OBESITY INDUCES METABOLIC ALTERATIONS OF T LYMPHOCYTES IN INFLUENZA VACCINATED ADULTS

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ABSTRACT

William D. Green: Obesity induces metabolic alterations of T lymphocytes in influenza-vaccinated adults
(Under the direction of Melinda Beck)

Obesity increases the risk for infection due to impairments in T cell activation and function. Although the metabolic profiles of T cells dictates their function, what remains unknown is if obesity alters T cell metabolism, thereby impairing function. This study examined the metabolic profiles of T cells from healthy weight and obese white women. We demonstrated that obesity alters the metabolic profile of quiescent and activated peripheral blood mononuclear cells, and isolated CD4 and CD8 T cells. T cells from obese non-diabetic subjects exhibited higher rates of aerobic glycolysis and mitochondrial respiration compared to obese metformin-treated diabetic subjects. This ability of metformin to lower T cell metabolism in obese diabetics to healthy weight levels, despite elevated serum glucose, suggests that metformin's mechanism of action directly impacts T cells. These results suggest that perturbations in T cell metabolism are the mechanism for impairment of T cell function in obese adults.

To my girlfriend Natalie, my father David, and brother Joseph,
these past two years would not have been possible without your
love and support; and to my late-mother Patricia, losing you was
an incredibly devastating and challenging time. But it forced me
to grow into the person I am today and I dedicate this work in your memory.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

PBMC	Peripheral blood mononuclear cell
TCR	T cell receptor
ATP	Adenosine triphosphate
IL-7	Interleukin-7 (naïve cell homeostasis cytokine)
IL-15	Interleukin-15 (memory cell homeostasis cytokine)
IL-2	Interleukin-2 (pro-growth, proliferation cytokine)
IL-10	Interleukin-10 (anti-inflammatory, repressor cytokine)
IFN-γ	Interferon gamma
T_{reg}	T regulatory cell
AMPK	Adenosine-mono-phosphate kinase
mTOR	Mammalian Target of Rapamycin
Bcl-2	B-cell lymphoma 2
HB	Hepatitis B
HA	Hepatitis A
$LepR^{H-/-}$	Hypothalamic Leptin Receptor knockout mouse
$LepR^{HFlox/Flox}$	Hypothalamic Leptin Receptor Flox mouse
Glut1	Glucose transporter 1
ATMs	Adipose Tissue Macrophages
APCs	Antigen presenting cells
MHCII	Major histocompatibility complex II
Akt	Protein kinase B (serine/threonine protein kinase)
Th1	T helper 1 subtype (CD4+ lineage)

Th2	T helper 2 subtype (CD4+ lineage)
Th17	T helper 17 subtype (CD4+ lineage)
VAT	Visceral adipose tissue
CD4+	Cluster of differentiation 4 – helper T cell marker
CD8+	Cluster of differentiation 8 – cytotoxic T cell marker
CD153+	Cluster of differentiation 153 – TNFα ligand or CD30 ligand
CD44+	Cluster of differentiation 44 – effector-memory T cell marker
PD-1	Programmed cell death protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
OXPHOS	Oxidative phosphorylation
OCR	Oxygen consumption rate (pmoles/min)
ECAR	Extracellular acidification rate (mpH/min)
OCR:ECAR	OCR/ECAR
FBS	Fetal bovine serum
RPMI-1640	Leukocyte culture media
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
BPL	B-propiolactone
H1N1	hemagglutinin 1 neuraminidase 1
H3N2	hemagglutinin 3 neuraminidase 2
OCT	Organic Cation Transporter
ConA	Conacanavalin A
ANOVA	Analysis of variance
ELISA	Enzyme-linked immunosorbent assay

ILI	Influenza-like illness
HAI	Hemagglutination inhibition assay
BMI	Body mass index

CHAPTER I - BACKGROUND

Introduction

Prevalence rates of obesity have exponentially increased within the past few decades [1]. This dramatic rise in cases of obesity increases the risk for a variety of chronic and infectious diseases. Of particular note, obesity increases the risk of morbidity and mortality to influenza virus infection independent of other co-morbidities [2]. In any given year, roughly 3,000 to 50,000 people die from influenza infection in the United States [3]. However, in pandemic years, as was the case in 2009 with the pandemic H1N1 influenza outbreak, the number of incident cases and resultant deaths from influenza infection greatly increases, especially in high risk populations like children, the elderly and the obese.

The immune response to infection involves a complex orchestra of diverse cell types, including dendritic cells, macrophages, natural killer cells, and B and T cells. As one of the most prominent and critical players in the response to infection, subsets of T cells range in activity from supporting the function and activation of other immune cells, as well as T cells themselves, to producing pro and anti-inflammatory proteins. Furthermore, cytotoxic T cell subsets are instrumental in the elimination of pathogen-infected host cells. This diversified set of immunologically adaptive immune cells plays a critical and central role in combating pathogens. A number of T cell knockout models have demonstrated that removal of any subset of T cells during an infectious challenge often results in higher rates of morbidity and mortality. For many years, T cell activation and function was believed to occur from a combination of antigen recognition, subsequent signaling cascades and micro-environmental cues [4,5]. However, recent

studies have clearly demonstrated that the cellular metabolism of the T cell is also a key player affecting differentiation, proliferation, function and its ultimate fate [6-11].

Recent publications of how metabolic fuels such as glucose, amino acids and fatty acids elicit distinct metabolic profiles depending on cell state (naïve, effector or memory) and subtype (Th1, Th2, Th17, Treg, etc.) [7,12-14], has led to a revolution in the understanding of T cell driven immunity. Furthermore, these findings highlights the metabolic plasticity of T cells to respond to the energetic and biosynthetic demands required to successfully fight infection. For primers on T cell metabolism, refer to the excellent reviews by MacIver *et al.* [4] and Buck *et al.* [5].

Although T cells respond to antigenic challenge by altering their metabolic state, what is not as well understood is how metabolic dysfunction may alter their ability to function. One such altered metabolic environment that may have a profound effect on T cell function is obesity. Obesity has classically been characterized as a state of excess adiposity and is associated with chronic inflammation and metabolic dysfunction such as hyperglycemia, hyperleptinemia and hormone resistance [15]. These uncontrolled metabolic alterations can lead to the development of chronic diseases such as type II diabetes, nephritis and/or chronic kidney disease, cardiovascular disease and specific cancers [16]. However, recent data from our lab and others demonstrate a link between obesity and increased incidences of infectious diseases, most likely through impaired cellular and humoral immune responses [16,17].

Considering recent findings on how T cell metabolism drives cellular function and survival, understanding how obesity impacts these processes in T cells remains critical. Here, we examine the impact of obesity on lymphocyte metabolism, specifically CD4+ and CD8+ T cells, in adult white women vaccinated against influenza virus. This work utilizes a unique high-

throughput method to determine metabolic profiles of cells to explore possible mechanisms for T cell impairment in the context of obesity. Given this exponential increase in the prevalence of obesity over the past few decades, coupled with the increasing threat of influenza epidemics and pandemics, understanding how the complex metabolic state of obesity influences immunity warrants investigation.

T cell metabolism supports cell survival and function

T cells display metabolic flexibility unlike other cells in the body. Upon stimulation of the T cell receptor (TCR) and costimulatory receptors, T cells undergo a dramatic metabolic shift from a quiescent to an activated state, highlighted by a change from primarily catabolic to anabolic metabolism (Figure 1) [7]. The shift from oxidative phosphorylation to glycolysis produces ATP and supports the generation of nucleotides and amino acids for the production of daughter cells necessary to mount an effective immune response [18]. Importantly, this glycolytic shift supports effector functions, which vary depending on T cell subtype [6,14,19]. After clearance of the infection, the majority of effector T cells undergo apoptosis, with a small subset remaining as long-lived memory T cells [20,21]. These memory T cells revert back to a relatively quiescent catabolic state. However, unlike their naïve cell counterparts, memory T cells quickly respond to antigenic challenge upon re-exposure through elevated metabolic activity, increased proliferation and production of cytokines [22,23].

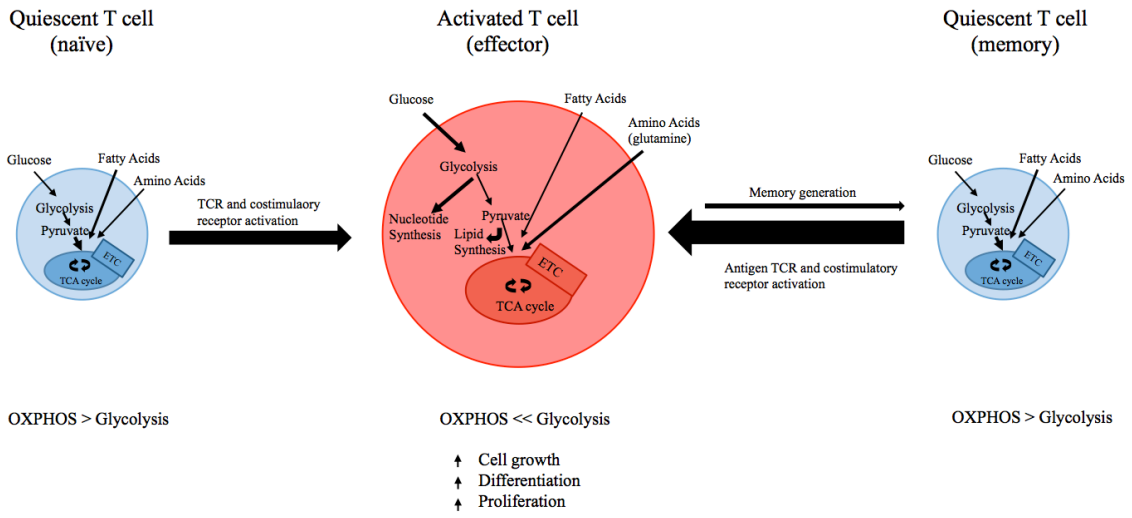

Figure 1: T cell metabolism and state of quiescent and activated T cells. Quiescent T cells utilize oxidative respiration of glucose-derived pyruvate as well as fatty acids and amino acids to produce ATP through the TCA cycle and electron transport chain. This energy production supports immune surveillance and homeostasis. Upon stimulation of the T cell receptor (TCR) and costimulatory receptors, T cells upregulate glycolysis and glutamine oxidation while reducing fatty acid oxidation to support cell growth, differentiation and the production of daughter cells. Following clearance of the pathogen, the majority of T cells undergo apoptosis with a subset surviving as memory T cells, which return to a quiescent state dependent on oxidative phosphorylation of fatty acids.

These distinct metabolic states of T cells require signaling molecules to support homeostasis, effector function and survival. Cytokines such as IL-7 and IL-15 sustain the catabolic survival functions of naïve and memory T cells, respectively [19,24,25]. Other cytokines, such as IL-2, elicit proliferation [26], while interferon gamma (IFN-γ) promotes pro-inflammatory T cell subtypes like Th1 and cells of innate immunity [27]. T cell subsets also help to down-regulate the inflammatory response once pathogen clearance has occurred. For example, regulatory T cells (T_{reg}) and the cytokine IL-10 help to suppress T cell activation and proliferation [27,28].

Aside from cytokine and growth factor signals, nutrients and hormone signaling also influence T cell metabolism [11,26,29]. These signals affect regulatory pathways controlled by enzymes such as the mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) to influence cell growth/proliferation or homeostasis [30,31]. In the absence of these extrinsic signals, T cells lose their ability to maintain homeostasis and eventually succumb to apoptosis through Bcl-2 mediated cell death [10,31].

Obesity impairs the immune response to infection

As of 2014, for the first time in recorded history, obese adults outnumber underweight adults worldwide, with the global prevalence of obesity equaling 10.8% in men and 14.9% in women [1]. In the United States, obese adults comprise 36.5% of the population [32]. Obesity has been linked to increased incidences of infectious diseases, including periodontal infections, influenza, bacterial pneumonia, nosocomial and surgical site infections, among others [2,16]. Furthermore, obesity has been shown to impair immunological responses in both adults and children [33-35].

Following the first influenza pandemic of the 21st century, obesity was identified as an independent risk factor for increased morbidity and mortality from pandemic H1N1 infection [2]. Our laboratory studied the response to influenza vaccination in obese adults and found that increasing BMI was associated with greater declines in influenza-specific antibody titers one-year post vaccination [34]. In addition, influenza-stimulated T cells from obese adults were less functional and less activated compared with T cells from vaccinated healthy weight adults [36].

Obesity reduces circulating levels of γδ T cells, and impairs their function through reduced IL-2 receptor expression and IFN-γ production [37]. Moreover, obesity has been

implicated in reduced function of T_{reg} cells in obese patients with asthma [38]. Finally, growing evidence suggests that obesity impairs immune responses to vaccines such as hepatitis B (HB), hepatitis A (HA), rabies and tetanus, and that it increases risk of several bacterial infections [39].

Despite growing evidence supporting the notion that obesity impairs immunological responses to infection, it is worth noting that some studies have found a protective effect of obesity against infection. Roth *et* al., in their review of over 20 epidemiological studies, found that obesity was associated with better outcomes from infections such as tuberculosis, community-acquired pneumonia and sepsis [40]. They argue that aspects of the metabolic syndrome often found in obesity provide an advantageous niche for immune cells to fight off infection. While there is controversy between findings on obesity's impact on the immune response to infection, it is important to note that obesity is a multifactorial condition that impacts a variety of tissue and organ systems.

Obesity alters the metabolome during infection

Studies using murine models of obesity have shown great similarity to obesity in humans, providing a translational model to study this complex condition [41]. Recent studies have shown altered metabolic profiles in diet-induced and genetically obese mice following influenza virus challenge *in vivo*. Using ^{1}H NMR and global liquid chromatography-mass spectrometry, Milner *et* al. reported specific alterations in the metabolic profiles of influenza-infected diet-induced obese mice when compared with infected lean mice in serum, liver, lung, mesenteric white adipose tissue, urine, feces and bronchoalveolar lavage fluid [42,43]. Changes in metabolites included increased levels of fatty acid, cholesterol and phospholipids in lung tissues isolated

from obese mice compared to lean mice, correlating with increased lung damage and mortality observed in the infected obese mice [43].

Notably, obese mice showed significant fold increases in glutamyl-proline, tetrahydrocortisol, 3-hydroxybutyric acid and numerous acyl-carnitine metabolites in lung tissues [43], suggesting that differential metabolism in these tissues may be driving immune dysregulation in the influenza-infected lung. Additionally, diet-induced obese mice had a 55-fold increase in p-cresol sulfate concentrations in lungs at 4 days post infection compared to lean mice [43]. P-cresol sulfate, a metabolite produced by gut microbiota during secondary metabolism of p-cresol, accumulates during kidney failure and can impact cell function [43]. This increase in secondary metabolites in obese mice suggests that the obese microbiome may differ from lean, and thus contribute to differences in immune function.

In order to differentiate between the effects of diet vs. obesity, mice lacking hypothalmic leptin receptors ($LepR^{H-/-}$) were utilized in the influenza infection model. $LepR^{H-/-}$ knockout mice were established by crossing fully floxed leptin receptor mice with Cre transgene expressing C57BL/6J-Tg(Nkx2-1-cre)2S mice driven by the Nkx2.1 promoter. These transgenic mice lacked leptin receptor signaling in hypothalamic neurons, resulting in obesity from excess consumption of a low fat chow diet thereby removing the influence of high fat diet on immune function. Compared to lean controls ($LepR^{HFlox/Flox}$ consuming an identical diet), obese mice that gained weight on the chow diet exhibited altered metabolic profiles. Similar to high fat fed diet-induced obese mice, the $LepR^{H-/-}$ obese mice had higher fatty acid, cholesterol and nucleic acid metabolites in urine and lung tissues following influenza virus infection [43]. Again, these variations in metabolites correlated with greater lung pathology and inflammation, as well as reduced levels of quiescent and activated $CD4^+$ and T_{reg} cells in the lung and bronchoalveolar

lavage fluid during the immune response to influenza virus infection [43]. These studies suggest that obesity itself, not the diet, alters metabolites both in circulation and in tissue specific regions impacted by infection. This work supports the notion that obesity alters the metabolic landscape of the host, thus impairing T cell function, leading to increased susceptibility to infectious disease.

Adipocytes in the obese state promote inflammatory T cell activation through altered metabolism

Another theory as to how obesity impairs the T cell response to infection involves the distorted cytokine and adipokine milieu brought about by excess adiposity. Leptin, a hormone involved in energy homeostasis, has been shown to be essential for glucose uptake in effector T cells [29]. Upregulation of Glut1 transporters by leptin signaling under normal conditions represents a bioenergetic advantage for T cells to prevent suppressed proliferative and functional responses when glucose concentrations become limited [10,26,29]. However, in obesity, when leptin secretion becomes systemic and chronic, leptin signaling may lead to altered $CD4^+$ T cell differentiation. Leptin signaling has been shown to promote pro-inflammatory T cell subtypes, Th_1 and Th_{17} [29,44,45], suggesting leptin plays a critical role in the development of an inflammatory adipose tissue microenvironment. Leptin's role in modulating T cell repertoires, leading to inflammatory adipose tissue microenvironments is further supported by the discovery of pro-inflammatory $CD8^+$ T cells precede adipose tissue macrophage (ATM) infiltration in visceral adipose tissue, with marked declines in $CD4^+$ helper T cell and T_{reg} cell populations in obese mice compared to lean [46].

Infiltration of T cells in adipose tissue has received attention for its suggested role in the development of insulin resistance [47]. Recent findings by Morris et al. identified a mechanism by which ATMs function as antigen presenting cells (APCs) to regulate the activation of $CD4^+$ T cells in mice [48]. This finding, reproduced by Cho *et* al., identified a novel MHCII-dependent activation loop between $CD4^+$ T cells and ATMs which supports T cell driven metainflammation in adipose tissue in mice [49].

However, large human adipocytes also activate $CD4^+$ T cells through MHCII-upregulation, thereby acting as APCs to stimulate T cell inflammatory effector functions [50]. This activation of adipose tissue associated T cells by MHCII-mediated APCs and leptin costimulation supports the notion of an inflammatory microenvironment in obese adipose tissue. These findings suggest the possibility that obesity leads to suppressed T cell response to infection through altered T cell populations caused by premature activation to pro-inflammatory T cell subtypes.

Adipocytes in the obese state promote T cell senescence

Senescence, typically associated with aging, is described as a fate in which cellular proliferation becomes halted but metabolic activity and function remain [51]. This cell phenotype represents a state of cellular exhaustion, with chronic activation of Akt and mTOR, thereby supporting cytokine production but limiting proliferation [51,52]. Recently, obesity was proposed to increase T cell senescence in visceral adipose tissue (VAT) of high fat fed obese mice [53]. These mice displayed increased accumulation of $CD153^+PD\text{-}1^+CD44^+CD4^+$ T cells in VAT, resembling the activity of senescent associated T cells, with increased osteopontin

secretion and VAT inflammation [53]. Previously, osteopontin has been shown to be a Th1 promoting cytokine that supports pro-inflammatory function [54].

This increased production of PD-1 (programmed cell death-1), an exhaustion/tolerance marker, [55], signals an exhausted state of visceral adipose T cells. This role of PD-1 as an immune regulator of TCR activation in effector T cells is evidenced by the establishment of PD-1 expressing $CD8^+$ effector memory T cells, and not central memory T cells [56]. Furthermore, strength of PD-1 expression is tied to signaling activation and effector function in T cells, such that high expression of PD-1 is needed for cell exhaustion but only low levels are required to disrupt some functions such as IL-2 or TNF-α secretion [57].

Finally, $CD4^+$ T cell metabolism alters PD-1 expression in mice cultured with galactose versus glucose [58]. This forced alteration in T cell metabolism from aerobic glycolysis to oxidative phosphorylation (OXPHOS) resulted in differences in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PD-1 expression, correlating with impairments in IFN-γ production [58]. Given aerobic glycolysis' critical requirement in optimal IFN- γ production, this connection between aerobic glycolysis and PD-1 expression demonstrates a novel mechanism through which T cell function is influenced by nutrition. How long-term exposure to obesogenic conditions impacts gene expression remains unresolved. However, some recent studies suggest that obesity can lead to the hypermethylation of lymphocyte DNA in humans and animals [59,60], thereby implicating an epigenetic component that supports the conditions of obesity associated T cell senescence.

Hypotheses

Despite the lack of direct evidence linking obesity and altered T cell metabolism with impaired immune response to infection, foundations from several studies over recent years support the hypothesis that obesity disrupts T cell metabolism, resulting in impaired function. Altered metabolism of the obese host has been shown both in the steady state and during infection. Hormone resistance, inflammation and alterations in nutrient levels all influence T cell activation, function and survival. A surfeit of nutrients such as glucose and fatty acids, along with excess leptin production in the obese state, may elicit the activation of T cells in the absence of specific pathogens, thus, skewing naïve and memory cells towards inflammatory Th1 & Th17 subtypes, while reducing anti-inflammatory T_{reg} repertoires (Figure 2). The response to infection in an obese environment most likely would result in disrupted T cell metabolism through increased glycolytic and oxidative flux and cause impaired T cell response by promoting inflammation and reducing anti-inflammatory immune surveillance.

Here, we examined the immunometabolic profile of isolated peripheral blood mononuclear cells (PBMCs) and T lymphocytes from adult white women vaccinated against influenza virus. Using high-throughput extracellular flux analysis, we show that obesity induced higher rates of aerobic glycolysis and mitochondrial respiration in PBMCs in both quiescent and activated states. Furthermore, obesity alters basal respiration and basal glycolysis of quiescent CD4+ and CD8+ T cells, with notably higher rates of OXPHOS compared to healthy weight controls. Given the reliance of effector T cells on glucose metabolism to facilitate effector functions, we hypothesized that obese non-diabetic subjects would differ from obese metformin-treated diabetic subjects with insulin resistance and resultant glucose dysregulation. Interestingly, obese metformin-treated diabetics had fasting glucose levels greater than 125mg/dl, indicating

that metformin treatment had not restored homeostatic glucose levels. However, CD4+ and CD8+ T cells from obese metformin-treated subjects exhibited levels of basal respiration and glycolysis similar to cells from healthy weight controls. This finding suggests metformin may work to restore homeostatic metabolism of quiescent CD4+ and CD8+ T cells. Finally, activated CD4+ and CD8+ T cells exhibited no differences in aerobic glycolysis or OXPHOS, but did show differences in spare respiratory capacity, suggesting that elevated glucose levels do not drive impairments in effector T cell function in the obese state.

Taken together, this investigation provides the first demonstration of obesity-driven metabolic alterations of T cells. These observed alterations in quiescent and activated T cell metabolism might account for impairments in T cell function. Furthermore, elevated glucose levels did not account for differences in obese subjects; suggesting that other factors associated with obesity, and not excess glucose, result in altered T cell metabolism. How chronic exposure to excess nutrients like lipids, hormones and/or inflammation influences gene regulation and immune function in lymphocytes remains unknown. However, trends from this work support the notion that obesity is a complex and multifactorial condition, most likely influencing immunity through a number of mechanisms. With the recent explosion in cases of obesity in the United States and around the globe, understanding how this diverse metabolic state influences immune responses to infection warrants further investigation.

The results of this investigation are expressed in Chapter 2 as a journal manuscript, with the intention of publication upon confirming results with future experiments.

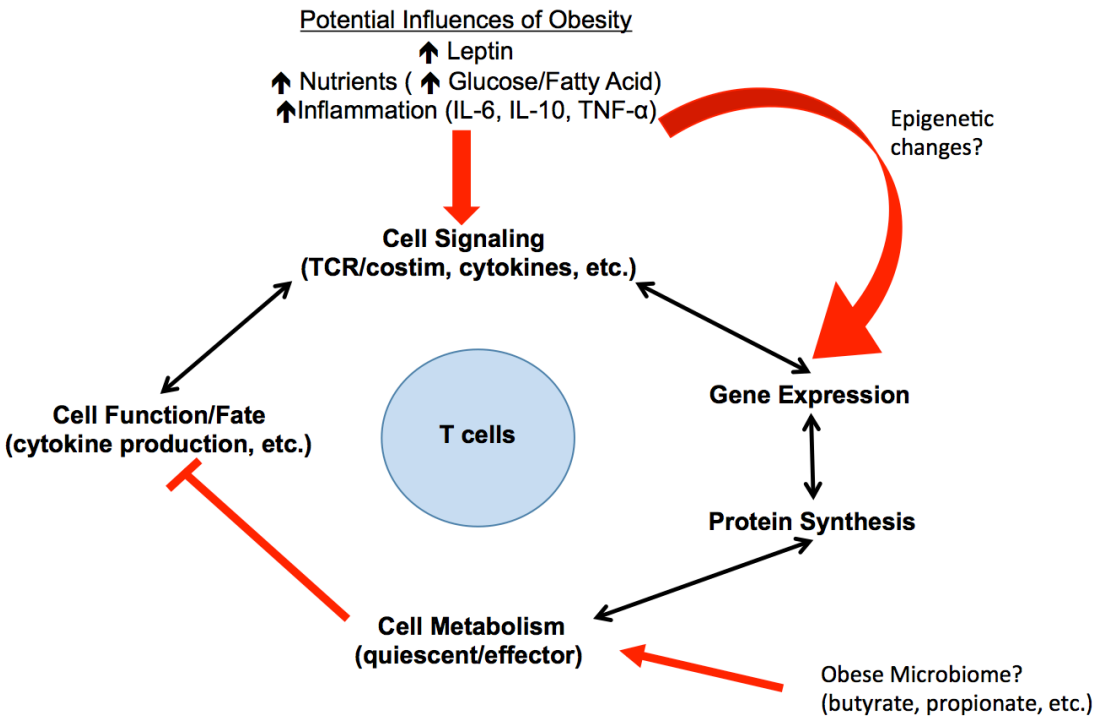

Figure 2: Potential mechanisms of impairment of normal T cell signaling and response to infection in obesity. Nutrients, leptin and inflammation signals cause a disruption of normal T cell metabolism, resulting in impaired cellular functions. Long-term exposure to these extrinsic signals alters the metabolic profile of T cells, impacting their feedback of cytokine secretion and functional response to infection. Whether or not long-term exposure results in epigenetic changes, or metabolite influence from obese microbiota has yet to be determined. TCR, T cell receptor. Costim, costimulatory receptor.

CHAPTER II – JOURNAL MANUSCRIPT

[1]Obesity induces metabolic alterations of T lymphocytes in influenza-vaccinated adults

Summary

An epidemic of obesity in both adults and children over the past three decades increases the risk of chronic and infectious diseases. Within the past few years, obesity has been shown to impair the adaptive immune response to infection through alterations in T cell functioning. Importantly, obesity has been shown to increase morbidity and mortality from influenza infection. Animal and human studies show that obesity impairs the activation and function of influenza-specific T cell response. This diverse cell population relies heavily on glucose and glutamine metabolism to support critical effector functions when pathogenically challenged. Recent findings in the fields of obesity and T cell mediated immunity demonstrate a unique relationship between altered mechanisms of T cell metabolic homeostasis and plasticity of adaptive immune responses in the obese setting. Here, we show that obesity alters the quiescent and activated metabolic profile of peripheral blood mononuclear cells, and more critically, $CD4^+$ and $CD8^+$ T cell lymphocytes in white women vaccinated against influenza virus. Furthermore, T cells from obese non-diabetic subjects with no metabolic dysregulation of glucose exhibit higher rates of aerobic glycolysis and mitochondrial respiration compared to obese metformin-treated diabetic subjects. This restorative effect of metformin occurs in the absence of restored serum glucose, suggesting that its mechanism of action impacts T cells directly and may offer

[1] Manuscript to be submitted

therapeutic benefits beyond hepatic and peripheral glucose homeostasis. While results from this study require further investigation to confirm its findings with larger sample sizes and in populations other than white women, these results support growing evidence that perturbations in T cell metabolism drive the impaired immune response of T cell lymphocytes, stemming from chronic nutrient, hormone and adipokine dysregulation in the obese. Our findings may provide the mechanistic link for obesity-driven impaired immune response to influenza infection and vaccination.

Introduction

Currently, obesity ranks as one of the largest growing epidemics in the United States and worldwide [61]. In the US alone, obese adults comprise 36.5% of the population with 15-20% of children and youth classified as obese [32,61]. Obesity has been linked with higher incidences of infectious diseases, including periodontal infections, influenza, bacterial pneumonia, nosocomial and surgical site infections, among others [2,16]. Classically characterized as a state of excess adiposity, obesity has been associated with chronic inflammation and metabolic dysfunctions such as hyperglycemia, hyperleptinemia and hormone resistance [15]. These uncontrolled metabolic conditions can lead to the development of chronic diseases such as type II diabetes, kidney disease, cardiovascular disease and specific cancers [16]. However, recent data demonstrates a link between obesity and increased incidences of infectious diseases, most likely through impaired cellular immune responses [16,17].

Following the first pandemic influenza outbreak of the 21st century, obesity was identified as an independent risk factor for increased morbidity and mortality from pandemic H1N1 infection [2]. Previous studies in our lab have shown impaired immunological responses

in obese adults compared to healthy weight adults. Using influenza vaccination as a model to study the immune response in obesity, our lab has demonstrated that increasing BMI was associated with greater declines in influenza-specific antibody titers one-year post vaccination [34]. Furthermore, when isolated peripheral blood mononuclear cells (PMBCs) from influenza vaccinated healthy weight, overweight and obese adults were stimulated with H1N1 influenza virus in vitro, CD4+ and CD8+ T cells from obese and overweight adults expressed fewer activation and functional markers compared to healthy weight adults [36]. These impairments included less expression of activation markers CD28, CD40 ligand, CD69 and IL-12R as well as reduced expression of IFN-γ and granzyme B, critical cytokines produced by effector T cells involved in influenza clearance [36]. Additionally, no differences were seen in dendritic cell activation or function, suggesting obesity impacts T cell activation and function independent of antigen presentation. Given obesity's numerous metabolic and micro-environmental effects through long-term net positive caloric intake and resultant hormone/cytokine dysregulation, understanding how these factors influence T cell function remains unresolved and critically important.

For many years, T cell activation and function was believed to occur from a combination of antigen recognition, subsequent signaling cascades and micro-environmental cues [4,5]. Within the past decade, cellular metabolism has been shown to critically influence T cell differentiation, proliferation, function and ultimately their cellular fate [6-11]. Reports of how metabolic fuels such as glucose, amino acids and fatty acids elicit distinct metabolic profiles depending on cell state (naïve, effector or memory) and subtype (Th1, Th2, Th17, Treg, etc.) [7,12-14], has led to a revolution in the understanding of T cell driven immunity. Furthermore, this newfound understanding highlights the metabolic plasticity of T cells to respond to the

energetic and biosynthetic demands required to successfully fight infection [8]. Although it is known how T cells respond to antigenic challenge by drastically altering their metabolic state, what is not as well understood is how altered metabolic conditions such as obesity may influence T cell metabolic programming, thus impairing T cell function and response to infection.

In this investigation, a subgroup of white women age 35 to 65 were recruited from the previously established prospective observational cohort study on influenza vaccination at the University of North Carolina at Chapel Hill's Family Medicine Center, Chapel Hill, NC. Subjects were recruited following enrollment from the traditional influenza study cohort for a one-time blood draw in March 2016 and February/March 2017. Subjects included three demographic classes: healthy weight non-diabetics (BMI 18.5 to 24.9; fasting glucose < 100mg/dL), obese non-diabetic (BMI ≥ 30.0; fasting glucose < 100mg/dL) and obese metformin treated diabetics (BMI > 30.0; fasting glucose > 125mg/dL). PBMCs, CD4+ and CD8+ T cells were isolated from each subject and metabolically profiled using high-throughput extracellular flux analyzers (XF24 and XFe96). This technique provides a measure of aerobic glycolysis and mitochondrial oxidative phosphorylation for cells types of interest, to allow for the rapid metabolic characterization of cells.

Given that T cells predominately rely on glucose and glutamine metabolism for homeostasis and effector functions, we hypothesized that obesity would increase the metabolic profile of freshly isolated quiescent CD4+ and CD8+ T cells, thereby giving a snapshot of the metabolic programming of these cell types within the host. Furthermore, upon stimulation in culture, when these cells become highly glycolytic and require glucose and glutamine to support proliferation, cell survival and effector function, we hypothesized that obesity would increase the rates of glycolytic and respiratory flux of glucose, pyruvate and glutamine fuels compared to

healthy weight subjects, suggesting that previously observed impairments in effector functions may be due to alterations in metabolic programming of isolated T cells, stemming from the obese environment. Finally, obese metformin-treated diabetics were included as a separate group to determine the effect of a metabolic drug on T cell metabolism in an uncontrolled glucose environment. Previous research has shown that metformin treatment facilitates memory T cell formation and function, possibly through restored T cell metabolic activity [62,63]. Recent data also suggests that metformin may be protective against cancer formation and beneficial also as a cancer therapeutic [64].

Here we show altered metabolic profiles of CD4+ and CD8+ T cells isolated from healthy weight, obese non-diabetic and obese diabetic subjects vaccinated against influenza virus. These differences in metabolism present a novel mechanism through which T cell function may be compromised due to non-optimal metabolic flux, supporting the notion of obesity inducing pro-inflammatory T cell subtype differentiation and impairment of resting or quiescent T cell populations. Furthermore, we demonstrate that influenza specific HAI antibody titers from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults respond to vaccine at 30 days, but fall at six months post vaccination, with no differences between groups. This data suggests that T cell mediated responses, and not antibody seroconversion or seroprotection, should be considered when assessing for correlates of protection from influenza infection.

Methods & Materials

Subjects. Participants were recruited from the prospective observational cohort study conducted by the Beck Lab on the investigation of BMI on influenza vaccination response at the University

of North Carolina at Chapel Hill Family Medicine Center, Chapel Hill, NC [36]. Adult white females age 35 to 65 vaccinated against influenza virus were recruited as a study cohort subgroup following written informed consent under approval of the University of North Carolina at Chapel Hill's Biomedical Institutional Review Board. Venous antecubitual serum and plasma was obtained using aseptic phlebotomy in overnight fasting subjects. Participants had previously received either the 2015-2016 or 2016-2017 quadrivalent influenza vaccination (FLUARIX® 481035) from UNC-Family Medicine as part of their normal preventative care prior to enrolling in the study subgroup.

The strains in the 2015-2016 influenza vaccine included: A/California/07/2009(pH1N1), A/Switzerland/9715293/2013(H3N2), B/Phuket/3073/2013, and B/Brisbane/60/2008. The strains in the 2016-2017 influenza vaccine included: A/Christchurch/16/2010 (H1N1) NIB-74XP (an A/California/7/2009 (H1N1) pdm09-like virus), A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, B/Phuket/3073/2013, and B/Brisbane/60/2008. Exclusion criteria included: immunosuppressive drugs or diseases like HIV, acute febrile illness, autoimmune disease, medical history of immunotherapy for cancer, history of Guillain-Barre syndrome, and any hypersensitivity to influenza vaccine components.

Subjects were recruited from March 2016 to March 2017, with blood draws no closer than two months apart to ensure compliance with IRB approved procedures. Blood draws occurred in two phases; the first in March 2016 for initial PBMC characterization (n=7), followed by a second wave of blood draws in February/March 2017 (n=9). Height and weight measurements were taken to calculate body mass index (BMI = (kg)/height$(m)^2$), as well as medical history to determine diabetes status and drug medications. Fasting glucose was measured using a glucometer immediately after whole blood collection (FreeStyle Precision

Neo). Three groups of participants were recruited: healthy weight with no history of diabetes (BMI 18.5 – 24.9; fasting glucose < 100 mg/dl), obese non-diabetic (BMI ≥ 30.0; fasting glucose < 100), and obese diabetic patients actively taking metformin (BMI ≥ 30.0; fasting glucose > 125). As part of the traditional influenza vaccine study, participants were monitored during flu season for cases of medically confirmed influenza and influenza-like illness (ILI), defined as a fever over 100^{o}F and a cough in the absence of any other medical diagnosis [65]. Demographics of subjects are displayed in Tables 1 & 2.

Cell Isolations & Stimulation. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh plasma using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient separation as previously described [36]. PBMCs were washed twice with Ca^{2+} and Mg^{2+} free HBSS before being re-suspended in complete RPMI-1640 containing 10mM glucose, 2mM glutamine, 10% FBS and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) and counted using a hemocytometer. PBMCs were cultured in complete RPMI-1640 for 72hrs at 37^{o}C and 5% CO_2 with or without treatment with a PBS control or 0.5μg/μL lectin mitogen, concanavalin A (Sigma-Aldrich), per 1x10^{6} PBMCs. Isolated CD4+ and CD8+ T cells were obtained using negative selection exclusion beads (StemCell Technologies, Vancouver, Canada) following PBMC isolation. Isolated T cells were stimulated using anti-CD3/anti-CD28 magnetic beads (1:1 cell to bead ratio) and 30IU IL-2 (ThermoFisher Scientific, Waltham, MA) in complete RPMI-1640 containing 10mM glucose, 2mM glutamine, 5% autologous plasma and penicillin/streptomycin for 72hrs at 37^{o}C and 5% CO_2. Isolated T cells were cultured using autologous plasma instead of FBS in order to more accurately recapitulate physiological culture

conditions of the host for each subject, thereby activating cells in their host milieu of fatty acid, hormones, growth factors, etc.

Metabolic Assays. Mitochondrial stress tests were performed on cells using Agilent's XF24 and XFe96 flux analyzers. Unstimulated (control) and concanavalin A stimulated PBMCs were assayed using the XF24 extracellular flux analyzer as previously described [6]. Cells were counted, attached to culture plates via CellTak adhesion at $1.2x10^6$ cells per well in non-buffered DMEM with 25mM glucose and 1mM glutamine (Sigma-Aldrich, St. Louis, MO). Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured at 37^{o}C under basal conditions and in response to 1μM oligomycin (an ATPase inhibitor), 0.5μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) (a mitochondrial uncoupling agent) and 0.75μM rotenone (complex I inhibitor) and1.5μM antimycinA (complex III inhibitor) (Sigma-Aldrich, St. Louis, MO).

Freshly isolated CD4+ and CD8+ T cells and 72hr CD3/CD28 + IL-2 stimulated CD4+ and CD8+ T cells were assayed using the XFe96 extracellular flux analyzer. Cells were plated as previously described at $2x10^5$ cells per well in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1mM pyruvate. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured at 37^{o}C under basal conditions and in response to 1μM oligomycin (an ATPase inhibitor), 1.5μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) (a mitochondrial uncoupling agent), 0.1μM rotenone (complex I inhibitor) and1.0μM antimycinA (complex III inhibitor) (Sigma-Aldrich, St. Louis, MO).

Metabolic parameters were calculated as follows. Basal respiration (OCR (pmoles/min) and basal glycolytic rate (ECAR (mpH/min)) were determined as the last time point

measurement before injection of oligomycin. Maximal respiration was determined as the peak OCR (pmoles/min) following injection of FCCP, while spare respiratory capacity was calculated as the Max OCR – Basal OCR. Finally, OCR:ECAR ratio was determined by dividing the basal respiration by basal glycolysis. Non-mitochondrial respiration (OCR following rotenone+antimycin A injection) was not subtracted from these observed readings, as commonly reported in previous studies, in order to reduce variance from drug injection effects and reduce differences in plate measurement between metabolic platforms, thus allowing for total representation of all O_2 consumption and H^+ production by cells in these metabolic parameters.

Influenza Specific Antibody Determination. Serum was obtained for each subject by allowing whole blood to clot at room temperature for 30mins prior to refrigeration at 4^oC over night and centrifugation at 800xg at 4^0C for 10mins. Serum antibody titers for A/California/7/2009(H1N1) were determined via hemagluttination inhibition assay as previously described [66]. Samples were assessed in duplicate and represent mean for each individual subject at day 0 (pre-vaccination), 30 days post vaccination, six months post vaccination and one year post vaccination.

Statistical Analysis. Values for oxygen consumption (OCR) and extracellular acidification (ECAR) from technical replicates were averaged for each well. Physiologically impossible values for OCR or ECAR (values ≤ 0) were excluded. Biological medians ± standard error was reported as shown to reduce effect of skew from outliers. Data was assessed for significance by one-way or two-way ANOVA followed by Tukey's multiple comparisons where denoted. Student's t test or Mann-Whitney sum rank test were used to determine significance between two

groups. Correlation coefficients were determined by Spearman's rank correlation. Data was determined to be statistically significant when $p < 0.05$. All analyses were conducted in Graphpad Prism 7.0 for Mac OS X.

Results

PBMCs from obese adults display altered metabolism compared to healthy weight adults. In order to assess if obesity alters the global metabolism of circulating immune cells, peripheral blood mononuclear cells (PBMCs) were isolated from healthy weight, obese non-diabetic and obese metformin-treated diabetic subjects and assayed using the mitochondrial stress test with an XF24 extracellular flux analyzer (Fig. 3). Quiescent (resting) PBMCs from both obese non-diabetic and obese metformin-treated diabetic subjects had a ~1.5x higher basal respiratory rates compared to healthy weight adults (Fig. 3C), with no differences in basal glycolysis (Fig. 3D). No significant differences were observed in the cells spare respiratory capacity (SRC) between groups, a measure of cellular ability to metabolically respond to energetic demands (Fig. 3E). Additionally, no differences were seen in the ratio of basal oxygen consumption to basal glycolysis (OCR:ECAR) (Fig. 3F). However, for both SRC and OCR:ECAR ratio, an upward trend was seen in both obese groups compared to healthy weight.

PBMCs include a diverse set of leukocytes including lymphocytes (T cells, B cells, NK cells) as well as monocytes and dendritic cells [67]. Upon treatment with concanavalin A (Con A), a T cell specific lectin mitogen, antigen presenting cells (APCs) display Con A peptides to CD4+ and CD8+ T cells causing global activation of PBMCs through a T cell mediated mechanism [68]. When cells were treated with Con A (activated) as opposed to a PBS control (quiescent), cells from all groups showed an increase in OCR and ECAR, indicating treatment

with Con A resulted in activation of these cells in culture, thus causing dramatic shifts in their metabolic programming (Fig. 3A-B). Interestingly, obese non-diabetic and obese metformin-treated diabetic subjects showed greater induction in basal OCR, with only obese non-diabetics having higher basal ECAR, compared to healthy weight adults (Fig. 3C-D). Again no significant differences between groups were observed in SRC or OCR:ECAR (Fig. 3E-F).

Quiescent CD4+ T cells display trends in elevated respiration in obese non-diabetic subjects, with obese metformin-treated diabetics more like healthy weight. Freshly isolated CD4+ T cells were subjected to a mitochondrial stress test using the XFe96 flux analyzer, measuring OCR and ECAR in response to olgiomycin, FCCP, and antimycin A + rotenone (Fig. 4A-B). Similar trends were seen in basal OCR, basal ECAR and SRC, with obese non-diabetic CD4+ T cells exhibiting higher rates compared to healthy weight and obese metformin-treated diabetic CD4+ T cells (Fig. 4C-E). No differences in trends were seen in OCR:ECAR (Fig. 4F). Despite a lack of significant differences between these measures, likely due to a low sample size, these trends suggest that freshly isolated quiescent CD4+ T cells from obese non-diabetic adults have a higher metabolic profile of aerobic glycolysis and mitochondrial respiration than healthy weight or obese metformin-treated diabetic CD4+ T cells.

Activated obese non-diabetic CD4+ T cells display trends in elevated basal respiration and SRC than healthy weight CD4+ T cells. Freshly isolated CD4+ T cells were activated using anti-CD3 and anti-CD28 antibody coated beads for 72hrs in the presence of IL-2. Compared to quiescent T cells (Fig 4.), activation of CD4+ T cells resulted in greatly increased rates of mitochondrial respiration and aerobic glycolysis (Fig. 5A-D). No differences were seen in

stimulated CD4+ T cells' glycolytic rates between groups, when these cells should be predominately glycolytic. However, trends suggest that activated obese non-diabetic CD4+ T cells have slightly higher rates of mitochondrial respiration compared to healthy weight and obese metformin-treated diabetics. Furthermore, obese non-diabetic CD4+ T cells had elevated levels of SRC, with obese diabetics falling slightly below but not quite returning to healthy weight levels (Fig. 5E). No differences were seen in OCR:ECAR in stimulated CD4+ T cells (Fig. 5F).

Quiescent CD8+ T cells display trends suggesting alterations in basal respiration, basal glycolysis and spare respiratory capacity between healthy weight, obese non-diabetic and obese metformin-treated diabetics. Quiescent CD8+ T cells display low levels of basal respiration and glycolysis following isolation, typical of resting CD8+ T cells (Fig. 6A-B). Interestingly, CD8+ T cells from both obese non-diabetic subjects and obese metformin-treated diabetics had higher trends in basal respiration (Fig. 6C). A negative trend in basal ECAR was observed with obese non-diabetics and obese metformin-treated diabetics having lower rates of basal glycolysis compared to healthy weight (Fig. 6D). Additionally, quiescent obese metformin-treated diabetic CD8+ T cells had lower trends for SRC (Fig. 6E) and higher OCR:ECAR ratio (Fig. 6F), signifying that obese diabetic CD8+ T cells were more respiratory in nature yet did not achieve the same maximal respiration after treatment with FCCP.

Activated obese metformin-treated diabetic CD8+ T cells displayed higher trends for aerobic glycolysis and suppressed SRC compared to healthy weight. As previously seen with activated CD4+ T cells (Fig. 5), activation of CD8+ T cells cause dramatic induction of OCR (Fig. 7A)

and ECAR (Fig. 7B) in all three demographic groups. However, obese metformin-treated diabetic CD8+ T cells had ~1.5x higher basal ECAR than healthy weight (Fig 7D), with only ~1.2x higher basal OCR induction than healthy weight (Fig. 7C). Trends in obese non-diabetic OCR (Fig. 7C) and ECAR (Fig. 7D) fell in between healthy weight and obese metformin-treated diabetics. Additionally, obese metformin-treated diabetics had a ~3 fold lower rate for SRC. This followed trends of quiescent obese metformin-treated diabetic CD8+ T cells, as they did not reach similar levels of maximal respiration signifying activated obese metformin-treated diabetic CD8+ T cells had suppressed metabolic potential for energetic demand following administration of FCCP (Fig. 7E). Finally, no differences were observed in the ratio of basal OCR to ECAR between groups (Fig 7F).

No significant differences between basal respiration and glycolysis for quiescent CD4+ T cells in euglycemic versus diabetic glucose. Despite taking the metabolic drug metformin to restore serum glucose levels, obese diabetic subjects still had elevated fasting glucose levels compared to healthy weight and obese non-diabetic subjects (Table 2). To determine if high glucose levels directly account for altered T cell metabolism, quiescent CD4+ T cells were assessed in euglycemic (5mM or 90mg/dl) or diabetic level glucose media (10mM or 180mg/dl). No significant differences were seen in basal OCR (Fig. 8A) or basal ECAR (Fig. 8B) for healthy weight CD4+ T cells cultured in either 5mM or 10mM glucose media. Healthy weight CD4+ T cells did exhibit higher maximal respiration and thus higher SRC in 10mM glucose, most likely due to increase nutrient availability (data not shown).

A/California/7/2009 antibody HAI titers increase following vaccination and decline at 6 months post vaccination. Influenza specific HAI antibody titers increased following vaccination (Fig. 9A-B), regardless of weight or diabetic status for both 2015-2016 and 2016-2017 study years. No differences were seen in seroconversion rates between demographic groups, defined as a four-fold or greater increase in antibody titer following vaccination, confirming previously reported seroconversion rates for influenza vaccination in healthy weight, obese and obese diabetic subjects [66]. However, while not significant due to the small sample size, obese non-diabetic subjects in for the 2015-2016 study year had a mean fold seroconversion rate of 4.25 compared to 7.5 fold increase for healthy weight subjects (Fig. 9A). Interestingly, influenza specific HAI antibody titers declined at six months post-vaccination to similar levels as observed at one year post-vaccination (Fig 9A). Additionally, there were no differences in influenza virus specific levels of seroprotection between groups, deemed as having an antibody titer of 40 or greater (Fig. 9A-B).

Age significantly correlates with quiescent CD8+ T cell basal ECAR, despite no significant differences in age for subject group. Using our demographic and metabolic data, we found that only age significantly correlated with quiescent CD8+ T cell basal glycolysis. However, there were no significant differences in age among subjects between the three demographic groups. Several measures approached significance, most notably quiescent CD8+ T cell basal glycolysis and incidence of influenza or ILI ($p = 0.0556$) (Table 3). Other notably correlations included incidence of Flu or ILI with activated CD4+ T cell ECAR ($p = 0.111$). Again, these correlations are influenced by the small sample size used in the study.

Discussion

Previous studies in our lab demonstrated that compared to influenza vaccinated healthy weight adults, T cells from obese adults vaccinated against influenza virus were impaired. Specifically, CD4+ and CD8+ T cells from obese adults, when exposed to pH1N1 *in vitro*, expressed less activation and functional markers. These impairments were shown to be independent of antigen presentation, suggesting alterations in T cells from obese adults may be due to their distorted metabolic environment [36]. Additionally, our lab has shown that, compared to healthy weight adults, obese adults have greater declines in antibody titers at one-year post vaccination [34]. Finally, we have shown that obese adults have twice the risk of influenza or influenza like illness despite equivalent levels of seroconversion and seroprotection from influenza vaccination (Neidich et al. 2017, under review). This recent finding challenges the current standard for correlates of protection, suggesting that a T cell mediated response and not antibody threshold is required to achieve vaccine protection in obese adults. Despite this newfound understanding, the cause of T cell impairment in obesity remains elusive.

Recent attention identifying the importance of cellular metabolism for lymphocyte function highlights a novel mechanism through which obesity may influence immunity. Technological advancements in the assessment of cellular metabolism using high-throughput, real-time measures allows for the diverse characterization of a wide array of cellular phenotypes and pathologies. As our understanding of how metabolism is altered in a number of pathological events such as infection or cancer, this rapid profiling of cellular metabolism offers tremendous benefits for drug screening, pathogenic phenotyping and more. In the case of obesity, this technological platform offers the opportunity to quickly and effectively screen for alterations in

aerobic glycolysis and mitochondrial respiration in live cells, thus generating insights that can be used to guide targeted assessments of metabolic and signaling pathways altered in obesity.

Here, compared to influenza vaccinated healthy weight adult white women, we report differences in metabolic profiles for obese non-diabetic and obese metformin-treated diabetic adults. Quiescent PBMCs from obese non-diabetic and obese metformin-treated diabetic subjects demonstrated higher basal respiratory rates, suggesting global metabolic programming is altered in obese adults compared to healthy weight adults. Additionally, upon stimulation with the T cell specific mitogen, Concanavalin A, PBMCs from obese non-diabetic and obese metformin-treated diabetic adults had higher respiratory rates but no differences in OCR:ECAR, indicating a greater induction in both aerobic glycolysis and mitochondrial respiration upon stimulation. This global dysregulation of metabolism in PBMCs from obese subjects towards a more glycolytic and respiratory environment supports the hypothesis of hyperactive immune cells contributing to obese-associated inflammatory environments. However, due to the diversity of cell types found in circulating PBMCS, whether or not this global alteration in metabolism was due to differences in T cells was unclear.

To better assess T cell specific metabolic differences between influenza vaccinated healthy weight, obese non-diabetic and obese metformin-treated diabetic adults, freshly isolated CD4+ and CD8+ T cells were further investigated. We found that compared to healthy weight, quiescent obese non-diabetic CD4+ and CD8+ T cells tended to have higher basal respiration. Additionally, CD4+ but not CD8+ T cells from obese non-diabetic subjects had higher trends in basal glycolysis. Quiescent T cells are primarily oxidative in nature, utilizing glutamine and fatty acid to drive catabolic metabolism in order to support homeostasis. Altered rates of OXPHOS and glycolysis in quiescent CD4+ and CD8+ T cells in obese non-diabetics may inhibit the

anabolic transition into effector T cells upon activation, thereby contributing to the impaired response of T cells from obese individuals to influenza virus challenge.

Interestingly, in several cases, T cells from obese metformin-treated diabetic subjects displayed metabolic profiles closer to T cells from healthy weight subjects than their obese non-diabetic counterparts. This lower metabolic profile occurred in spite of persistent elevated serum glucose in obese diabetic subjects. This restoration of T cell metabolism to "healthy" levels signifies metformin's direct action on T cell metabolism independent of its proposed mechanisms of action through glucose homeostasis. Recent publications suggest that metformin may work to restore T cells to a homeostatic quiescent state through blockage of mTOR cascade signaling, thereby suppressing pro-growth metabolic signaling [63]. Treatment of T cells with metformin promote the development of memory CD8+ T cells through induction of fatty acid oxidation and suppression of glycolytic pathways via AMPK [63]. However, other studies show metformin's inhibition of mTOR's pro-growth signaling and resultant metabolic switch from catabolic to anabolic metabolism in CD4+ T cells occurs through an AMPK-independent manner [62]. Regardless of direct or indirect action, our findings demonstrate trends suggesting a restorative effect of metformin treatment in obese diabetic CD4+ and CD8+ T cells to their homeostatic quiescent state.

Given the widespread use of metformin in type-2 diabetic patients, and its early evidence of potential therapeutic benefit in the prevention and treatment of cancer [64], understanding how metformin's impact on T cell metabolism, especially in immune-compromised populations such as the obese, remains a critical and unresolved question. Our data suggests that metformin treatment may offer therapeutic benefits for obese adults by suppressing hyperactive metabolic programming in quiescent and activated CD4+ and CD8+ T cells. Given previously seen

impairments in T cell function of influenza vaccinated obese adults, this finding has significant public health implications as obese adults are at heightened risk of morbidity and mortality from influenza infection. Furthermore, metabolic impairments in T cell function may exist in early-onset or childhood obesity when immune development and vaccination efforts are at a critical time period. Thus, therapeutic benefits of metformin treatment in obese children warrant further study for the possible restorative metabolic effects on T cells, thereby influencing metabolic impairment of T cell function for infections such as influenza.

While much of the data shown here does not meet significance due to the small sample size, the trends suggest critical differences in both mitochondrial respiration and aerobic glycolysis between our demographic groups. Using the largest variance observed in obese non-diabetics, as this demographic had the largest variation in metabolic parameters compared to healthy weight and obese metformin-treated diabetics, conservative power calculations would require a sample size of 15 per group to adequately assess significant differences between activated T cells, with quiescent T cells requiring a sample size of 9 per group. Even still, this report offers the first identification of altered metabolic programming in obese adults compared to healthy weight, suggesting obesity impacts T cell metabolism and function through altered metabolic flux. The metabolic difference between healthy weight and obese adults warrants further study as these highly metabolic cells play a critical role in supporting innate and adaptive immune protection and recovery from influenza virus infection.

Table 1: PMBC subject demographics

	Healthy Weight	Obese Non-Diabetic	Obese Metformin-treated Diabetic	Total
Participants	2	2	3	7
BMI	20.2 ± 0.14	36.3 ± 6.8	42.2 ± 8.1	
BMI Range	20.1 - 20.3	31.5 - 41.1	33 - 48.3	
Age	36.5 ± 0.8	55.5 ± 0.7	57.8 ± 7.1	
Age Range	36.0 - 37.0	55.0 - 56.0	50.0 - 64.0	
Race	Caucasian	Caucasian	Caucasian	
Sex	Female	Female	Female	
Diabetes	No	No	Yes	
Metformin	No	No	Yes	
Mean Fasting Glucose	69.0 ±4.2	91.5 ± 0.7	158.0 ± 34.0	
Fasting Glucose Range	66 - 72	91 - 92	124 - 192	
Menopausal (%)				
yes	0 (0.0%)	2 (100%)	3 (100%)	5 (71.4%)
no	2 (100%)	0 (0.0%)	0 (0.0%)	2 (28.6%)
Smoking History (%)				
yes	0 (0.0%)	0 (0%)	1 (33.33%)	1 (14.3%)
no	2 (100%)	2 (100%)	2 (66.67%)	6 (85.7%)
Confirmed Flu or ILI (%)				
yes	0 (0.0%)	0 (0.0%)	1 (33.33%)	1 (14.3%)
no	2 (100%)	2 (100%)	2 (66.67%)	6 (85.7%)

Table 2: CD4+ and CD8+ T cell subject demographics

	Healthy Weight	Obese Non-Diabetic	Obese Metformin-treated Diabetic	Total
Participants	3	3	3	9
BMI	22.7 ± 1.4	37.6 ± 3.8	37.2 ± 5.0	
BMI Range	20.4 - 24.11	31.3 - 41.0	30.2 - 42.7	
Age (years)	48 ± 7.4	50.3 ± 5.1	48.2 ± 7.8	
Age Range (years)	40.0 - 58.0	44.0 - 57.0	37.0 - 58.0	
Race	Caucasian	Caucasian	Caucasian	
Sex	Female	Female	Female	
Diabetes	No	No	Yes	
Metformin	No	No	Yes	
Mean Fasting Glucose (mg/dL)	82 ± 2.0	82.7 ± 7.0	146 ± 19.7	
Fasting Glucose Range (mg/dL)	80 - 84	76 - 90	125 - 164	
Menopausal (%)				
yes	1 (33.33%)	1 (33.33%)	1 (33.33%)	3 (33.33%)
no	2 (66.67%)	2 (66.67%)	2 (66.67%)	6 (66.67%)
Smoking History (%)				
yes	0 (0%)	0 (0%)	1 (33.33%)	1 (11.11%)
no	3 (100%)	3 (100%)	2 (66.67%)	8 (88.89%)
Confirmed Flu or ILI (%)				
yes	1 (33.33%)	0 (0%)	1 (33.33%)	2 (22.22%)
no	2 (66.67%)	3 (100%)	2 (66.67%)	7 (77.78%)

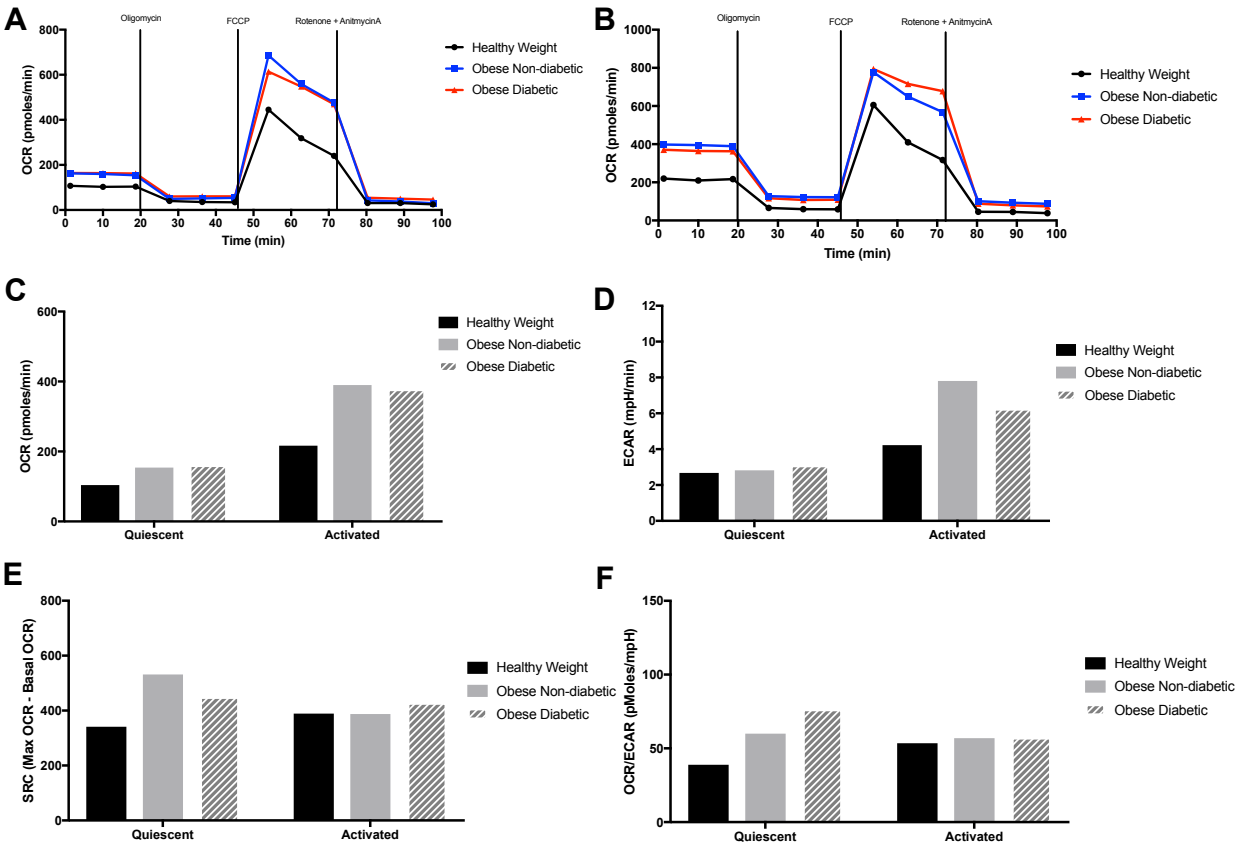

Figure 3. Quiescent and Concanavalin A activated PBMCs from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Quiescent (A) and ConA activated (B) PBMCs from healthy weight (n=2), obese non-diabetic (n=2) and obese metformin-treated diabetic (n=3) adults exhibit changes in basal OCR and ECAR in response to 1.0 µM olgiomycin, 0.5µM FCCP, 1.5µM antimycinA and 0.75µM rotenone (A-B). Quiescent PBMCs had higher basal respiration (C) in obese non-diabetic and obese metformin-treated diabetic cells compared to healthy weight. Activated PBMCs had higher rates of basal respiration and glycolysis for obese non-diabetics (C-D), but only higher rates for basal ECAR in activated obese non-diabetics (D). Obese non-diabetic had higher trend of SRC in quiescent PBMCs compared to healthy weight (ns) (E), and saw an increasing trend in OCR:ECAR from healthy weight to obese non-diabetic and obese metformin-treated diabetic PBMCs (F). Data are biological medians. Two-way ANOVA with Tukey's multiple comparisons.

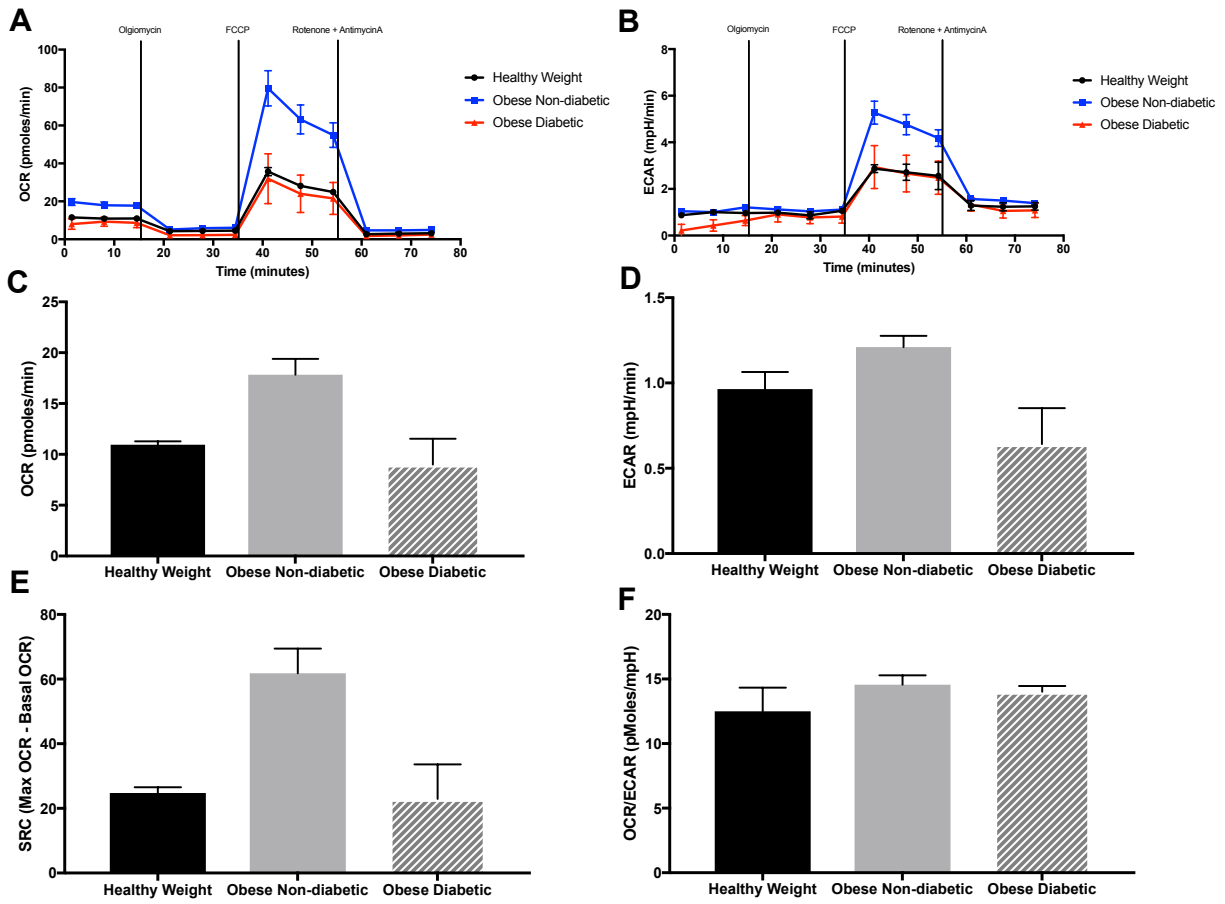

Figure 4. Metabolic profile of quiescent CD4+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of freshly isolated CD4+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects exhibits changes in OCR and ECAR in response to 1.0 μM olgiomycin, 1.5μM FCCP, 1.0μM antimycinA and 0.1μM rotenone (A-B) in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1mM pyruvate. Obese non-diabetics had higher trends in basal OCR (C), basal ECAR (D) and spare respiratory capacity (E). No differences in trends were seen for OCR:ECAR (F). Data are median ± SE. No significance, one-way ANOVA with Tukey's multiple comparisons. Individual comparisons were made using Mann-Whitney sum rank test, no significance found.

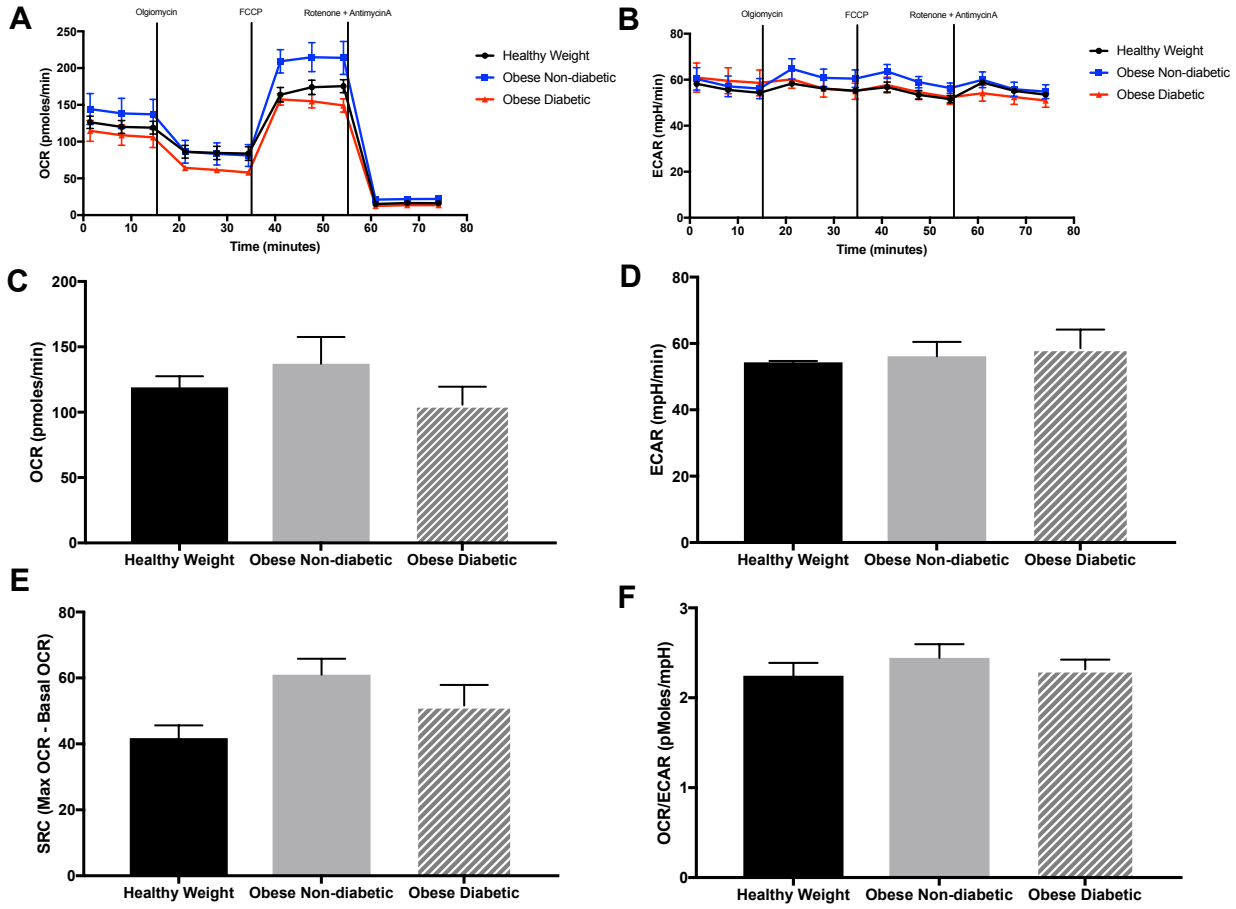

Figure 5. Metabolic profile of activated CD4+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of anti-CD3/anti-CD28 + IL-2 stimulated CD4+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects cultured in 5% autologous plasma exhibits changes in OCR and ECAR in response to 1.0 µM olgiomycin, 1.5µM FCCP, 1.0µM antimycinA and 0.1µM rotenone (A-B) in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1mM pyruvate. No differences in trends were seen for basal OCR (C), basal ECAR (D) or OCR:ECAR (F). Obese non-diabetic and obese metformin-treated diabetic stimulated CD4+ T cells had higher trends in spare respiratory capacity (E). Data are median ± SE. No significance, one-way ANOVA with Tukey's multiple comparisons. Individual comparisons were made using Mann-Whitney sum rank test, no significance found.

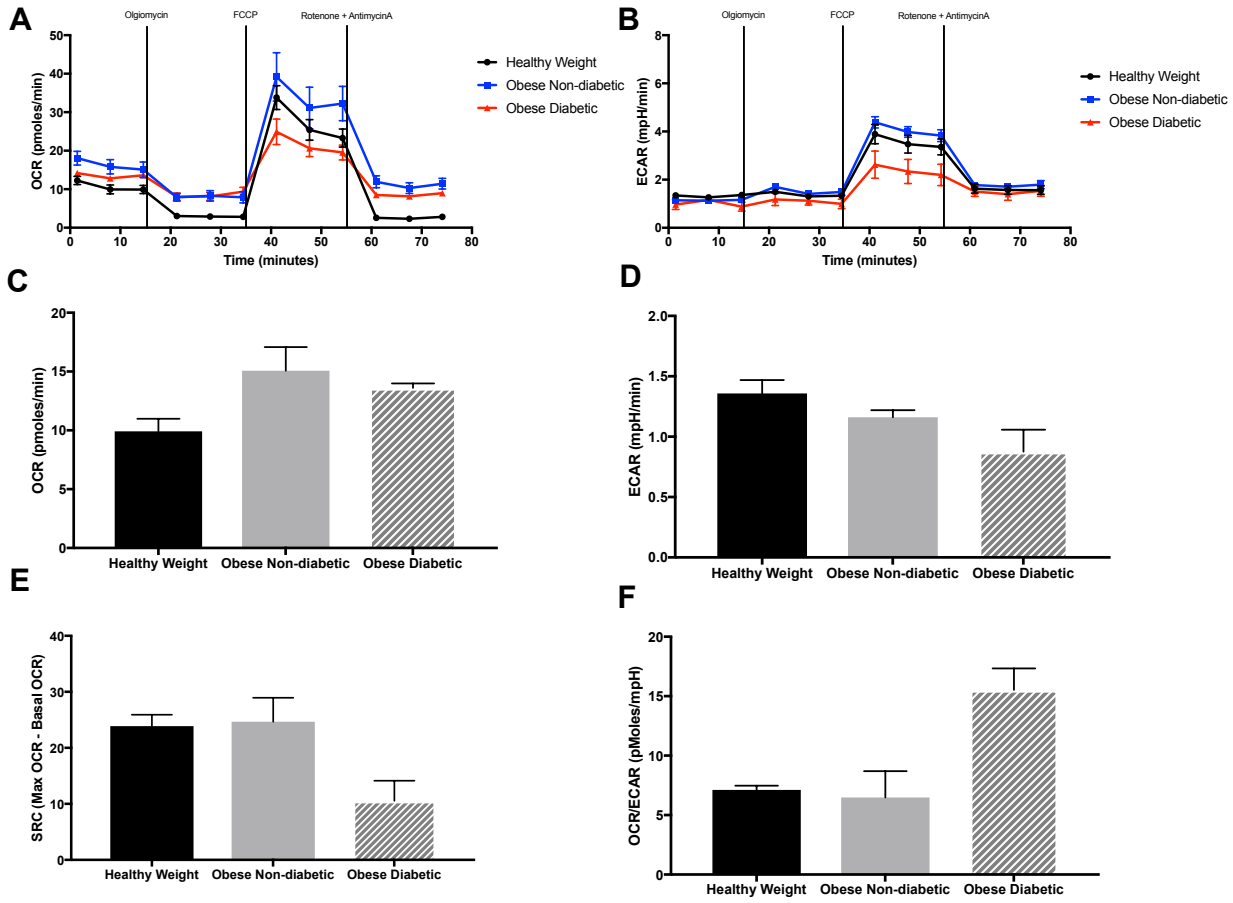

Figure 6. Metabolic profile of quiescent CD8+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of fresh CD8+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects exhibit changes in OCR and ECAR in response to 1.0 µM olgiomycin, 1.5µM FCCP, 1.0µM antimycinA and 0.1µM rotenone (A-B) in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1mM pyruvate. Higher trends in basal OCR were seen in obese non-diabetic and obese diabetic CD8+ T cells compared to healthy weight (C). A decreasing trend was seen in basal ECAR (D) between groups. Obese metformin-treated diabetic CD8+ T cells had lower SRC and nearly two-fold higher OCR:ECAR than healthy weight and obese non-diabetic resting CD8+ T cells. Data are median ± SE. No significance, one-way ANOVA with Tukey's multiple comparisons. Individual comparisons were made using Mann-Whitney sum rank test, no significance found.

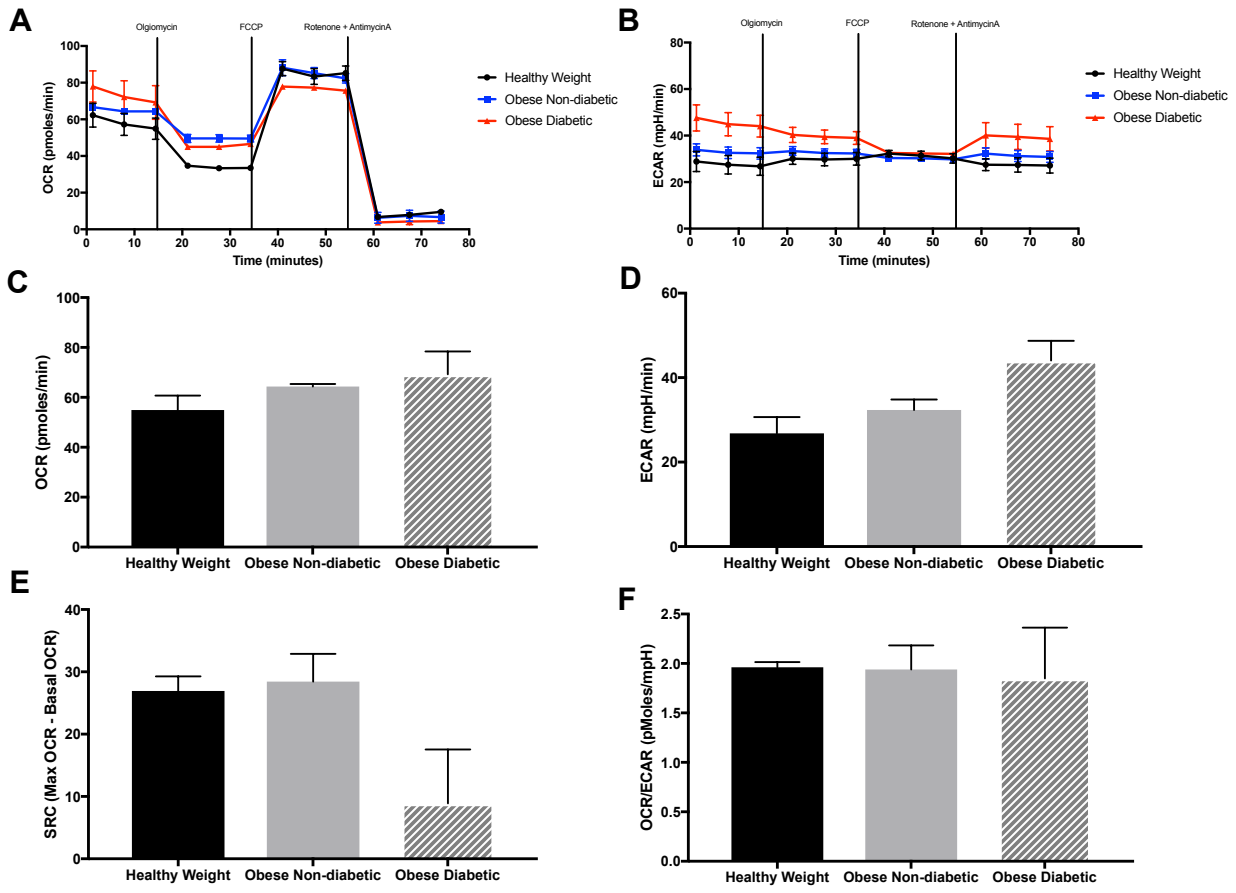

Figure 7. Metabolic profile of activated CD8+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of anti-CD3/anti-CD28 + IL-2 stimulated CD8+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects cultured in 5% autologous plasma exhibits changes in OCR and ECAR in response to 1.0 µM olgiomycin, 1.5µM FCCP, 1.0µM antimycinA and 0.1µM rotenone (A-B) in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1mM pyruvate. No differences in trends were seen for basal OCR (C), basal ECAR (D) or OCR:ECAR (F). Obese metformin-treated diabetic stimulated CD8+ T cells had greatly reduced spare respiratory capacity (E). Data are median ± SE. No significance, one-way ANOVA with Tukey's multiple comparisons. Individual comparisons were made using Mann-Whitney sum rank test, no significance found.

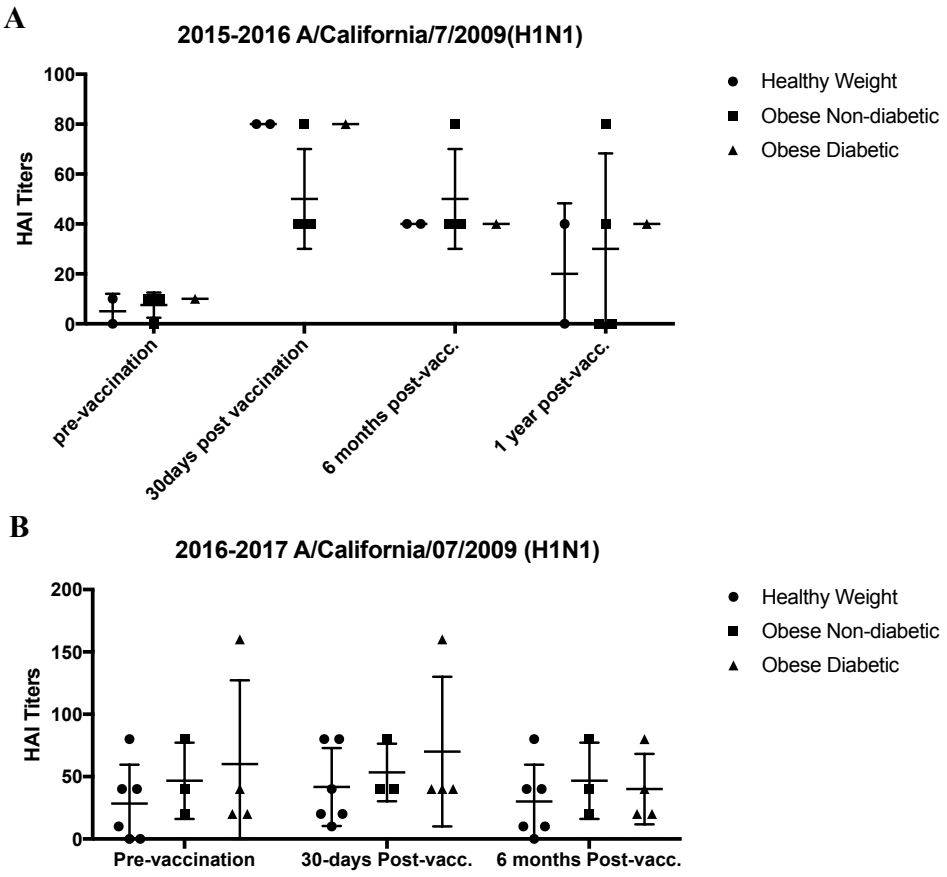

Figure 8. Influenza specific HAI antibody titers for A/California/7/2009(H1N1) from 2015-2016 and 2016-2017 vaccine years. Virus specific antibody titers from healthy weight (n=3), obese non-diabetic (n=3) and obese diabetic (n=3) adult subjects vaccinated against Influenza virus increase following vaccination from day 0 to30-days post vaccination (A-B). Obese diabetic's had a greater increase in IgG1 antibody titer at 30 days post vaccination than obese non-diabetic ($p<0.05$), but difference from healthy weight (B). Antibody titers decline marginally by 6 months post vaccination. Data from both vaccine years were combined, as the same strain was included in both vaccines. Data are mean ± SEM. Two-way ANOVA and Tukey's multiple comparisons were used to determine significance.

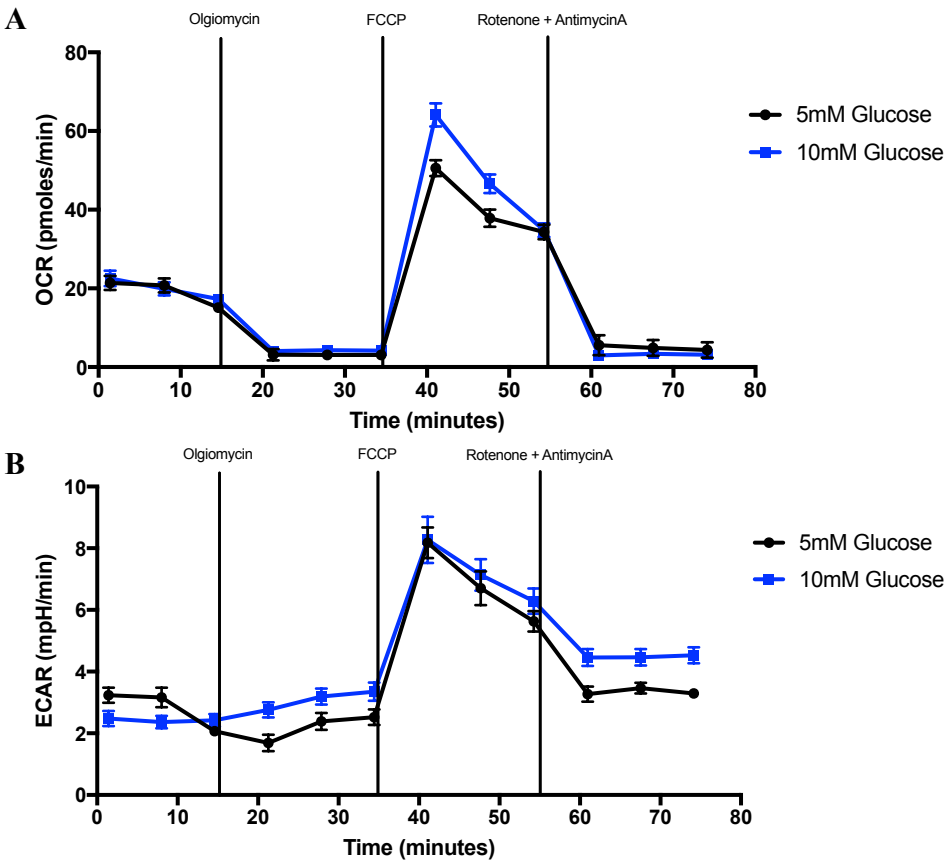

Figure 9. Metabolic profile of quiescent CD4+ T cells from influenza vaccinated healthy weight adults in euglycemic or diabetic glucose conditions. Quiescent CD4+ T cells in euglycemic (5mM or 90mg/dl) glucose versus diabetic (10mM glucose or 180mg/dl) glucose conditions. No significant differences were seen in basal OCR (A) or ECAR (B) depending on glucose concentration. SRC was significantly higher, most likely due to excess nutrient availability suggesting glucose concentrations can become limits in culture. Data are mean ± SEM. Student's T test.

Table 3. Summary data from Spearman's correlation coefficients. Age was found to positively correlate with quiescent CD8+ ECAR data despite no significant differences between group ages. Other values may contribute in larger samples sizes.

Table 3: Spearman's correlation coeficients for metabolism vs demographic data

Metabolic Paramter		Age	BMI	Fasting glucose	Smoking Status	Metformin Use	Flu or ILI
Quiescent CD4+ OCR	Spearman r	-0.06723	0.01667	-0.4184	0.5477	-0.2739	0.5175
	p value (two-tailed)	0.8685	0.9816	0.2627	0.2222	0.5476	0.2222
Quiescent CD4+ ECAR	Spearman r	0.06723	0.03333	-0.5188	0.5477	-0.1826	0.414
	p value (two-tailed)	0.8685	0.9484	0.1558	0.2222	0.7143	0.3333
Activated CD4+ OCR	Spearman r	-0.1933	-0.05	-0.159	-0.5477	-0.2739	-0.414
	p value (two-tailed)	0.6176	0.9116	0.6835	0.2222	0.5476	0.3333
Activated CD4+ ECAR	Spearman r	-0.05883	0.3833	0.09205	-0.5477	0.09129	-0.6211
	p value (two-tailed)	0.8862	0.3125	0.8182	0.2222	0.9048	0.1111
Quiescent CD8+ OCR	Spearman r	-0.06723	0.4167	-0.1255	-0.2739	0	-0.1035
	p value (two-tailed)	0.8685	0.2696	0.7512	0.6667	>0.9999	0.8889
Quiescent CD8+ ECAR	Spearman r	0.7479	0.08333	-0.2343	0.5477	-0.09129	0.7246
	p value (two-tailed)	**0.0247**	0.8432	0.5401	0.2222	0.9048	0.0556
Activated CD8+ OCR	Spearman r	-0.02395	0.07143	0.2635	0.2474	0.6299	-0.252
	p value (two-tailed)	0.9642	0.882	0.524	0.75	0.1429	0.6429
Activated CD8+ ECAR	Spearman r	-0.2156	-0.1667	0.08383	0.2474	0.6299	0
	p value (two-tailed)	0.6091	0.7033	0.8493	0.75	0.1429	>0.9999

CHAPTER III – SIGNIFICANCE & FUTURE DIRECTIONS

This investigation provides the first evidence of metabolic alterations of human T cells in the context of obesity. Quiescent CD4+ and CD8+ T cells from obese non-diabetic subjects showed trends of higher mitochondrial respiration, with CD4+ T cells from obese non-diabetics also exhibiting higher levels of basal respiration. Interesting, quiescent CD8+ T cell from obese non-diabetics had slightly lower rates of basal glycolysis, suggesting obesity's impact on T cell metabolism is a complex multifactorial mechanism that differs based on individual cellular demand. Furthermore, obese metformin-treated diabetics, who despite medication still maintained elevated fasting glucose levels, had trends in CD4+ and CD8+ T cell metabolism similar to healthy weight controls. This finding suggests that metformin acts directly on T cell metabolism, possibly helping to restore it to normal homeostatic levels.

Metformin has been implicated as a metabolic therapeutic for the prevention and treatment of cancer [64]. Given the incredible similarity between cancer metabolism and T cell metabolism, it is possible that metformin works to restore "normal" metabolic programming of these highly metabolic cell lineages. In the context of influenza and obesity, metformin treatment may restore homeostatic T cell metabolism to an optimal quiescent range, such that upon exposure to influenza virus, obese cells can then appropriately respond without over-responding to a hyper-inflammatory state. Furthermore, metformin treatment may support anti-inflammatory T regulatory cells, thereby helping to reduce the inflammatory microenvironment associated with excess adiposity. These initial findings provide a potential mechanistic link between previously

shown functional impairments in influenza specific T cell responses and metabolic dysregulation in obese adults.

In 2014, influenza ranked as the 8th leading cause of death in the United States, with a population mortality rate of 1.4 deaths per 100,000 occurring due to influenza infection [69]. Given the growing population of adults classified as obese, this increase in obesity puts a increasing proportion of adults at risk of severe influenza infection and poor health outcomes due to T cell driven metabolic alterations of immunity against influenza virus. In the context of public health, these findings provide a potential mechanistic cause for obesity associated immune impairments, resulting in increased susceptibility to severe influenza outcomes. Furthermore, obesity's impact on the immune system may influence developing immune systems like those of children classified as obese, increasing the susceptibility to influenza as well as other infectious diseases.

The major strength of this work involves its novel identification of metabolic alterations of T cell metabolism in obese adults. It provides the first look into altered metabolic profiles of CD4+ and CD8+ T cells in healthy weight and obese adults. Furthermore, it provides evidence to support the metabolic drug metformin as a direct therapeutic target, impacting T cell metabolism. Finally, this work demonstrates that obesity driven alterations in T cell metabolism is most likely not glucose driven, as previously thought. Instead, other factors of obesity such as dysregulations in pro-growth tyrosine kinases such as insulin and EGF, as well as hormone adipokines like leptin may account for the altered metabolic programming of T cells from obese adults.

This work also had several limitations. The sample size in this investigation did not yield enough power to accurately identify statistically significant differences between several metabolic conditions. Furthermore, the sample population was limited based on subject

availability and thus in an effort to minimize potential variability between subjects, a tightly defined study population was used. This limits the diversity of the study and reduces generalizability to males, other races, younger or older adults, etc. Secondly, this work was unable to directly tie alterations in T cell metabolism to impairments in T cell function. Finally, this work did not assess any other metabolic components of obesity aside from glucose metabolism.

Future experiments should address several of these limitations by expanding the sample size as well as metabolic and functional parameters assessed to establish a stronger understanding of obesity's influence on T cells. For example, creating a flow cytometry panel to correlate metabolic parameters with T cell activation and function would provide a more direct understanding of physiologic effects of obesity on T cell metabolism. Several key markers that should be assessed include CD40, CD69 and CD28, which are activation markers of activated T cells. Other expression markers like PD-1, an exhaustion signal upregulated during senescence, would indicate an exhausted state of T cells. These markers coupled with metabolic data would elicit a deeper understanding of how metabolic profiles of mitochondrial respiration and glycolysis influence T cell activation. Additionally, by including functional markers such as IFN-γ, granyzme B, and IL-12R (an IFN-γ receptor which promotes the differentiation of Th1 T cells), cells from healthy weight and obese adults can be further characterized into more distinct optimally functional or impaired phenotypes.

Metabolic conditions should also be further investigated. We showed trends in heighted mitochondrial respiration of glucose derived pyruvate and glutamine in quiescent CD4+ and CD8+ T cells. Additionally, we showed there was no difference in basal respiration of quiescent CD4+ T cells in euglycemic (5mM glucose) versus diabetic (10mM glucose) conditions.

However, quiescent T cells also utilize fatty acid for homeostatic metabolism. Future experiments should investigate whether oxidation of fatty acids differs between T cells from healthy weight and obese adults in both a quiescent state and activated state. By using radiolabeled palmitate or oleate in combination with non-labeled fatty acid, glucose and glutamine, beta-oxidation and/or incorporation of ^{13}C-palmitate or ^{13}C-oleate would demonstrate how T cells from healthy weight and obese adults utilize fatty acids in quiescent and activated states. Given the potential for significantly higher circulating levels of fatty acids in obese adults, this type of experiment would elicit a more accurate physiological understanding of how T cells from obese adults use lipid to sustain homeostasis, or perhaps explain why in an activated state, functional production of cytokines cannot occur as very little fatty acid oxidation should occur.

Finally, looking at other factors such as hormones (insulin, letpin, adiponectin), cytokines (IL-2, IL-12, etc.) or microbiome-produced metabolites (short-chain fatty acids, etc.) would provide a more-thorough mechanistic understanding of how dyregulated factors of obesity influence T cell metabolism. Using techniques such as ELIS to quantify serum cytokines or hormones might account for higher or lower metabolic profiles of quiescent and activated T cells. Other future experiments might include measuring the distribution of microbiota in obese adults and coupling this information to metabolic factors in obese sera and resultant profiles of healthy weight and obese T cells to see if particular metabolites influence immune cell metabolism.

Taken together, this work builds on previously shown impairments in T cell responses to influenza vaccination in obese adults. T cell metabolic differences between healthy weight and obese adults present a novel mechanism through which T cell function may be compromised; supporting the notion of obesity associated pro-inflammatory T cell subtype differentiation and

impairment of resting or quiescent T cell populations. Furthermore, we demonstrate that influenza specific HAI antibody titers from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults respond to vaccine at 30 days, but fall at six months post vaccination, with no differences between groups. This data suggests that T cell mediated responses, and not antibody seroconversion or seroprotection, should be considered when assessing for correlates of protection from influenza infection in obese adults. The metabolic difference between healthy weight and obese adults warrants further study as these highly metabolic cells play a critical role in supporting innate and adaptive immune protection and recovery from influenza virus infection.

REFERENCES

1. Collaboration NRF: **Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants**. *The Lancet* 2016, **387**:1377-1396.

2. Louie JK, Acosta M, Samuel MC, Schechter R, Vugia DJ, Harriman K, Matyas BT, California Pandemic Working G: **A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1)**. *Clin Infect Dis* 2011, **52**:301-312.

3. **Estimates of Deaths Associated with Seasonal Influenza -- United States, 1976-2007**. *Morbidity and Mortality Weekly Report* 2010, **59**:1057-1089.

4. van Panhuys N, Klauschen F, Germain RN: **T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo**. *Immunity* 2014, **41**:63-74.

5. Brownlie RJ, Zamoyska R: **T cell receptor signalling networks: branched, diversified and bounded**. *Nat Rev Immunol* 2013, **13**:257-269.

6. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, Sullivan SA, Nichols AG, Rathmell JC: **Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets**. *J Immunol* 2011, **186**:3299-3303.

7. MacIver NJ, Michalek RD, Rathmell JC: **Metabolic regulation of T lymphocytes**. *Annu Rev Immunol* 2013, **31**:259-283.

8. Buck MD, O'Sullivan D, Pearce EL: **T cell metabolism drives immunity**. *J Exp Med* 2015, **212**:1345-1360.

9. Pearce EL, Poffenberger MC, Chang CH, Jones RG: **Fueling immunity: insights into metabolism and lymphocyte function**. *Science* 2013, **342**:1242454.

10. MacIver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC: **Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival**. *Journal of Leukocyte Biology* 2008, **84**:949-957.

11. Jacobs SR, Herman CE, MacIver NJ, Wofford JA, Wieman HL, Hammen JJ, Rathmell JC: **Glucose Uptake Is Limiting in T Cell Activation and Requires CD28-Mediated Akt-Dependent and Independent Pathways**. *The Journal of Immunology* 2008, **180**:4476-4486.

12. Pearce EL, Pearce EJ: **Metabolic pathways in immune cell activation and quiescence**. *Immunity* 2013, **38**:633-643.

13. Lochner M, Berod L, Sparwasser T: **Fatty acid metabolism in the regulation of T cell function**. *Trends Immunol* 2015, **36**:81-91.

14. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, Anderson SM, Abel ED, Chen BJ, Hale LP, et al.: **The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function**. *Cell Metab* 2014, **20**:61-72.

15. Gregor MF, Hotamisligil GS: **Inflammatory mechanisms in obesity**. *Annu Rev Immunol* 2011, **29**:415-445.

16. Huttunen R, Syrjanen J: **Obesity and the risk and outcome of infection**. *Int J Obes (Lond)* 2013, **37**:333-340.

17. Milner JJ, Beck MA: **The impact of obesity on the immune response to infection**. *Proc Nutr Soc* 2012, **71**:298-306.

18. Park BV, Pan F: **Metabolic regulation of T cell differentiation and function**. *Mol Immunol* 2015, **68**:497-506.

19. Jacobs SR, Michalek RD, Rathmell JC: **IL-7 is essential for homeostatic control of T cell metabolism in vivo**. *J Immunol* 2010, **184**:3461-3469.

20. Weinberg SE, Chandel NS: **Futility sustains memory T cells**. *Immunity* 2014, **41**:1-3.

21. O'Sullivan D, van der Windt GJ, Huang SC, Curtis JD, Chang CH, Buck MD, Qiu J, Smith AM, Lam WY, DiPlato LM, et al.: **Memory CD8(+) T cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development**. *Immunity* 2014, **41**:75-88.

22. van der Windt GJ, Everts B, Chang CH, Curtis JD, Freitas TC, Amiel E, Pearce EJ, Pearce EL: **Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development**. *Immunity* 2012, **36**:68-78.

23. van der Windt GJ, O'Sullivan, D., Everts, B., Ching-Cheng Huang, S., Buck, M.D., Curtis, J. D., Chang, C., Smith, A. M., Ai, T., Faubert, B., Jones, R. G., Pearce, E. J., Pearce, E. L.: **CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability**. *PNAS* 2013, **110**:14336-14341.

24. Cui G, Staron MM, Gray SM, Ho PC, Amezquita RA, Wu J, Kaech SM: **IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory CD8+ T Cell Longevity**. *Cell* 2015, **161**:750-761.

25. Dong W, Ding T, Wu L, Ren X, Epling-Burnette PK, Yang L: **Effect of IL-7 and IL-15 on T cell phenotype in myelodysplastic syndromes**. *Oncotarget* 2016, **7**:27479-27488.

26. Renner K, Geiselhoringer AL, Fante M, Bruss C, Farber S, Schonhammer G, Peter K, Singer K, Andreesen R, Hoffmann P, et al.: **Metabolic plasticity of human T cells: Preserved**

cytokine production under glucose deprivation or mitochondrial restriction, but 2-deoxy-glucose affects effector functions. *Eur J Immunol* 2015, **45**:2504-2516.

27. Battaglia A, Buzzonetti A, Baranello C, Fanelli M, Fossati M, Catzola V, Scambia G, Fattorossi A: **Interleukin-21 (IL-21) synergizes with IL-2 to enhance T-cell receptor-induced human T-cell proliferation and counteracts IL-2/transforming growth factor-beta-induced regulatory T-cell development**. *Immunology* 2013, **139**:109-120.

28. Sheng J, Chen W, Zhu HJ: **The immune suppressive function of transforming growth factor-beta (TGF-beta) in human diseases**. *Growth Factors* 2015, **33**:92-101.

29. Saucillo DC, Gerriets VA, Sheng J, Rathmell JC, Maciver NJ: **Leptin metabolically licenses T cells for activation to link nutrition and immunity**. *J Immunol* 2014, **192**:136-144.

30. Pollizzi KN, Powell JD: **Integrating canonical and metabolic signalling programmes in the regulation of T cell responses**. *Nat Rev Immunol* 2014, **14**:435-446.

31. Blagih J, Coulombe F, Vincent EE, Dupuy F, Galicia-Vazquez G, Yurchenko E, Raissi TC, van der Windt GJ, Viollet B, Pearce EL, et al.: **The energy sensor AMPK regulates T cell metabolic adaptation and effector responses in vivo**. *Immunity* 2015, **42**:41-54.

32. Ogden CL, Carroll MD, Fryar CD, Flegal KM: **Prevalence of Obesity Among Adults and Youth- United States, 2011–2014**. *NCHS data brief* 2015, **no 219**.

33. Park HL, Shim SH, Lee EY, Cho W, Park S, Jeon HJ, Ahn SY, Kim H, Nam JH: **Obesity-induced chronic inflammation is associated with the reduced efficacy of influenza vaccine**. *Hum Vaccin Immunother* 2014, **10**:1181-1186.

34. Sheridan PA, Paich HA, Handy J, Karlsson EA, Hudgens MG, Sammon AB, Holland LA, Weir S, Noah TL, Beck MA: **Obesity is associated with impaired immune response to influenza vaccination in humans**. *Int J Obes (Lond)* 2012, **36**:1072-1077.

35. Carolan E, Hogan AE, Corrigan M, Gaotswe G, O'Connell J, Foley N, O'Neill LA, Cody D, O'Shea D: **The impact of childhood obesity on inflammation, innate immune cell frequency, and metabolic microRNA expression**. *J Clin Endocrinol Metab* 2014, **99**:E474-478.

36. Paich HA, Sheridan PA, Handy J, Karlsson EA, Schultz-Cherry S, Hudgens MG, Noah TL, Weir SS, Beck MA: **Overweight and obese adult humans have a defective cellular immune response to pandemic H1N1 influenza A virus**. *Obesity (Silver Spring)* 2013, **21**:2377-2386.

37. Costanzo AE, Taylor KR, Dutt S, Han PP, Fujioka K, Jameson JM: **Obesity impairs gammadelta T cell homeostasis and antiviral function in humans**. *PLoS One* 2015, **10**:e0120918.

38. Mascitelli L, Pezzetta F, Goldstein MR: **Leptin and regulatory T cells in obese patients with asthma**. *Thorax* 2008, **63**:659.

39. Tagliabue C, Principi N, Giavoli C, Esposito S: **Obesity: impact of infections and response to vaccines**. *Eur J Clin Microbiol Infect Dis* 2016, **35**:325-331.

40. Roth J, Sahota N, Patel P, Mehdi SF, Wiese MM, Mahboob HB, Bravo M, Eden DJ, Bashir MA, Kumar A, et al.: **Obesity paradox, obesity orthodox, and the metabolic syndrome: An approach to unity**. *Mol Med.* 2016, **22**.

41. Nilsson C, Raun K, Yan FF, Larsen MO, Tang-Christensen M: **Laboratory animals as surrogate models of human obesity**. *Acta Pharmacol Sin* 2012, **33**:173-181.

42. Milner JJ, Wang J, Sheridan PA, Ebbels T, Beck MA, Saric J: **1H NMR-based profiling reveals differential immune-metabolic networks during influenza virus infection in obese mice**. *PLoS One* 2014, **9**:e97238.

43. Milner JJ, Rebeles J, Dhungana S, Stewart DA, Sumner SC, Meyers MH, Mancuso P, Beck MA: **Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice**. *J Immunol* 2015, **194**:4846-4859.

44. Reis BS, Lee K, Fanok MH, Mascaraque C, Amoury M, Cohn LB, Rogoz A, Dallner OS, Moraes-Vieira PM, Domingos AI, et al.: **Leptin receptor signaling in T cells is required for Th17 differentiation**. *J Immunol* 2015, **194**:5253-5260.

45. Deng T, Lyon CJ, Minze LJ, Lin J, Zou J, Liu JZ, Ren Y, Yin Z, Hamilton DJ, Reardon PR, et al.: **Class II major histocompatibility complex plays an essential role in obesity-induced adipose inflammation**. *Cell Metab* 2013, **17**:411-422.

46. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S, et al.: **CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity**. *Nat Med* 2009, **15**:914-920.

47. McLaughlin T, Liu LF, Lamendola C, Shen L, Morton J, Rivas H, Winer D, Tolentino L, Choi O, Zhang H, et al.: **T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans**. *Arterioscler Thromb Vasc Biol* 2014, **34**:2637-2643.

48. Morris DL, Cho KW, DelProposto JL, Oatmen KE, Geletka LM, Martinez-Santibanez G, Singer K, Lumeng CN: **Adipose Tissue Macrophages Function As Antigen-Presenting Cells and Regulate Adipose Tissue CD4+ T Cells in Mice**. *Diabetes* 2013, **62**:2762-2772.

49. Cho KW, Morris DL, DelProposto JL, Geletka L, Zamarron B, Martinez-Santibanez G, Meyer KA, Singer K, O'Rourke RW, Lumeng CN: **An MHC II-dependent activation loop between adipose tissue macrophages and CD4+ T cells controls obesity-induced inflammation**. *Cell Rep* 2014, **9**:605-617.

50. Xiao L, Yang X, Lin Y, Li S, Jiang J, Qian S, Tang Q, He R, Li X: **Large adipocytes function as antigen-presenting cells to activate CD4(+) T cells via upregulating MHCII in obesity**. *Int J Obes (Lond)* 2016, **40**:112-120.

51. Wang CY, Kim HH, Hiroi Y, Sawada N, Salomone S, Benjamin LE, Moskowitz MA, Liao JK: **Obesity Increases Vascular Senescence and Susceptibility to Ischemic Injury Through Chronic Activation of Akt and mTOR**. *Science Signaling* 2009, **2**:1-11.

52. Palmer AK, Tchkonia T, LeBrasseur NK, Chini EN, Xu M, Kirkland JL: **Cellular Senescence in Type 2 Diabetes: A Therapeutic Opportunity**. *Diabetes* 2015, **64**:2289-2298.

53. Shirakawa K, Yan X, Shinmura K, Endo J, Kataoka M, Katsumata Y, Yamamoto T, Anzai A, Isobe S, Yoshida N, et al.: **Obesity accelerates T cell senescence in murine visceral adipose tissue**. *J Clin Invest* 2016, **126**:4626-4639.

54. Lund SA, Giachelli CM, Scatena M: **The role of osteopontin in inflammatory processes**. *J Cell Commun Signal* 2009, **3**:311-322.

55. Jin HT, Ahmed R, Okazaki T: **Role of PD-1 in regulating T-cell immunity**. *Curr Top Microbiol Immunol* 2011, **350**:17-37.

56. Charlton JJ, Chatzidakis I, Tsoukatou D, Boumpas DT, Garinis GA, Mamalaki C: **Programmed death-1 shapes memory phenotype CD8 T cell subsets in a cell-intrinsic manner**. *J Immunol* 2013, **190**:6104-6114.

57. Wei F, Zhong S, Ma Z, Kong H, Medvec A, Ahmed R, Freeman GJ, Krogsgaard M, Riley JL: **Strength of PD-1 signaling differentially affects T-cell effector functions**. *PNAS* 2013:E2480-E2489.

58. Chang CH, Curtis JD, Maggi LB, Jr., Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, et al.: **Posttranscriptional control of T cell effector function by aerobic glycolysis**. *Cell* 2013, **153**:1239-1251.

59. Simar D, Versteyhe S, Donkin I, Liu J, Hesson L, Nylander V, Fossum A, Barres R: **DNA methylation is altered in B and NK lymphocytes in obese and type 2 diabetic human**. *Metabolism* 2014, **63**:1188-1197.

60. Jacobsen MJ, Mentzel CM, Olesen AS, Huby T, Jorgensen CB, Barres R, Fredholm M, Simar D: **Altered Methylation Profile of Lymphocytes Is Concordant with Perturbation of Lipids Metabolism and Inflammatory Response in Obesity**. *J Diabetes Res* 2016, **2016**:8539057.

61. Mitchell NS, Catenacci VA, Wyatt HR, Hill JO: **Obesity: overview of an epidemic**. *Psychiatr Clin North Am* 2011, **34**:717-732.

62. Zarrouk M, Finlay DK, Foretz M, Viollet B, Cantrell DA: **Adenosine-mono-phosphate-activated protein kinase-independent effects of metformin in T cells**. *PLoS One* 2014, **9**:e106710.

63. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y: **Enhancing CD8 T-cell memory by modulating fatty acid metabolism**. *Nature* 2009, **460**:103-107.

64. Kasznicki J, Sliwinska A, Drzewoski J: **Metformin in cancer prevention and therapy**. *Ann Transl Med* 2014, **2**:57.

65. **Overview of Influenza Surveillance in the United States**. *Centers for Disease Control and Prevention, National Center for Immunizaiton and Respiratory Diseases (NCIRD)* 2016.

66. Sheridan PA, Paich HA, Handy J, Karlsson EA, Schultz-Cherry S, Hudgens M, Weir S, Noah T, Beck MA: **The antibody response to influenza vaccination is not impaired in type 2 diabetics**. *Vaccine* 2015, **33**:3306-3313.

67. Ganeshan K, Chawla A: **Metabolic regulation of immune responses**. *Annu Rev Immunol* 2014, **32**:609-634.

68. Lijen P, Petrov, S. V.: **In vitro proliferative response of human peripheral blood mononuclear cells to concnavalin A**. *Clinica Chimica Acta* 1997, **264**:91-101.

69. Kochanek KD, Murphy SL, Xu J, and, Tejada-Vera B: **Deaths: Final Data for 2014**. *National Vital Statistics Report* 2016, **65**.