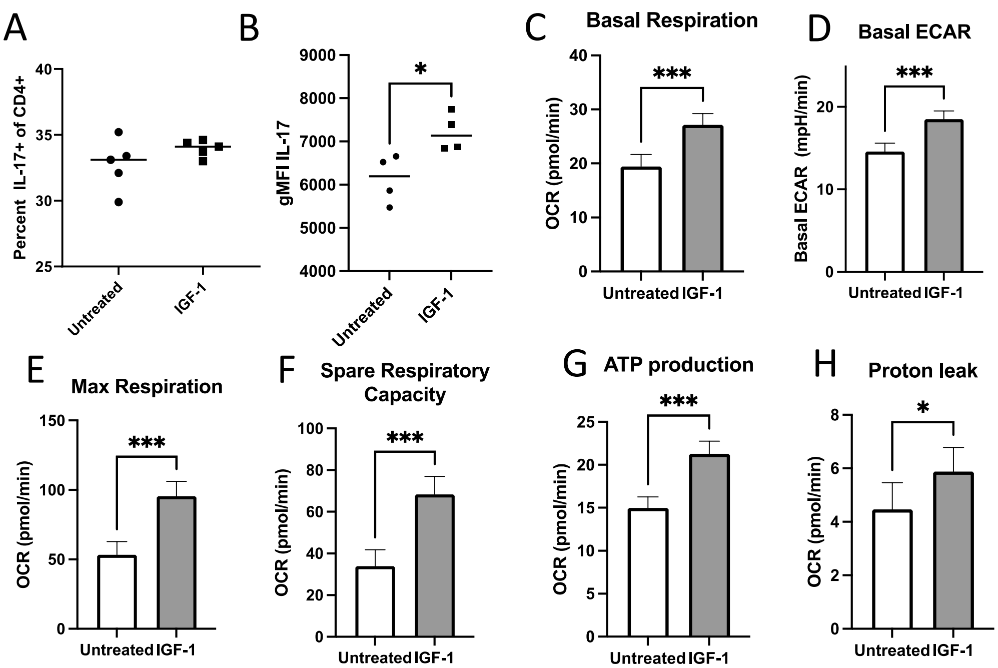

Figure 15: IGF-1 specifically influences Th17 metabolism and function

CD4+ T cells from C57BL/6 mice were differentiated in vitro to Th17 cells in neuronal serum-supplemented media for three days, and then treated with or without IGF-1 (50ng/mL) for an additional 48 hours. (A-B) Cells were analyzed by flow cytometry for percent IL-17+ cells (A) and IL-17 expression by MFI (B). (C-H) Extracellular flux analysis was used to measure basal OCR, basal ECAR, max OCR, SRC, ATP production, and protein leak. Data representative of 3 independent experiments. n=4-5 per experiment. Data analyzed using student's t test (*p<0.05; **p<0.01, ***p<0.001)

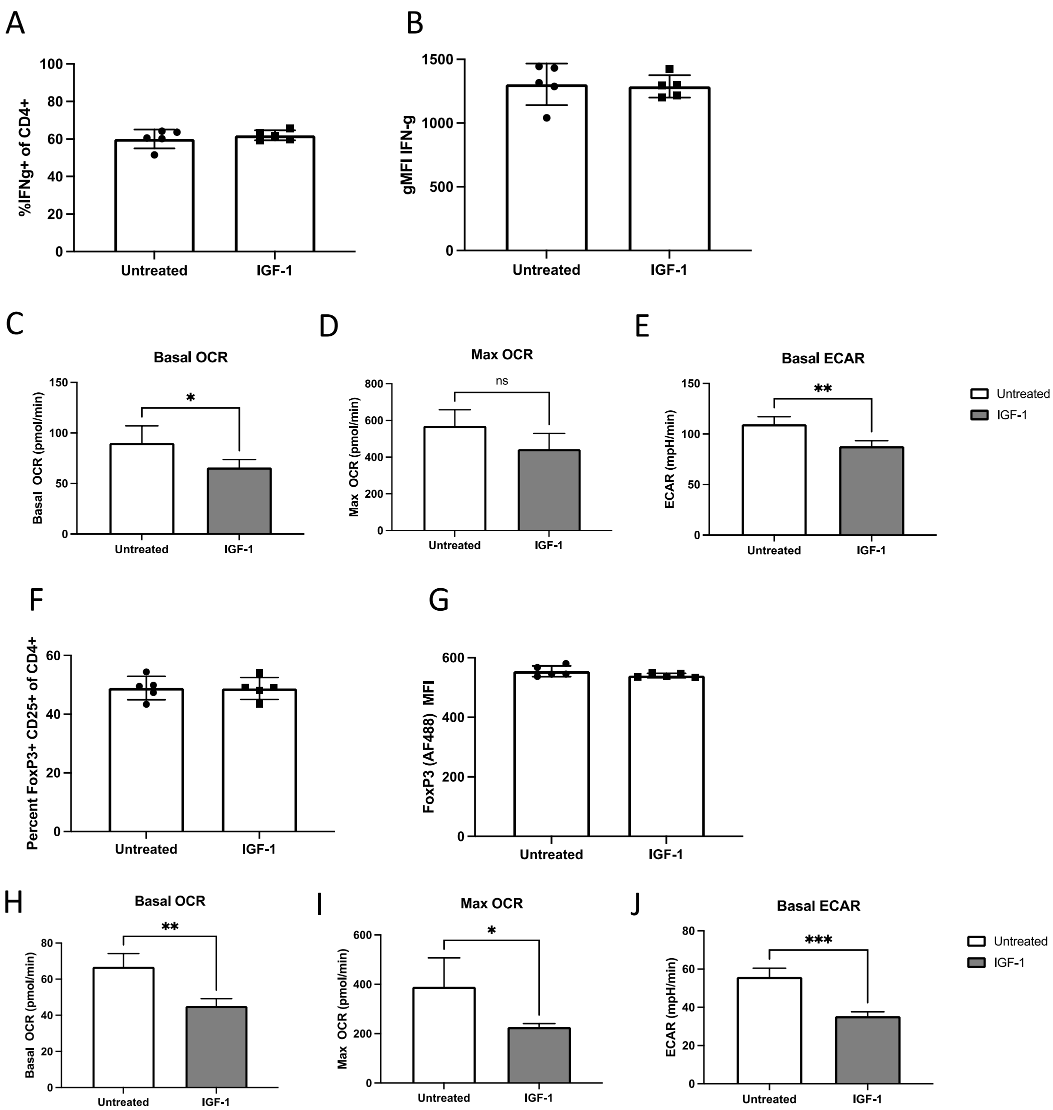

Figure 16: Th1 and Treg cells do not respond to IGF-1 in the same way as Th17 cells

$CD4^+$ T cells from C57BL/6 mice were differentiated in vitro to Th1 or Treg cells in neuronal serum-supplemented media for three days, and then treated with or without IGF-1 (50ng/mL) for an additional 48 hours. (A-B) Cells were analyzed by flow cytometry for percent IFN-γ positive cells (A) and IFN-γ expression by MFI (B). (C-E) Extracellular flux analysis was used to measure basal OCR, max OCR, and basal ECAR. (F-G) Cells were analyzed by flow cytometry for percent FoxP3+ CD25+ positive cells (F) and FoxP3 expression by MFI (G). (H-J) Extracellular flux analysis was used to measure basal OCR, max OCR, and basal ECAR. Data representative of 2 independent experiments. n=4-5 per experiment. Data analyzed using student's t test (*p<0.05; **p<0.01, ***p<0.001)

3.2.6 IGF-1 treatment of Th17 cells reduces mitochondrial membrane potential and mitochondrial ROS levels

IGF-1 treated Th17 cells also had decreased mitochondrial membrane potential (MMP) compared to untreated Th17 cells, as measured by TMRE staining (Figure 17A), but showed no significant difference in mitochondrial mass (Figure 17B). Decreased membrane polarization can be an indicator that the mitochondria are uncoupled, meaning that protons are translocating across the inner mitochondrial membrane through a mechanism other than ATP synthase, and are thus not used to make ATP. This mechanism of uncoupling can be a used by cells to protect against oxidative stress because increased mitochondrial polarization causes an increase in ROS production. To test whether IGF-1 treatment of Th17 cells decreased mitochondrial ROS along with the decrease in MMP, we used Mitosox to stain for ROS in the mitochondria. Indeed, we saw that Th17 cells treated with IGF-1 had reduced Mitosox staining (Figure 17C). Furthermore, cellular ROS production, as measured by Cellrox staining, also showed decreased ROS production in IGF-1 treated Th17 cells (Figure 17D), suggesting that IGF-1 exerts a cytoprotective effect in addition to a metabolic effect.

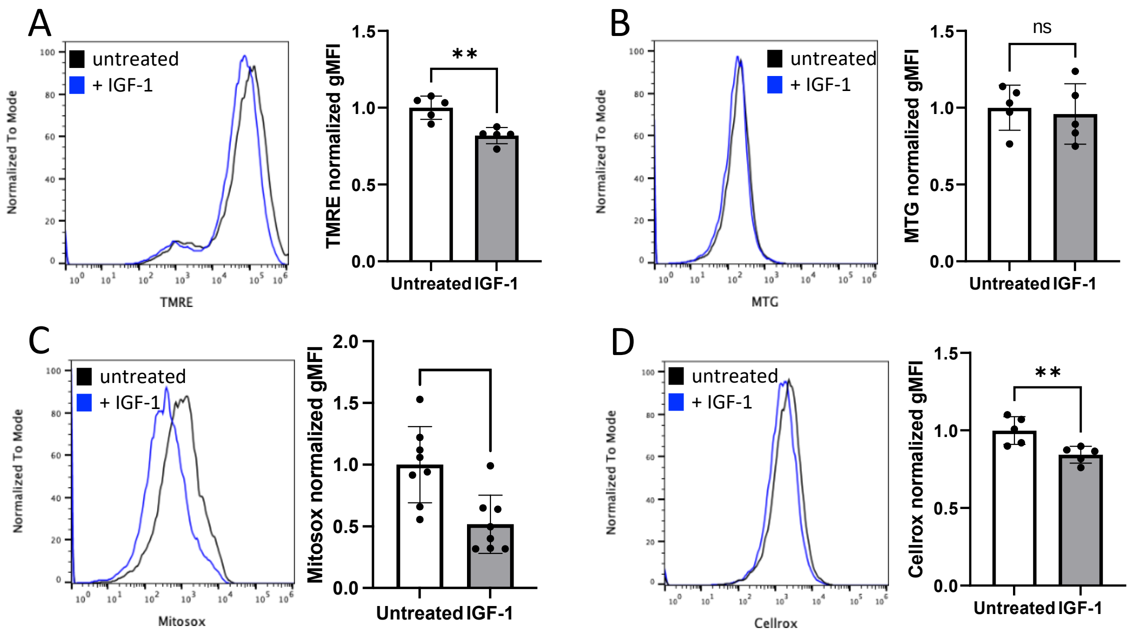

Figure 17: IGF-1 treatment decreases mitochondrial membrane potential and reactive oxygen species in Th17 cells

CD4⁺ T cells from C57BL/6 mice were differentiated in vitro to Th17 cells in neuronal serum-supplemented media for three days, and then treated with or without IGF-1 (50ng/mL) for an additional 48 hours. (A) Mitochondrial membrane potential measured by TMRE staining, normalized to untreated gMFI. (B) Mitochondrial mass measured by mitotracker green staining, normalized to untreated gMFI. (C) Mitochondrial reactive oxygen species production measured by Mitosox staining, normalized to untreated gMFI. (D) Cellular ROS production measured by Cellrox staining, normalized to untreated gMFI. Data combined from 2-3 independent experiments. Representative histograms shown. n=2-3 per experiment. Data analyzed using student's t test (*p<0.05; **p<0.01, ***p<0.001)

3.2.7 Cytoprotective effect of IGF-1 on Th17 cells requires both IR and IGF-1R

To determine if these IGF-1-mediated changes on Th17 cell mitochondrial membrane potential and mitochondrial ROS require signaling by IR, IGF-1R, or both, we differentiated Th17 cells in vitro from IGF-1R cKO mice and littermate controls. Following differentiation, Th17 cells were treated with IGF-1 for 48 hours. We observed that IGF-1 treatment of Th17 cells from control mice decreased TMRE staining but had no effect on TMRE in Th17 cells from IGF-1R cKO mice (Figure 18A). Consistent with our earlier results, Mitotracker Green staining was not significantly changed in either control or IGF-1R cKO Th17 cells treated with IGF-1 (Figure 18B). Similar to TMRE, Mitosox staining was decreased on control Th17 cells but not on Th17 cells generated from IGF-1RcKO mice (Figure 18C). These data suggest that the effect of IGF-1 on Th17 cell mitochondrial membrane polarization and mitochondrial ROS production is IGF-1R dependent.

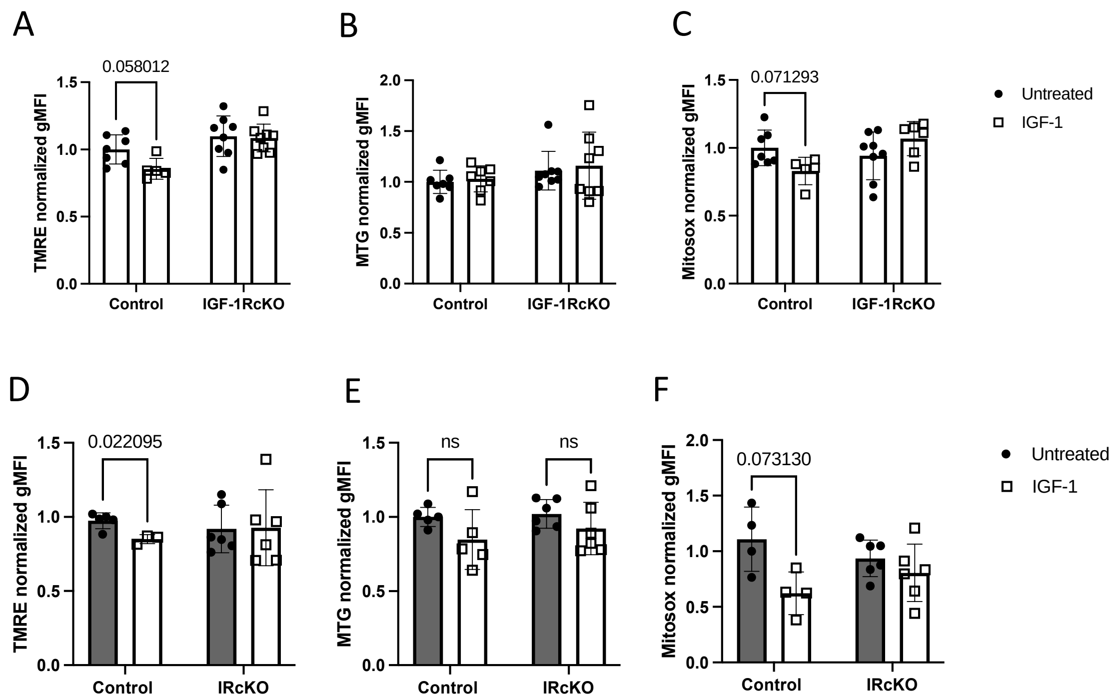

Figure 18: IGF-1 treatment of Th17 cells decreases membrane potential and mtROS production in an IGF-1R and IR dependent manner

(A-C) CD4+ T cells were isolated from spleens of control and IGF-1RcKO mice and differentiated *in vitro* to Th17 cells in neuronal serum-supplemented media for three days, then treated with or without IGF-1 (50ng/mL) for an additional 48 hours. Mitochondrial membrane potential measured by TMRE staining. Normalized geometric mean fluorescence intensity shown *(A)*. Mitochondrial reactive oxygen species production measured by Mitosox staining. Normalized geometric mean fluorescence intensity shown *(B)*. Mitochondrial mass measured by mitotracker green staining. Normalized geometric mean fluorescence intensity shown *(C)*. **(D-F)** CD4+ T cells were isolated from spleens of control and IRcKO mice and differentiated *in vitro* to Th17 cells in neuronal serum-supplemented media for three days, then treated with or without IGF-1 (50ng/mL) for an additional 48 hours. Mitochondrial membrane potential measured by TMRE staining *(D)*. Normalized geometric mean fluorescence intensity shown. Mitochondrial reactive oxygen species production measured by Mitosox staining. Normalized geometric mean fluorescence intensity shown *(E)*. Mitochondrial mass measured by mitotracker green staining. Normalized geometric mean fluorescence intensity shown *(F)*. Data combined from three independent experiments. (A-C) n= 3-9 per group (D-F) n=3-6 per group. Data analyzed using students t-test (p values shown)

Insulin and IGF-1 are closely related hormones that bind to receptors from the same tyrosine kinase receptor family. Insulin and IGF-1 signal through insulin receptor (IR) and IGF-1 receptor (IGF-1R), respectively. However, because these hormones and receptors are so closely related, insulin and IGF-1 can signal through each other's receptors, albeit at a lower efficiency. Furthermore, IR and IGF-1R chains can heterodimerize to form a hybrid receptor, through which both hormones can signal, although IGF-1 has a higher affinity.

Since IGF-1R and IR are highly homologous, we also investigated the requirement for IR in mediating the effect of IGF-1 on mitochondrial membrane potential and mROS production in Th17 cells. While IGF-1 treatment decreased the mitochondrial membrane potential in Th17 cells from control mice, this effect was lost in IR cKO Th17 cells (Figure 18D). Mitochondrial mass was not significantly changed following IGF-1 treatment of Th17 cells from either control or IR cKO mice (Figure 18E). IGF-1 treatment reduced mROS production by Th17 cells from control mice, but not by Th17 cells from IR cKO mice (Figure 18F). Interestingly, these results are similar to those in Th17 cells from IGF-1R cKO mice, meaning that the effect of IGF-1 on Th17 cell mitochondrial membrane polarization and mitochondrial ROS production is both IR and IGF-1R dependent. This indicates a potential role for the IR/IGF-1R hybrid receptor in mediating effects of IGF-1 on Th17 cell mitochondrial function.

3.2.8 Uncoupling protein 2 mediates the cytoprotective effect of IGF-1 on Th17 cells

Since IGF-1 treatment of Th17 cells was shown to reduce mitochondrial membrane potential and mROS production, we next set out to understand how IGF-1 signaling could be mediating this effect. One mechanism by which the mitochondrial membrane potential can be decreased is through uncoupling proteins, which move protons back across the inner mitochondrial membrane, bypassing ATP synthase.

Uncoupling protein 2 (UCP2) is a mitochondrial transmembrane protein which functions to allow protons to flow across the inner mitochondrial membrane, thus uncoupling ATP synthesis (188). UCP2 is closely related to uncoupling protein 1, which is involved in brown fat thermogenesis. UCP2 is known to be expressed on immune cells and is upregulated in $CD4^+$ and $CD8^+$ T cells during activation (189). In $CD8^+$ T cells, UCP2 was shown to inhibit glycolysis and fatty acid synthesis, while reducing ROS (190). Total body knockout of UCP2 results in more severe disease in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (191). This is due to the increase in ROS produced by innate immune cells (191). Interestingly, UCP2 has been shown to be upregulated by IGF-1 treatment in cancer cell lines (192).

We therefore investigated whether IGF-1 treatment of Th17 cells increased UCP2 expression. Indeed, we found that IGF-1 upregulated UCP2 mRNA in both activated $CD4^+$ T cells and in differentiated Th17 cells (Figure 19A-B). Furthermore, we observed an increase in UCP2 protein levels after IGF-1 treatment (Figure 19C). We next used an inhibitor of UCP2, genipin, to probe whether UCP2 activity was required for IGF-1 effects on mitochondrial metabolism. When Th17 cells were treated with IGF-1 in the presence of the UCP2 inhibitor, the IGF-1 induced changes in mitochondrial membrane potential and mROS production were reversed, without affecting mitochondrial mass (Figure 19D-F). UCP2 inhibition also reversed IGF-1 effects on oxygen consumption, proton leak, and ATP production (Figure 19G-I). These data suggest that the cytoprotective effects of IGF-1 are mediated, at least in part, through UCP2 activity.

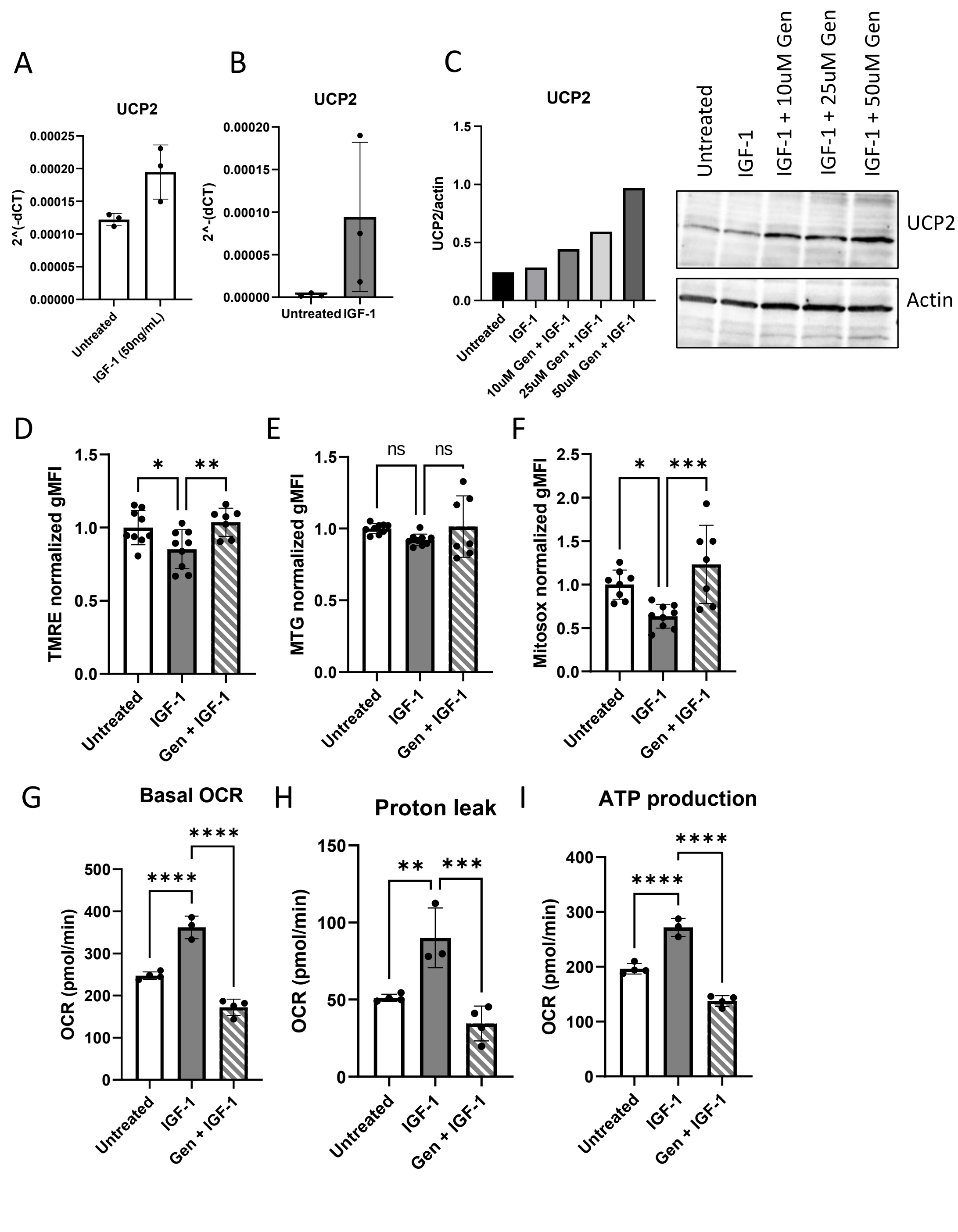

Figure 19: Inhibition of UCP2 with Genipin reverses the effect of IGF-1 on mitochondria and metabolism

(A) $CD4^+$ T cells were activated for 48 hours on anti-CD3/CD28 coated plates in serum free conditions and treated with IGF-1 for the last 24 hours of activation. Expression of UCP2 was analyzed by qRT-PCR. (B-I) $CD4^+$ T cells from C57BL/6 mice were differentiated in vitro to Th17 cells in neuronal serum-supplemented media for three days, and then treated with or without IGF-1 (50ng/mL) or IGF-1 + Genipin (Gen; 10uM, 25uM, or 50uM) for an additional 48 hours. (B) Expression of UCP2 was analyzed by qRT-PCR. (C) Expression of UCP2 was analyzed by immunoblot (D) Mitochondrial membrane potential measured by TMRE staining. Normalized geometric mean fluorescence intensity shown (E) Mitochondrial mass measured by mitotracker green staining. Normalized geometric mean fluorescence intensity shown. (F) Mitochondrial reactive oxygen species production measured by Mitosox staining. Normalized geometric mean fluorescence intensity shown. (G-H) Extracellular flux analysis was used to measure basal OCR, proton leak, and ATP synthesis. (A-C,G-I) Data representative of two independent experiments n=3-4 per experiment (D-F) Data combined from two independent experiments. n=7-9 per group. Data analyzed using one way ANOVA (*p<0.05; **p<0.01, ***p<0.001, p<0.0001)

3.3 Discussion and Conclusions

Previous studies have independently shown the effects of insulin and IGF-1 on $CD4^+$ T cell metabolism and function. Given the high degree of homology of IR and IGF-1R, the overlapping signaling pathways, and the potential for insulin and IGF-1 to signal through both receptors, we set out to understand the relationship between these effects. Here we have presented data suggesting that IGF-1 receptor expression is upregulated upon $CD4^+$ T cell activation at both the protein and transcript level. This is in contrast to previous studies that indicate increased insulin receptor expression upon activation using ligand binding assays. Since insulin can bind to IGF-1 receptor, it is possible that at saturated staining conditions, IGF-1 receptor was detected using this method.

We also show side by side the metabolic and functional effect of insulin and IGF-1 on activated $CD4^+$ T cells. Our data suggest that insulin has a more potent metabolic effect on $CD4^+$ T cells at physiologic concentrations. In contrast, IGF-1 has a more specific functional effect regarding increasing IL-17 production in both bulk activated $CD4^+$ T cells and Th17 cells. Furthermore, our data demonstrate that the metabolic reprogramming downstream of insulin or IGF-1 signaling is maintained for days, which adds color to the previous studies that involve serum starvation followed by acute hormone treatment. This suggests that nutritional status communicated to T cells results in a metabolic and functional program that is persistent and maintained.

While investigating whether IGF-1 impacted mitochondrial biogenesis and function in $CD4^+$ T cells, we were surprised to find that the mitochondrial membrane potential (TMRE staining) was reliably decreased downstream of IGF-1 treatment. Polarization of the mitochondria is very tightly controlled, and depolarization could indicate proton leakage back across the mitochondrial membrane and thus decoupling of the electron transport chain from ATP production by ATP synthase. Reduced mitochondrial membrane potential could indicate non-functional mitochondria, however our extracellular flux analyses demonstrate that the mitochondria are performing oxidative phosphorylation at an increased rate in IGF-1 treated cells. Another potential effect of decreased mitochondrial membrane potential is protection against mitochondrial ROS generation. Indeed, we found that IGF-1 treatment reduced mROS levels in Th17 cells.

In order to depolarize the membrane, protons must be allowed to flow back across the inner mitochondrial membrane through some mechanism. While uncoupling protein 1 is highly expressed in brown adipose tissue, UCP2 is known to be expressed in immune cells and many others cell types. We observed that UCP2 mRNA and protein levels were responsive to IGF-1 treatment in Th17 cells, suggesting that perhaps UCP2 could be participating in the uncoupling of the mitochondria downstream of IGF-1 signaling. To test this hypothesis, we used an inhibitor of UCP2, genipin, to demonstrate that the effects of IGF-1 on mitochondrial membrane potential and ROS production are dependent on UCP2 activity. Indeed, our data suggest that IGF-1 mediates a cytoprotective effect on Th17 cells by uncoupling the mitochondria through expression of UCP2.

Our model is as follows: IGF-1, signaling through IR and/or IGF-1R, signals to the cell to increase mitochondrial metabolism as well as glycolytic metabolism. In the setting of Th17 cells, in particular, IGF-1R signaling includes an increase in uncoupling protein expression that reduces membrane potential and reduces ROS production, which is particularly damaging to Th17 cells. Inhibition of this uncoupling protein reverses the metabolic and mitochondrial effects of IGF-1 treatment of Th17 cells. This mechanism could be very important in Th17 cells. According to Gerriets et al. and others, Th17 cells are sensitive to ROS signaling, which could explain why this mechanism occurs specifically in Th17 cells (34). IGF-1 signaling on Th17 cells could have evolved to protect the Th17 response from potential damage due to high metabolic flux.

Interestingly, both IR and IGF-1R appear to be required for the effects of IGF-1 on Th17 cells, which could indicate that the hybrid receptor is required for mediating the effects of IGF-1 on Th17 cells in particular. The hybrid receptor has been shown to form in various cell lines, however the presence of hybrid receptor on $CD4^+$ T cells has not been established. The IR/IGF-1R hybrid receptor has a greater affinity for IGF-1 than insulin but may have independent action of either IR or IGF-1R. Further studies using double knockout mice and co-immunoprecipitation/proximity ligation assay are required to establish the presence of hybrid receptor and the requirement for both IR and IGF-1R in this process.

3.4 Materials and Methods

3.4.1 Animals

T cell-specific IGF-1 receptor (IGF-1R) knockout mice were generated by crossing CD4Cre transgenic mice with IGF-1R-floxed mice (Jackson Laboratory). The mice were backcrossed for ten generations to the C57B/L6 background. The following genotypes were studied: $CD4Cre^{+}IGF\text{-}1R^{fl/fl}$ (IGF-1 receptor knockout mice; IGF-1RcKO) and

CD4Cre⁻IGF-1R$^{fl/fl}$ (controls; Ctrl). Mice were group housed (up to 5 per cage), maintained at ambient temperature, and given ad libitum access to food and water. Experimental mice were used at 8-12 weeks of age. All animal protocols were approved by the Institutional Animal Care and Use Committees at Duke University or the University of North Carolina at Chapel Hill.

3.4.2 Tissue collection, processing, and cell culture

Mice were euthanized using CO_2 inhalation. Spleens were mashed and strained in PBS, washed, and resuspended. $CD4^+$ T cells were isolated from splenocytes using the StemCell $CD4^+$ T cell isolation kit (StemCell technologies, Vancouver, BC, Canada). Isolated $CD4^+$ T cells were activated for 48 hours on a plate that was coated overnight with anti-CD3 (1ug/mL; Biologend) and anti-CD28 (5ug/mL; Biolegend). $CD4^+$ T cells were treated with IGF-1 (50ng/mL) after 24 hours of activation. Activated $CD4^+$ T cells were analyzed by flow cytometry, extracellular flux analysis. Serum free conditions were used to minimize the impact of growth factors in media influencing results. RPMI was supplemented with P/S, Glutamine, Hepes, BME, and either 0.35% BSA, or N21 media supplement (1x), which is certified insulin free (Neuronal serum). $CD4^+$ T cells were plated at 1 million cells per mL.

To skew $CD4^+$ T cells to functional subsets of T helper cells, the protocol described by Espinosa et al (193) was followed.

3.4.3 Metabolic flux assays

CD4$^+$ T cells were washed with Seahorse XF RPMI 1640 media (Agilent, Santa Clara, CA) and plated at a density of 250,000 cells/well (50 μL) in a Seahorse XFe96 plate (Agilent) pre-coated with Cell-Tak (Corning, Corning, NY). After spinning down the plate at 200 rpm for 1 min, the plate was incubated for 30 min in a humidified 37°C incubator in the absence of CO_2. Seahorse XF RPMI 1640 media (130 μL) was added, and the plate was incubated for an additional 20 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe96 Analyzer (Agilent). Mitostress test was performed by programmed injection of oligomycin (final concentration 1μM), FCCP(0.5μM), and Rotenone(0.75μM)/Antimycin A (1μM).

3.4.4 Flow cytometry

At least 1 million splenocytes were stimulated for 4.5 h in complete media containing Golgi Plug (2 μg/ml) (BD Biosciences), PMA (50 ng/ml) (Sigma-Aldrich, St. Louis, MO), and ionomycin (1 μg/ml) (Sigma-Aldrich), then permeabilized and fixed with Cytofix/Cytoperm kit (BD Biosciences) and stained for IFN-γ and IL-17A following the manufacturer's protocol. Samples were acquired on a ThermoFisher Attune NxT flow cytometer or a BD Canto flow cytometer, and data were analyzed using FlowJo (Treestar, Ashland, OR).

3.4.5 Metabolic dyes

To look at mitochondrial mass, 100,000 cells were plated in a V-bottom 96-well plate and stained with Mitotracker green (50nM final concentration) for 30 minutes at 37°C. To look at mitochondrial membrane potential, cells were stained with TMRE (100nM) for 30 minutes at 37°C. To look at mitochondrial ROS production, cells were stained with Mitosox Red (1.25μM) for 30 minutes at 37°C. To look at nutrient uptake, cells were stained in either Bodipy (10μM) or 2NBDG (100μM) for 30 minutes at 37°C. Samples were acquired on a ThermoFisher Attune NxT flow cytometer, and data were analyzed using FlowJo (Treestar, Ashland, OR).

3.4.6 ELISA

Supernatants were collected from cell culture samples at the time of cell collection. Samples were retained at -80°C until ELISA was performed. IL-17 ELISAs (Biolegend) were performed according to manufacturer's instructions at sample dilutions of either 2x or 5x. IFN-γ ELISAs (Biolegend) were performed according to manufacturer's instructions at sample dilutions of either 50x or 100x.

3.4.7 Western blot

Cell pellets were lysed with RIPA buffer plus protease inhibitors and phosphatase inhibitors (PhosStop) for 20-40 minutes on ice, vortexing every 5 minutes.

The pellets were then spun down for 30 minutes at high speed and the supernatants collected. Protein concentration was determined by colorimetric protein assay. Protein was diluted to 1ug/uL in lysis buffer and 5x sample buffer. 35uL of protein was loaded onto an 8-15% gel, with 8uL ladder on either side of samples. Gel was run for 40 minutes at 200V. Gel was transferred onto a PVDF membrane (BioRad) using the BioRad transfer case. Membrane was blocked overnight in 5% BSA in TBST on a shaker at 4°C. Membrane was then blotted with antibodies to either IR, IGF-1R, actin, UCP2, or FOXO1 (Cell Signaling) for 1 hour at RT, up to overnight at 4°C. Blots were imaged on BioRad gel imager and analyzed using BioRad ImageLab software.

3.4.8 qRT-PCR

RNA was isolated from cell pellets using RNeasy kits (Qiagen) following manufacturer's instructions. cDNA was made from isolated RNA using BioRad iScript cDNA synthesis kits. qPCR reaction using Syber Green included 1uL sample cDNA and 3uL of Syber, primers, water mix in a 386 well plate. qPCR was run on BioRad CFX Maestro.

4. Conclusions

4.1 Conclusions

Despite the evidence that leptin contributes significantly to the inflammatory T cell phenotype in obesity and that inflammatory T cells promote aberrant glucose metabolism in obesity, our work demonstrates that loss of leptin receptor on T cells is not sufficient to reverse the systemic metabolic dysfunction seen in obesity. We did find however, that leptin signaling to $CD4^+$ T cells promotes some of the inflammatory phenotype of obese T cells, including increased oxidative metabolism and increased IFN-γ production by $CD4^+$ T cells. Together, these data suggest that other hormones, adipokines, and cytokines could be contributing to T cell inflammation in obesity. For example, insulin and IL-6 are known to be upregulated in obesity and both promote activation and inflammatory phenotypes in T cells. Furthermore, insulin, IGF-1 and leptin have overlapping signaling pathways such that insulin and IGF-1 may be able to compensate for the loss of leptin signaling to some extent.

Our work here adds to the growing body of work describing the response of T cells to nutritionally regulated hormone signaling, in particular insulin and IGF-1. T cells require upregulated metabolism upon activation to grow, divide and produce cytokine. Interestingly, the very same anabolic pathways T cells require for proper activation are those promoted by insulin and IGF-1 signaling. This signals that nutritionally regulated hormones could potentially license T cells for activation, providing additional stimulus (in addition to the TCR/costimulation and cytokine) to promote the metabolic remodeling needed to support ideal T cell function. Lending credence to this idea is the evidence that hormone levels must be finely tuned. High levels as in obesity or low levels as in undernutrition result in aberrant T cell metabolism and function. Additionally, our newly discovered cytoprotective role for IGF-1 in Th17 cells further supports this claim, such that IGF-1 licenses the activation of Th17 cells, while simultaneously protecting them from the consequences of massive metabolic activity. This is a fascinating mechanism that connects total body nutritional status to the intricate mitochondrial processes of regulating membrane potential.

4.2 Future directions

This work opens the possibility that T cells are sensitive to environmental signals beyond cytokines. Future work should continue to explore what other systemic signals T cells are sensitive to that could promote activation, metabolic changes, or functional outcomes that are yet unexplored. For example, our lab is looking into the metabolic effects of IL-6 signaling. As the connections between the immune system and other body systems such as the endocrine system emerge, our understanding of the regulation of the immune response must expand to the signaling molecules produced by these systems.

First, given that the presence of both insulin and IGF-1 receptors are required for the cytoprotective effect of IGF-1, as shown here, elucidating the presence of the hybrid receptor on the T cell surface must be a priority. Understanding the receptors through which these signals are being mediated will clarify downstream effects and help us to understand how insulin and IGF-1 signal to T cells. The development of antibodies specific for mouse insulin receptor and IGF-1 receptor will be critical to this effort, allowing the examination of cell surface expression by flow cytometry, and the presence of hybrid receptors by proximity ligation assay. Additionally, generating an IR/IGF-1R double knockout mouse would remove the possibility of compensation by signaling through the other receptor.

Future studies will further elucidate the connection between IGF-1 signaling and UCP2 expression and activity. UCP2 is known to be regulated by glutathionylation. Since IGF-1 is known to affect the glutathione pathway (194), it would be interesting to understand whether this is the mechanism by which IGF-1 regulates UCP2, or whether it is by direct transcriptional control, or some combination of these mechanisms. Furthermore, UCP2 is known to have a role as a metabolic sensor that regulates a metabolic switch from glycolysis to glutaminolysis and fatty acid oxidation. Given that Th17 cells rely greatly on glutamine metabolism, the role of UCP2 in Th17 metabolism would be interesting to explore.

Finally, there is some evidence for direct regulation of gene expression by nuclear translocation of activated insulin receptor and IGF-1 receptor. Whether this mechanism occurs in T cells is unknown. Given this possibility, it would be interesting to understand whether direct transcriptional control of metabolic genes is occurring with activation of IR and IGF-1R, or whether canonical signaling pathways are solely responsible in T cells.

4.3 Final Remarks

The immune system is informed by the nutritional status of the organism. Mounting an immune response is energetically expensive, so resources are tightly controlled, and energy availability must be signaled to immune cells prior to activation. Therefore, nutritionally regulated hormones license the activation and metabolic reprogramming of T cells. In the context of obesity, leptin promotes the inflammatory and metabolically dysfunctional T cells that are associated with metabolic syndrome. However, leptin signaling to T cells is not sufficient to drive obesity-associated metabolic dysfunction systemically. Other nutritionally regulated mediators, insulin and IGF-1 promote anabolic metabolism of activated T cells. IGF-1 specifically exerts a cytoprotective effect on Th17 cells, whereby mitochondrial membrane potential and mitochondrial ROS are reduced by the action of UCP2. These studies explore the role that nutritionally regulated hormones play in regulating and licensing the immune response and provide new avenues for exploration of T cell metabolism and function.

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Biography

Kaitlin Kiernan McConnell graduated from the University of Pittsburgh in 2016 with a Bachelor of Science degree in Molecular Biology. Later that year, she joined the Department of Immunology at Duke University as a graduate student where she received the James B. Duke Fellowship upon matriculation. Kaitlin joined the laboratory of Dr. Nancie MacIver in spring of 2017. Kaitlin worked on several projects in the lab that led to several laboratory publications. A first author manuscript titled "Effects of T cell leptin signaling on systemic glucose tolerance and T cell responses in obesity" is currently in revision. Kaitlin's first author manuscript based on her insulin and IGF-1 thesis work is in preparation and will be submitted in the coming weeks. Kaitlin also wrote a review article titled "The role of the adipokine leptin in immune cell function in health and disease," which was published in Frontiers in Immunology in 2021, and contributed to another review, "Changes in Nutritional Status Impact Immune Cell Metabolism and Function," which was published in Frontiers in Immunology in 2018. Kaitlin also wrote two comment articles titled "A Novel Mechanism for Th17 Inflammation in Human Type 2 Diabetes Mellitus" published in Trends in Endocrinology and Metabolism in 2020, and "Viral Infection "Interferes" with Glucose Tolerance," which was published in Immunity in 2018.