INFLUENZA-SPECIFIC T CELL MEMORY: INFLUENCE OF OBESITY, WEIGHT LOSS, WEIGHT GAIN

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Nutrition (Nutritional Biochemistry) in the Gillings School of Global Public Health.

Chapel Hill
2017

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ABSTRACT

Jennifer Rebeles: Influenza-Specific T Cell Memory: Influenza of Obesity, Weight Loss, Weight Gain
(Under the direction of Melinda A. Beck)

Obesity is a global epidemic, with 10% of men and 14% of women obese worldwide. Obesity is a known risk factor for increased complications and death from infection with influenza virus, and impairs the T cell response to both influenza infection and vaccination. As obesity is primarily a metabolic disorder, and immune cell function is dictated by metabolism of the immune cell, the effect of obesity on memory T cell metabolism following a secondary influenza infection was investigated. This dissertation addressed whether the metabolic environment at the time of memory T cell generation or at the time of re-challenge would influence T cell metabolism and function.

C57BL/6J high fat diet-induced obese mice were infected with X-31 influenza virus to generate memory T cells, then switched to a low-fat diet to induce weight loss. Following weight loss and normalized fasting glucose levels, mice were re-infected with influenza Puerto Rico/8/34 (PR8) to activate the memory T cells in a newly generated lean state. Conversely, lean mice were infected with X-31 to generate memory T cells followed by a diet switch to a high fat diet to induce obesity. Following weight gain and elevated fasting glucose levels, mice were re-exposed to PR8. Compared with mice that were always lean, mice that were obese for both primary and secondary influenza infections had impaired T cell metabolism and function. Mice that lost weight maintained a metabolic phenotype that paralleled the always obese metabolic phenotype along with dysregulated frequencies of central memory, effector memory, and tissue resident memory T cell populations and decreased function of influenza-specific memory T cell subsets. Mice that

had gained weight, and were previously lean, maintained a metabolic profile similar to the mice that were always lean, although also had T cell subset alterations and diminished function.

Altogether, this data demonstrates that metabolic environment present at the time of memory T cell generation and at time of secondary immune challenge both impact T cell function. For the first time, obesity has been shown to alter T cell metabolism, and we demonstrate that weight loss will not restore T cell metabolism or function.

To my mother Rose, my father David, and my brother Sam, thank you for the unwavering support, love, and encouragement all these years in achieving my biggest dream. I dedicate this work to my grandmothers Esperanza Almeida and Castula Rebeles, who showed me the discipline of hard work, perseverance, and the values of faith and family.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Melinda A. Beck, for the support, faith, and encouragement in my graduate studies. Dr. Beck provided me with invaluable guidance, and helped me cultivate my techniques, skills, and critical thinking to become a better scientist. She gave me the opportunity to pursue research in immunology which is a dream of mine, and for that, I will forever be grateful. I would also like to thank my committee members for their time and support in the development of my dissertation: Rosalind Coleman, Liza Makowski, Stephen Hursting, and Jason Whitmire. The discussions, suggestions and contributions to my project was greatly appreciated. I would also like to recognize Qing Shi and Kim Bartholomew for their help with the daily lab duties that made working with them such a pleasure and positive environment. I also would like to acknowledge Will Green for the tremendous amount of technical help, as well as the stimulating scientific discussions which enhanced my dissertation. Finally, I would like to thank my parents, brother, family and friends for the constant laughs and encouragement.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B (serine/threonine protein kinase)
AMPK	5'-adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMI	Body mass index
C57BL/6J	general purpose mouse model
CD	Chow diet
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CD11a	T cell migration factor LFA-1
CD127	IL-7Rα chain
CPT1	Carnitine palmitolytransferase 1a
CVD	Cardiovascular disease
db/db	mutation in leptin receptor mouse model
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA

ECAR	Extracellular acidification rate (mpH/min)
EID_{50}	400 egg infectious dose 50%
ELISA	Enzyme linked immunosorbent assay
EM	Effector memory
FACS	Fluorescence activated cell sorting
FAO	Fatty acid oxidation
FFA	Free fatty acid
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GATA3	Transcription factor GATA binding protein 3
Glut1	Glucose transporter 1
H&E	Hematoxylin and eosin
HA	Hemaggluttinin protein
HAI	Hemaggluttinin inhibition assay
HDL	High density lipoprotein
HEV	High endothelial venules
HFD	High fat diet
IL	Interleukin
IFN	Interferon
IKKβ	Inhibitor of nuclear factor kappa B kinase beta
iNKT	Invariant Natural Killer T

IFNs	Type I interferons
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IRF3	IFN-regulatory factor 3
IRS-1	Insulin receptor substrate 1
JNK	Janus kinase
LAIV	Live attenuated influenza vaccine
LAL	Lysosomal acid lipase
LCMV	Lymphocytic choriomeningitis virus
LepR	Leptin receptor
LFD	Low fat diet
M1	Influenza matrix protein 1
M2	Influenza matrix protein 2
MCP-1	Macrophage chemoattractant protein-1
MDCK	Madin-Darby canine kidney epithelial cells
MEM	Minimum essential medium
MHCI	Major histocompatibility complex I
MHCII	Major histocompatibility complex II
mLN	mediastinal lymph node
MOI	Multiplicity of infection
mTOR	Mammalian Target of Rapamycin

NA	Neuraminidase
NF-κB	Nuclear factor κB
NK	Natural killer T cells
NLRP3	NOD-like receptors
NOS2	Nitric oxide synthase 2
NP	Nucleoprotein
NS	Non-structural protein
ob/ob	mutation in leptin gene mouse model
OCR	Oxygen consumption rate (pmoles/min)
OCR:ECAR	OCR/ECAR
OXPHOS	Oxidative phosphorylation
PA	RNA polymerase A
PAMPS	Pathogen associated molecular patterns
PB1	RNA polymerase B1 subunit
PB2	RNA polymerase B2 subunit
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cells
pH1N1	Influenza A/Puerto Rico/8/1934 (H1N1)
PNAD	Protein N-terminal asparagine amidohydrolase

PR8	Influenza virus A/Puerto Rico/8/34
PRR	Pattern recognition receptors
RANTES	Chemokine, regulated on activation, normal T cell expressed and secreted
RIG-1	retinoic acid inducible gene I
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SLC	Solute carrier
SLE	Systemic lupus erythematosus
SRC	Spare respiratory capacity
ssRNA	Single stranded RNA
STAT6	Signal transducers and activators of transcription 6
TAG	Triacylglycerol
$TCID_{50}$	Tissue culture infectious dose 50
TCR	T cell receptor
TIV	Trivalent inactivated vaccine
TNF-α	Tumor necrosis factor alpha
TLR	Toll like receptor
Tfh	T follicular helper cells
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells

TEMRA	Terminally differentiated effector memory T cells
Th1	T helper 1 subtype
Th2	T helper 2 subtype
Th17	T helper 17 subtype
TRAF6	Tumor necrosis factor receptor 6
T_{RM}	Tissue resident memory T cells
Treg	Regulatory T cells
X-31	Recombinant influenza virus A/Aichi/2/68 (H3N2)

CHAPTER I: OVERVIEW AND SPECIFIC AIMS

Overview

Over the past few decades, obesity has emerged as a worldwide epidemic and presents an increased risk for a constellation of other diseases including metabolic syndrome. Of particular note, obesity is recognized as an independent risk factor for increased morbidity and mortality from infection with influenza virus. Each year, 3,000 to 50,000 people in the US die from infection with influenza, and the approximate 500 million individuals worldwide who are obese are at an increased risk. It is imperative that we understand how obesity contributes to a poor outcome following influenza infection in order to devise strategies to limit morbidity and mortality in this expanding at-risk population.

Using a well-defined mouse model of both obesity and influenza infection, we have shown that obesity drives a dysfunctional immune response to influenza challenge. Our lab has demonstrated that following infection with influenza, diet induced obese mice have increased morbidity and mortality, decreased lung effector memory CD8+ T cells, and impaired activation and function of memory T cells to a secondary challenge with influenza virus. Previously, all of our infection studies have been carried out in obese mice. What is missing is whether or not weight loss can restore immune function or if weight gain, after memory T cells have been generated, inhibits their function. Can weight loss restore the function of memory T cells that were generated during the obese state? Do memory T cells, generated in a lean state, fail to function in an obese state?

The memory T cell response is critical for protection against subsequent infection and if infection occurs, to rapidly clear the pathogen. Memory T cells can more rapidly initiate effector functions to kill infected cells, secrete inflammatory cytokines, or provide help to other immune

cells for pathogen clearance. Effector T cells give rise to the memory T cell phenotype, but the differentiation pathways of these cells are not clearly defined, and even less is known about obesity and its role in the differentiation and maintenance of memory T cells. While effector T cells can live for days or weeks, memory T cells can last for months, years, or a lifetime. The reprogramming of a naïve T cell to become an effector cell and then a memory cell involves changes in chromatin structure and transcription factors to drive the cell to a specific fate. What has not been thoroughly investigated is whether obesity, at the time of reprogramming, determines the memory T cell fate indefinitely.

Nutrient conditions can influence the fate of T cell immune responses. Naïve CD4+ and CD8+ T cells utilize oxidation of glucose and fatty acids through beta-oxidation and the oxidative phosphorylation pathways. Upon exposure to antigen to activate these cells to an effector T cell phenotype, the metabolic demands increase in order to support rapid proliferation and effector function to clear the pathogen. Activation of CD4+ and CD8+ T cells results in a metabolic switch to utilize more glycolysis and glutaminolysis, and less oxidative phosphorylation. After the pathogen has been cleared and during the T cell contraction phase, the metabolic needs change to differentiate the effector cell to a long-lasting memory cell, which utilizes the oxidative phosphorylation pathway. Nutrient conditions in the obese state favor high serum glucose and fatty acids. Normal physiologic conditions utilize these metabolic fuels to differentiate T cell fate, but abnormal levels present in obesity may affect the ability of T cells to differentiate into the appropriate phenotype when activated. Furthermore, insulin resistance contributes to impaired glucose uptake in T cells. It is essential to understand if metabolic dysfunction in obesity contributes to the altered T cell metabolism and immune response during infection, and if these processes are reversed upon weight loss. The overarching hypothesis of this proposal is that the obese environment reprograms T cell metabolism, leading to impaired function, which can be revered with weight loss.

Specific AIMS

AIM 1: Determine if obesity impairs the metabolism of resting and activated memory T cells following a secondary influenza infection?

Hypothesis: Compared to lean mice, the metabolism of memory T cells from obese mice will be more active at rest, thereby limiting their metabolic potential when activated.

AIM 2: Determine if weight loss restores T cell metabolism and function to secondary influenza infection.

Hypothesis: Weight loss will restore T cell metabolism to a “lean” metabolism and improve function of memory T cells, impairing the response to a secondary influenza infection.

AIM 3: Determine if weight gain will induce metabolic dysfunction and impair the function of memory T cells generated in a lean state.

Hypothesis: The obese environment will alter the metabolism and function of the formally lean T cells.

CHAPTER II: BACKGROUND AND SIGNIFICANCE

Obesity introduction

Over the past 25 years, obesity, defined as a body mass index (BMI) over 30 (weight in kilograms divided by the square of height in meters) [1] has emerged as a worldwide growing public health concern, and continues to remain a contributing factor for chronic disease [2, 3]. The United States is ranked 18th in the world with the highest percentage of obesity in the adult population [4]. Within the United States alone, the rise of obesity has increased dramatically over [5]several decades. In 1985, no state had obesity rates higher than 15%. Currently, there are no states that remain under 20% with the majority of states above 25% [6]. The prevalence of obesity in the adult population from 2011-2014 was 36.5% [1], and childhood obesity at 17% [7] is paralleling the adult trends and continuing to rise as well. Obesity as a chronic condition can manifest into more serious, costly, and disabling conditions. However, obesity is also one of the top modifiable and preventable disease conditions.

Obesity is caused by excess calories consumed and not expended through activity, resulting in lipid storage of the excess calories. Several factors such as energy imbalance, genetics, endocrine, and medical conditions may be contributing co-morbidities including obesity [8]. Obesity in itself can be a debilitating disease, and is also a risk factor for several diseases such as cardiovascular disease, diabetes, musculoskeletal disease, hypertension, asthma, osteoarthritis, chronic obstructive pulmonary disease and some cancers [1, 9, 10]. In addition to contributing to chronic disease, obesity may overtake tobacco as the leading preventable cause of cancer risk [11]. Overweight (BMI 25-29.9) and obese are correlated with increased cancer incidence in men and women, with 28,000 and 72,000 reported in 2012, respectively [12]. While obesity has been associated with increasing risk for chronic diseases, it has also been noted as

an independent risk factor for increased risk of respiratory hospitalizations during influenza season [13], along with increased morbidity and mortality to the 2009 pandemic influenza A H1N1 infection [14].

Metabolic syndrome

Obesity often leads to the development of metabolic syndrome, a combination of risk factors that contribute to cardiovascular disease or the onset of type II diabetes. These risk factors include glucose intolerance, central adiposity, dyslipidemia, decreased high density lipoprotein and hypertension [15]. There have been many suggestions for defining metabolic syndrome, which has led to confusion and inconsistencies in collecting data [16], therefore the International Diabetes Federation proposed a world-wide definition to classify metabolic syndrome as having central obesity and 2 or more of the following factors: raised triglycerides, reduced high density lipoprotein (HDL)-cholesterol, raised blood pressure, and raised fasting plasma glucose [16-18].

There has been some debate as to whether all obesity overlaps with metabolic syndrome, as there are metabolically healthy obese individuals that have normal insulin and glucose levels [19], which deviates from the metabolically unhealthy obese phenotype. To date, there is no consensus on a definition of a metabolically healthy phenotype, however, most studies suggest that the absence of insulin resistance, lipid disorders and hypertension would constitute this phenotype [15]. While the term metabolically “healthy” obese is emerging in the literature, epidemiological studies suggest the need for stricter parameters and guidelines assessed to make such a definition. A systematic review on 20 studies in the literature and the association with cardiovascular disease (CVD) were assessed. They found a slight increase in cardiovascular risk of metabolically healthy obese adults compared to healthy weight adults, but it was difficult to assess whether this was a true risk or due to differences in methodology from the studies evaluated [20]. It is not currently known whether metabolically healthy adults have decreased risk of CVD compared to metabolically unhealthy adults, so more studies are needed.

Conversely, other studies suggest that the metabolically unhealthy obese phenotype can be reversed through lifestyle modifications or surgical means [21-23]. Lifestyle modifications with weight loss have decreased insulin resistance [24], prevented the onset of type II diabetes with impaired glucose tolerance [25, 26] and improved hypertension [27] and lipid values [28, 29]. Therefore, inducing weight loss by physical means improves metabolic health parameters. Bariatric surgery has been used as an intervention tool to induce weight loss, decreasing long-term morbidity and mortality compared to obese participants that did not undergo surgery [30-33], and decreased the development of new obese related conditions [30, 34]. Thus, weight loss through lifestyle modifications or surgical means improves metabolic health in previously obese adults.

Obesity and inflammation

The complications of obesity do not arise from a single tissue, but an orchestrated crosstalk among various tissues that results in a state of low-grade, chronic inflammation. While the mechanisms are not fully understood, it is suggested that liver, adipose, muscle, pancreas, brain, and gut [35-37] all contribute to obesity-induced chronic inflammation through various mechanisms [38]. This type of chronic inflammation differs from the normal physiological response of acute inflammation that is initiated by responding to an invading pathogen, or to an injury that requires the immune system to repair and restore the body to homeostasis. Acute inflammation is characterized by redness, increased heat, swelling, pain, and loss of function [39], whereas obesity-induced chronic inflammation results in dysregulated cytokine production, acute phase reactants, and increased activation of inflammatory signaling cascades [40, 41].

Adipose tissue is an active, metabolic, endocrine organ with a central role in in regulating energy homeostasis. Pre-adipocytes, adipocytes, endothelial cells, fibroblasts, stem cells, and many immune cell subsets such as CD4+, CD8+, and regulatory T cells (Tregs), B cells, macrophages, mast cells, eosinophils, and neutrophils make up the cellular environment of adipose tissue [42]. While adipocytes can expand and increase the volume of adipose tissue,

adipose tissue primarily consists of stromal vascular cells in which ~65% are leukocytes [43]. As adipocytes increase in volume (hypertrophy), they accumulate in subcutaneous (depots under the skin) or visceral locations (depots surrounding the organs). The accumulation of adipocytes in visceral depots increases risk for developing type 2 diabetes and cardiovascular disease [44-46].

Adipocytes secrete leptin, adiponectin, resistin, other adipokines which regulate energy homeostasis and pro-inflammatory cytokines such as IL-6 and TNF-α [47]. Leptin is an adipokine that is secreted in proportion to adiposity, and primarily regulates appetite and body weight regulation through the hypothalamus. However, leptin has various other roles related to immunity, reproduction, insulin sensitivity and growth [48-50]. Levels of adiponectin are higher in leaner individuals, and act on many tissues to improve insulin sensitivity [51] by inducing fatty oxidation in the liver, decreasing glucose production from the liver, and improving beta cell function [42].

Adipocytes are insulin sensitive tissues, and obesity contributes to the development of insulin resistance. Insulin resistance can be defined as the contributions of decreased effects of insulin on glucose uptake, metabolism and storage [52]. Manifestations of insulin contribute to the decreased uptake of glucose by tissues, and impaired suppression of hepatic glucose [52].

Under normal physiological conditions, free fatty acids are stored as triglycerides in adipocytes, and are mobilized by lipolysis to free fatty acids that can be released into the circulation and travel to tissues, such as muscle, to be used for mitochondrial beta oxidation in order to produce ATP. With obesity, the chronic consumption of excess calories results in increased adipocyte size and accumulation of triglycerides that metabolically overload the cell. This often induces hypertrophy, resulting in the secretion of cytokines to recruit immune cells such as macrophages [53]. These recruited macrophages secrete pro-inflammatory cytokines thereby creating a pro-inflammatory environment [53]. The pro-inflammatory environment inhibits additional lipid disposition, increasing the mobilization of fatty acids into the circulation. The accumulation of lipids in other tissues decreases the expression of genes that regulate

mitochondrial function, activating kinases that inhibit signaling of the insulin receptor to decrease glucose mediated transport into the cell, leading to insulin resistance [53].

Free fatty acids released from visceral adipose are taken up by the liver and may contribute to increased synthesis of triglyercides and released as very low-density lipoproteins which may contribute to hyperlipidemia. Conversely, the free fatty acids released from subcutaneous adipose tissue can be oxidized by muscle, and do not contribute as much to the synthesis of triglycerides compared to visceral adipose tissue. Additionally, the release of excess fatty acids can activate components of the innate system and activate toll like receptor 4 (TLR4), stimulating macrophages to secrete inflammatory cytokines further creating a more pro-inflammatory environment [54].

The role of adipocytes and the immune cells that reside within the adipose tissue have greatly contributed to understanding the contributions of chronic inflammation and insulin resistance in obesity [55]. Tumor necrosis factor alpha (TNF-α) was one of the first cytokines found to be secreted by the adipose tissue that induced local and systemic effects and promoted insulin resistance [56]. Additional cytokines such as leptin, macrophage chemoattractant protein-1 (MCP-1), resistin, angiotensinogen, IL-6, serum amyloid A, plasminogen activator inhibitor-1 and many others have since been discovered to be secreted by adipocytes and contribute to low-grade inflammation [41, 57-61]. T cells have been implicated in regulating the pro-inflammatory state and insulin sensitivity through a variety of potential mechanisms [55, 62-65]. T cell secretion of IFNγ polarizes macrophages to an M1 phenotype, inducing the secretion of pro-inflammatory cytokines IL-6 and TNF-α, whereas T cells that secrete IL4, IL-13, and IL-10 polarize macrophages to an anti-inflammatory M2 phenotype, which secretes high levels of IL-10 [54]. Increasing this pro-inflammatory M1 macrophage phenotype can negatively affect insulin sensitivity [66, 67], and the secretion of the inflammatory cytokines from these cells contributes to the chronic, unresolved inflammation [40]. Increased adiposity additionally results in decreased invariant Natural Killer T (iNKT) cell populations [55], decreased regulatory T cell populations (Treg) [65, 68], and a disproportionate ratio of CD8+ to CD4+ T cells [64], contributing to an

increase in an activated immune population that is favorable to maintain obesity induced inflammation.

Inflammatory cytokines, lipids, endoplasmic reticulum stress, and reactive oxygen species activate intracellular signaling cascades by different mechanisms, resulting in the activation of the IKKβ/NF-κB and JNK pathways [40, 41, 54, 69, 70] in metabolic cells such as adipocytes, hepatocytes and macrophage subtypes [54]. Excess nutrient intake can dysregulate the innate immune response via pattern recognition receptors (PRR)[71]. Toll like receptors (TLRs) are a type of PRRs that are normally activated by pathogens and initiate the appropriate immune response to clear the pathogen, however, in obesity, adipocyte TLRs can be activated by lipids [19, 72]. Activation of TLRs by lipids can lead to increased expression of TNF-α, IL-6, and other inflammatory mediators [19]. IKKβ/NF-κB activation leads to the translocation of NF-κB to the nucleus, whereas JNK activates the transcription factor activator protein-1 (AP-1), and both of these transcription factors increase expression of inflammatory genes [40]. Kinases IKK and JNK also contribute to inhibition of insulin signaling by serine phosphorylation of the insulin receptor substrate 1 (IRS-1), resulting in ubiquitination and degradation, and inhibiting downstream signaling [73]. The continued production of these inflammatory mediators maintains a feed forward loop, with no complete resolution of inflammation.

Obesity and vaccination

Immunization is the best method of protection from many infectious diseases. To date, there are limited studies reporting vaccine effectiveness in obesity. The response to hepatitis B vaccine has been the most studied. Several groups have consistently reported decreased response of protective levels of antibodies to hepatitis B surface antigen in obese adults [74-76], and poor hepatitis B antibody response in obese pre-adolescents [77].

Obesity in humans and in mouse models has shown impaired immune response to influenza vaccination [78-80]. A prospective, observational study enrolled adult participants with

various BMI's to acquire serum and peripheral blood mononuclear cells (PBMCs) pre and 30 days post influenza vaccination. In addition, as this study was conducted over multiple years, some participants also had a 1 year blood sample. Influenza antibody titers measured by ELISA at 30 days post vaccination did not yield any significant differences between healthy weight, overweight, and obese groups, however, at 1-year post vaccination there was a decline in antibody titers in obese participants measured by ELISA and hemagglutination inhibition assays (HAI), the standard measure of influenza antibody titers [79]. Compared with CD4+ and CD8+ T cells from healthy weight individuals, PBMCs from obese adults stimulated with vaccine strains of influenza showed decreased activation markers and functional markers IFNγ and Granzyme B [81] .

Together these data demonstrate that compared to healthy weight adults, both humoral and cellular vaccine-induced immune responses are altered in obese adults. To circumvent the decreased effectiveness of influenza vaccination in obesity, the use of adjuvants has been explored. Lean and obese mouse models were administered H7N9 vaccine alone, or vaccine with alum or squalene adjuvant and then challenged with A/Anhui/1/2013 H7N9 virus. Obese mice were able to mount an immune response producing neutralizing and nonneutralizing antibodies, however, compared with lean mice, viral clearance was still impaired increasing morbidity and mortality [78].

The literature is even more scarce in the effectiveness of vaccination in obese children. Compared to healthy weight children, antibody titers to tetanus toxoid were significantly reduced in overweight children, ages 8-17 [82]. Inflammatory cytokine IL-6 was found be significantly increased in the overweight group, contributing to low-grade inflammation, corroborating other studies that suggest that low grade inflammation is present in overweight children [83].

Animal models of obesity

There are many types of animal models used to study obesity, including rodents, pigs, canines, and non-human primates [84]. Rodents are the most commonly used models in studying obesity, but the choice of model depends on the scope and goals of the study. Two of the primary rodent models used in obesity studies are the monogenic and diet-induced mouse models.

The monogenic mouse models have spontaneous, single gene mutations or genetically engineered mutations that results in obesity. The more commonly used monogenic models are the *ob/ob* and *db/db* mouse models [85]. The *ob/ob* mouse model contains a spontaneous, single base pair deletion in the leptin gene that inserts a premature stop codon [84, 86, 87], resulting in a loss of function mutation [85, 88]. It is important to note, however, that the genetic background of these mice will dictate the obesity phenotype. For example, *ob/ob* on the C57BL/6J background will show a phenotype with extreme obesity, hyperinsulinemia, insulin resistance and hyperglycemia, whereas, on a C57BL/KS background, *ob/ob* mice will develop hyperglycemia and diabetes, reach maximum weight at 3-4 months of age, followed by gradual weight loss then death by 6 months of age [89].

All genetic backgrounds of *ob/ob* mice will result in a leptin deficiency that induces hyperphagia and reduced energy expenditure, resulting in obesity [85]. The *db/db* mouse model, or “diabetes mouse” contains a spontaneous mutation of a G to T point mutation in the leptin receptor gene that results in leptin receptor deficiency [86, 89]. These mice have very similar phenotype to that of the *ob/ob*, however, with high levels of leptin proportional to adiposity [89]. Monogenic models offer great value in testing potential therapeutics due to the distinct phenotype [85, 88], yet polygenic models are more similar to the human obese phenotype as the most common forms of human obesity are the result of the effects of multiple genes.

The most commonly used polygenic model is the C57BL/6J mouse strain, in which feeding a high caloric diet (typically high fat) induces obesity, hyperinsulinemia, and insulin resistance [86, 88, 90]. There are different diet strategies to induce obesity, but the 45% and

60% kcal fat content of soybean oil and lard are more commonly chosen depending on the experimental design and desired phenotype outcome.

Influenza virus epidemiology

Influenza virus is a highly contagious respiratory virus. Symptoms include fever, chills, cough, sore throat, runny or stuffy nose, muscle or body aches, headache, fatigue, and possibly vomiting and diarrhea [91]. It can take up to a couple of weeks to recover from the illness, and can lead to pneumonia and exacerbations of preexisting conditions such as asthma or chronic heart disease [91]. Most healthy people that contract influenza will recover within two weeks, however, certain populations are at higher risk for developing flu complications that can lead to severe outcomes including death. Populations at higher risk include pregnant women, children 6-59 months, adults over 65 years old, and immunocompromised individuals [92].

Outbreaks and pandemics of the influenza virus have been observed for the past three hundred years [93]. In 1918-1919 the worst pandemic of influenza infection emerged, with an estimated one-third of the world's population infected [94], and estimates of 50-100 million deaths [95, 96]. From 1979-1994, the crude percent overall death rate caused by influenza and pneumonia increased 59% [97], and flu continues to remain one of the top ten leading causes of death in the United States [98]. In order to understand the methods of transmission and contribution to epidemics and pandemics, it is necessary to understand the structure of the influenza virus.

Influenza virus structure

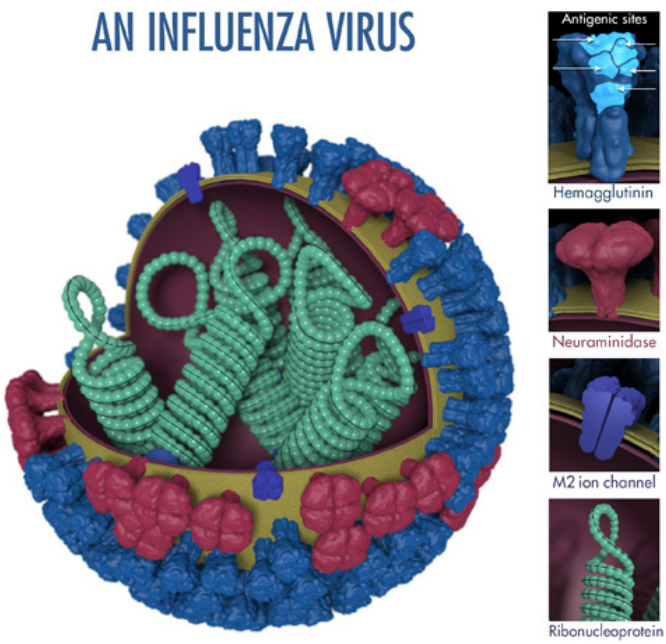


Figure 1. Structure of influenza virus.

Taken from CDC public health image library. Photo credit, illustrator Dan Higgins.

Influenza virus is an enveloped, negative stranded, segmented RNA virus that belongs to the Orthomyxoviridae family. There are four types of influenza viruses: A, B, C and D [99]. In humans, influenza viruses A and B are typically responsible for seasonal infections with strain A associated with more serious illness. Influenza C strains result in mild illness and D strains are isolated to cattle and not known to currently be transmitted to humans [99]. Influenza strains A and B contain 8 RNA segments, whereas influenza C contains 7 segments[100].

Influenza A viral proteins include hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), RNA polymerase B1 subunit (PB1), RNA polymerase B2 (PB2), RNA polymerase A (PA), nucleoprotein (NP), and non-structural protein (NS) [100]. The outer structure of the virus consists of a lipid bilayer, which is obtained from the host during virus budding from an infected host cell. The lipid membrane contains viral encoded glycoproteins HA and NA [99]. HA is the most abundant protein in the lipid bilayer and forms trimeric spikes that bind to sialic acid receptors on

host cells[101]. The HA also contains neutralizing antibody binding sites. The binding of the HA to host sialic acid receptors facilitates entry of the virus into the cell. Cells of the upper respiratory tract of humans primarily contain α2,6-linked sialic acids, with cells of the lower respiratory tract consisting of α2,3-linked sialic acids. This receptor specificity is one of the factors that contributes to zoonotic transmission of the virus, as avian influenza viruses preferentially bind to α2,3-linked sialic acids, which can be found in ducks, chickens, and migrating birds, but can still infect humans by infecting the lower respiratory tract [102, 103].

NA forms tetrameric structures that span the lipid bilayer and function as enzymes that cleave the sialic acids from the HA proteins to allow budding and release of the virion to infect neighboring cells [102]. Selected point mutations in these two proteins contribute to antigenic drift that can create influenza epidemics every 1-2 years [100]. However, when a cell is infected with more than one influenza strain and combines different HA and NA proteins to create a new subtype, a pandemic can occur [104]. The NA protein is the target of oseltamivir (Tamiflu) and zanamavir (Relenza), antiviral medications used to treat influenza infection [100].

The M1 protein is present in Influenza A viruses, and constitutes the matrix layer of the virus, that protects the viral RNAs. To release the viral RNAs upon entry into the host cells, the M2 ion channel protein pumps ions from the endosome to the interior of the virion, decreasing the pH, initiating the fusion of the inner layer of the endosome to release the viral RNAs. The release of the bound viral RNAs can then enter the nucleus and begin replication. The M2 channel is the target of adamantine family of antiviral drugs to inhibit viral replication, however, mutations in the virus result in resistance to this class of drugs [105].

Once the negative stranded viral RNAs enter the nucleus, the RNA dependent RNA polymerase complex made up of PA, PB1, and PB2 produces positive strand mRNA, to serve as templates for the transcription of viral proteins in the cytoplasm. These newly synthesized viral proteins are transported back to the nucleus and associate with NP to form viral ribonucleoproteins [102]. Other glycosylated proteins modified by the golgi apparatus and

endoplasmic reticulum are transported to the cell membrane, and when enough proteins aggregate at the plasma membrane, they bud off and form new virions.

Innate Immune response to influenza

The immune system is composed of innate and adaptive immune system of cells, tissues and organs that form a complex network to initiate an immune response to any invading pathogens, or injury. Upon infection, the innate system is activated immediately to provide the first line of host defense. The innate immune system is composed of chemical and physical barriers such as mucosal epithelium; innate immune cells such as macrophages, neutrophils, dendritic cells and natural killer cells (NK); and circulating proteins and cytokines that coordinate the interactions with immune cells and the environment to provide a constant layer of immunity [106]. The coordinated actions of innate immunity also provides support for the activation of the adaptive immune system, which is specific for the invading pathogen.

Influenza infection initiates the innate network upon initial infection. Influenza is more commonly spread in droplets when infected people cough, sneeze, or talk, but can also be transmitted when a person touches infected objects such as door knobs and then touch their nose or mouth [107]. Influenza virus is exposed to a series of physical barriers before it can infect the host cellular machinery. Tears from the eye contain surfactant proteins which can prevent influenza virus from infecting the eye [108-110]. The oral cavity is another source of entry for influenza virus, but the saliva provides another physical barrier with scavenger receptor cysteine rich glycoproteins, mucins, immunoglobulin A, and surfactant proteins that inhibit influenza infectivity [109, 111, 112]. The virus has to bypass the mucus coating the respiratory epithelium to continue to attach and infect the respiratory epithelial cells. The mucus layer contains highly glycosylated mucins containing sialic acid receptors [113], however, “mimic” sialyted structures that resemble these receptors bind influenza virus and trap them within the mucus layer to eliminate the virus and prevent infection [114-117]. When influenza virus binds to sialic acid receptors, neuraminidase sialidase activity cleaves these bound interactions to release

the virus from the mucus layer to gain entry into the respiratory epithelium [118]. Upon entry into the respiratory epithelium, innate cells such as neutrophils, dendritic cells, monocytes, macrophages and NK cells coordinate actions to limit infection and damage [101].

The innate system recognizes conserved regions of microbial agents, or pathogen associated molecular patterns (PAMPS) that are recognized by pattern recognition receptors (PRRs) on the infected cell. Influenza virus is recognized by specific PRRs: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I), and NOD-like receptors (NLRP3) [101]. Several innate immune cells work together to kill and clear the virus and any infected or dead host cells by PAMP activated signaling mechanisms. TLR3 which recognizes double stranded RNA (dsRNA) is expressed in many innate cells such as macrophages, and dendritic cells in humans and mice [119-123]. For example, when infected cells are phagocytosed by macrophages, TLR3 present in the phagosome will recognize viral dsRNA, viruses produce dsRNA during part of the infection cycle [124]. This activation induces a signaling cascade, culminating in the activation of nuclear factor κB (NF-κB) and IFN-regulatory factor 3 (IRF3), transcription factors that produce pro-IL-1β, pro-IL-18, TNF-a, IL-6, and IL-1 and other pro-inflammatory cytokines [125, 126].

TLRs 7 and 8 recognize ssRNA, and both are closely related proteins that can recognize the same ligand [127]. TLR7 is primarily expressed in innate plasmacytoid dendritic cells (pDCs) and IFN stimulated B cells [74], whereas TLR8 is expressed in macrophages and myeloid dendritic cells [128] and present in the endosomes from these innate cell types. The activation of TLR7/8 induces signaling that activates IRF7 and NF-κB pathway also producing type I interferons and pro-inflammatory cytokines [129].

RIG-I is expressed in most cells, and detects viral 5' tri-phosphate RNA to activate IRF3 and NF-κB pathways, resulting in production of type I interferons and pro-inflammatory cytokines [101]. NLRP3 is mainly expressed on neutrophils, macrophages, monocytes and conventional dendritic cells, with negligible expression on lymphocytes, eosinophils, and pDCs [130]. Infected cells such as macrophages, or cells of myeloid lineage express NLRP3 [125] . Proton flux of the viral protein M2 channel in the trans golgi network of infected cells activates the formation of a

complex of proteins making up the NLRP3 inflammasome. The formation of this complex activates caspase-1 which cleaves pro-IL-1β, pro-IL-18 into their bioactive forms [101, 125].

The end result of initiating these signaling cascades are the production of pro-inflammatory cytokines, chemokines, and secretion of type I interferons (IFNs) [125]. Type I IFNs induce expression of hundreds of IFN stimulated genes that function as anti-viral genes to protect uninfected cells, and limit infection of infected cells [101]. IL-1β is crucial to recruit innate immune cells to the site of infection [131], while IL-18 enhances production of IFNγ from NK and T cells to enhance cytotoxic activity [132, 133].

Adaptive immune response to influenza

The adaptive immune system consists of the humoral and cellular immune response that correlates with B and T cell driven immunity, respectively. In the context of influenza infection, vaccination strategies more commonly target the humoral response, to initiate the production of antibodies from antibody secreting B cells knows as plasma cells. Antibodies can bind to surface proteins of the virus to prevent the virus from infecting host cells (neutralization) and can bind to proteins of the complement system to activate opsonization and phagocytosis. Antibodies can also bind to infected cells to target the cell for antibody dependent cellular cytotoxicity carried out by innate cells [106, 134]. Dimeric, secretory immunoglobulin A (IgA) is the dominant mucosal antibody produced locally in the upper respiratory tract that offers protection by immune exclusion, intracellular neutralization, and antigen excretion [135]. Immunoglobulin G (IgG) is the most abundant circulating antibody in the serum, but is also found in the lower respiratory tract and facilitates cytotoxicity from NK cells and aids in opsonization of infected cells for clearance by alveolar macrophages [101, 134].

As mentioned previously, the innate system provides the first line of defense to influenza infection. The innate immune cells coordinate efforts to minimize damage to the host, and work with the adaptive immune system to initiate the most effective and efficient response to resolve

the infection. Alveolar macrophages reside in the alveolar lumen while dendritic cells reside in the airway epithelium and together these cells function to provide immune surveillance in the lung. Alveolar macrophages phagocytose infected cells [136], or secrete nitric oxide synthase 2 (NOS2) and TNF-α [137, 138] to limit the spread of infection. Antigen presenting cells (APC) such as dendritic cells endocytose viral components and migrate to the draining lymph nodes to present viral peptides through specialized cell surface receptors called major histocompatibility complexes (MHC), to be recognized by specific T cells. It is these interactions that bridge the coordination of the innate and adaptive immune arms to enhance a more specific and targeted immune response to influenza infection.

There are two classes of MHCs that bind to different subsets of T cells. The uptake of an extracellular virus or proteins in lysosome/endosomal vesicles are degraded by proteases, bound to MHC class II receptors, transported to the cell surface and presented to CD4+ T cells to proliferate and initiate helper functions [106]. Cytosolic viral proteins are degraded by proteasomes and transported to the endoplasmic reticulum where they bind to MHC class I receptors to be exported to the cell surface and presented to CD8+ T cells to activate them to proliferate and initiate cytotoxic functions [106]. T cells can only recognize specific amino acid sequences along with portions of the MHC molecule. In contrast, B cells can recognize peptides, proteins, nucleic acids, carbohydrates, lipids and small chemicals [106]. The presentation of viral peptides by APCs to T cells in secondary lymphoid organs initiates B and T cell signaling cascades. When there has been no previous exposure to the virus, the primary immune response is initiated that relies heavily on the innate immune response before the adaptive immune response can be activated, however, immunological memory to the infection is generated following a primary infection, allowing for a more rapid and efficient response following re-exposure.

Memory cell generation

Immunological memory is the process of the adaptive immune system that produces a more efficient and faster immune response to a previously encountered antigen. The premise for effective vaccination relies on the ability of the immune response to maintain immunological memory. Vaccination or a primary influenza infection initiates the adaptive immune system to respond in three phases: expansion, contraction, and memory cell generation and maintenance.

Naïve T cells recirculate in the blood and lymphoid tissues until recognition of cognate antigen presented by APCs in the lymph node. Naïve T cells initially enter the lymph nodes through high endothelial venules (HEV) via their homing cell surface receptors CD62L and CCR7, which bind to epithelial PNAD and SLC, respectively [139]. Upon sufficient antigen presentation and co-stimulation by APCs, CD8+ T cells proliferate and expand, acquiring antiviral effector functions such as producing IFNγ, TNFα, and upregulating expression of perforin and granzymes [140]. CD8+ T cell activation and expansion is dependent on adequate T cell receptor (TCR) and co-stimulatory signals, but also requires IL-12 and IFN-α to generate effector and memory CD8 T cells [141].

CD4+ T cell activation and expansion is dependent on the cytokine environment. Following influenza infection, Th1 subset of CD4+ T cells produces IFNγ, TNFα, and IL-2 along with T follicular helper cells (Tfh) and Th2 helper subsets that promote antibody production through B cell interactions [142]. Effector T cells clonally expand and proliferate to generate sufficient cell numbers to fight infection. Within the naïve CD8+ T cell pool in the mouse, it is estimated that there are about 1 out of 10^5 T cells for a given peptide/MHA complex, and once activated, these can expand 10^4 to 10^5 fold [143]. The CD8+ population is usually much lower than the CD4+ T cell population in the mouse, but it can expand disproportionately, at least 5-fold when exposed to certain viruses, such as lymphocytic choriomeningitis virus (LCMV) [144]. Other murine studies have reported an estimated 15 to 1000 antigen specific CD8+ T cells [145-148], and 10 to 200 CD4+ antigen specific cells for a given MHC/peptide epitope combination, with the ability to respond to 10^6-10^7 peptides[141].

Activation and expansion induced by antigen stimulation differentiates naïve T cells into effector CD4+ or CD8+ T cells. Effector cell expansion peaks at about 7 days post infection, followed by T cell contraction (90% of effector cells removed by apoptosis) and resolution of the viral infection. The cells that do not undergo apoptosis differentiate into long-lived antigen-specific memory T cells. As naïve T cells differentiate into effector cells, changes in chromatin structure and expression of transcription factors occurs in addition to changes in the expression of cell surface receptors [149].

The memory pool created in response to initial primary exposure to a specific pathogen consists of a heterogenous population of effector memory T cells (T_{EM}), central memory T cells (T_{CM}) [150], and tissue resident memory T cells (T_{RM}) [151]. T_{EM} and T_{CM} have been identified based on the expression of CD62L and CCR7 cell surface receptors. T_{EM} are primarily found in peripheral tissues coinciding with effector functional properties of cytotoxicity and inflammatory reactions where T_{CM} are primarily found in lymphoid tissues and display more helper functions to stimulate dendritic cells, help B cells and rapidly proliferate into effector cells following antigen stimulation [150]. Additionally, the high expression of IL-7Rα chain (CD127) identifies memory T cells [152, 153].

T_{EM} and T_{CM} are circulating T cell populations throughout peripheral and secondary lymphoid tissues, but another memory population, the tissue resident memory T cells (T_{RM}) are a non-circulating memory population residing in peripheral tissues with different cell surface markers than T_{EM} and $T_{CM.}$ In mouse models of influenza infection, T_{RM} can be found in the lungs, with CD4+ T_{RM} expressing CD69 and CD11a [151, 154], and CD8+ T_{RM} expressing CD69 and CD103 cell surface molecules [155]. These cells display specific homing and retention properties: polyclonal influenza specific CD4+ T_{RM} derived from lung and placed in another recipient will migrate back to the lung [151].

Memory cell metabolism

T cell metabolism is a dynamic process, with metabolic needs changing with the environment and stress signals. Before an encounter with antigen, naïve T cells are smaller in size, and maintain survival and homeostasis through T cell receptor (TCR) and IL-7 receptor signaling, which regulate expression of the glucose transporter, *Slc2a1* [156]. Glut1 is the dominant glucose transporter expressed in T cells [157], with increased Glut1 expression leading to increased glucose uptake. Changing metabolic needs in response to pathogen exposure are indicative of function. Naïve T cells circulate in secondary lymphoid tissues maintaining low levels of glycolysis, obtaining energy needs through fatty acid oxidation (FAO) and oxidation of pyruvate and glutamine through oxidative phosphorylation (OXPHOS) [73, 157]. These energy needs change during recognition of cognate antigen for the transition from naïve T cell to an activated effector T cell that proliferates and acquires effector functions to clear the pathogen.

Effector T cells utilize anabolic metabolism to produce lipids, nucleic acids, and proteins to support rapid proliferation of daughter cells and acquire effector functions to clear out the infection switching from a preference for OXPHOS to aerobic glycolysis and glutaminolysis [73]. Aerobic glycolysis was initially proposed by Otto Warburg to describe the unique metabolic needs of cancer cells and their metabolism of glucose metabolized to lactate despite presence of sufficient oxygen [158]. This Warburg effect has also been used to describe the metabolism of activated T cells [159]. After the pathogen has been cleared, the majority of effector T cells undergo apoptosis to yield a small population of long-lived memory T cells.

Memory T cells have their own distinct metabolic profile. While they have similar metabolic needs to naïve T cells, memory T cells rely on FAO and OXPHOS [160], requiring tumor necrosis factor receptor-associated factor 6 (TRAF6) [161], and IL-15 [162] to maintain long lived memory CD8+ T cells. Mice with a T cell specific deletion of TRAF6 were found to have impaired CD8+ T cell memory generation to antigen stimulation, however, metformin restored AMPK (5’ adenosine monophosphate-activated protein kinase) activation to promote FAO and memory T cell generation [161]. Rapamycin, the inhibitor of the intracellular kinase mammalian

target of rapamycin (mTOR) which regulates cell growth and metabolism, also regulates CD8+ T cell memory generation [163].

Unlike naïve T cells, memory T cells need to be metabolically primed to initiate a faster immune response upon re-encounter with antigen. IL-15 promotes mitochondrial biogenesis and increases the expression of carnitine palmitoyltransferase 1a (CPT1a) which transports long-chain fatty acids into the mitochondria to be used for FAO [162]. Enhanced mitochondrial biogenesis and increased CPT1a expression contribute to the spare respiratory capacity (SRC) of memory T cells [164], where SRC is the difference of the ATP produced at the basal level by oxidative phosphorylation compared to maximal potential [165]. Van der Windt, et al., proposed a model how SRC regulates memory T cell survival in infection, where increased mitochondrial mass increased use of fatty acids for OXPHOS, inducing memory cells to quickly switch their metabolism for pathogen clearance [162]. In contrast to effector T cells, memory T cells do not use stored extracellular fatty acids nor do they contain lipid droplets, but rather to fuel FAO, rely on extracellular glucose to synthesize triacylglycerol (TAG), utilizing lysosomal acid lipase (LAL) to release fatty acids to fuel FAO [166].

In sum, naïve, effector, and memory T cells have very different metabolic needs. Memory T cells rely on extracellular glucose for TAG synthesis and cell intrinsic lipolysis generate fatty acids to fuel FAO for OXPHOS [166]. Likewise, mitochondrial function and biogenesis are important for memory T cell development, with memory T cells having more mitochondrial mass than naïve cells [167]. Increased mitochondrial mass enables memory cells to have faster recall when re-stimulated with antigen. It is also speculated that this increase in mitochondrial mass driven by IL-15 is key to memory T cell survival and longevity [162]. In contrast, CD8+ T cells and CD4+ Th1 effector T cells express high surface levels of Glut1 and are highly glycolytic, while inducible Tregs favor fatty acid oxidation like that of memory T cells [168]. The development and metabolism of long-lived memory T cell generation and maintenance is still not fully understood. Because FAO and OXPHOS are the driving metabolism of memory T cell generation and

maintenance, it is important to investigate whether nutrient conditions of high serum glucose and fatty acids present with obesity dysregulate memory T cell metabolism.

Obesity effects on the immune response to influenza infection

Studies in obese mice infected with influenza virus demonstrate impaired innate and adaptive immune defenses resulting in increased morbidity and mortality [78, 169-173]. Impairments in the primary immune response were first demonstrated by Smith, et al., by inducing obesity in C57BL/6J mice with diet and infecting them with a mouse adapted strain of influenza virus A/Puerto Rico/8/34 (PR8). The mortality rate of obese mice to PR8 was 6.6 fold greater than the lean mice, and was associated with reduced NK cytotoxicity. At day 3 post infection, obese mice had reduced mRNA expression of anti-viral cytokines IFNα and IFNβ in the infected lung [169]. Lung cytokines TNF-α, IL-6, and IL-1β, normally produced during the inflammatory response to influenza, were decreased in obese mice compared to lean mice at day 3, along with chemokines MCP-1 and RANTES, which function in immune cell recruitment. In addition, obese PR8 infected mice have delayed mononuclear infiltration in the lung and decreased dendritic cell numbers [173]. Dendritic cells from infected obese mice displayed impaired function, as they were not able to properly stimulate CD8+ T cells to produce IFNγ [173]. Together these data suggest that obesity results in decreased immune response to a primary infection with PR8 influenza virus, resulting in increased mortality.

Based on the findings that obesity impairs the primary immune response, Karlsson and colleagues utilized DIO mouse models to investigate whether the memory T cell response to secondary influenza infection was impaired [170]. Lean and diet induced-obese mice were infected with X-31, a mouse adapted H3N2 influenza strain, and 4 weeks later infected with PR8. Although lean mice were fully protected from the secondary infection, obese mice had a 25% mortality rate. Lungs from influenza infected obese mice had low expression of antiviral cytokines IFNα and IFNβ [170]. In addition, obese mice had dysregulated lung cytokine and chemokine

expression and reduced influenza specific CD8+ T cells expressing IFNγ. Memory T cells had decreased IFNγ response to antigen presentation by DCs [170]. This set of experiments provided evidence that the memory response to secondary influenza infections was also impaired.

DIO also affects the maintenance of T_{EM} and T_{CM} populations. T_{CM} were maintained and remained constant with a slight increase in the obese group 84 days post infection in the lungs and spleen. However, compared to lean mice, T_{EM} cell number and percent in the lungs decreased in the obese mice at 84 days post infection [171]. Cytokines specific to memory homeostasis and survival had dysregulated expression in obese mice compared to lean mice, and inflammatory cytokines TNF-α and IL-6 were significantly increased in the lungs of obese mice 84 days post infection, despite the clearance of virus from the lungs [171]. The inability of obese mice to maintain influenza specific memory T cells suggest that impairments contribute to ineffective immune responses to subsequent exposures of influenza virus.

These findings of impaired memory T cell responses and maintenance of memory T cell populations led to further studies to determine the mechanisms contributing to these results. The 2009 influenza pandemic revealed one caveat that shed light on potential mechanisms of influenza immunity. Prior exposure to influenza virus can generate cross protective immunity where T cells recognize the internal conserved regions of the influenza A virus and can recognize and aid in recovery and decrease morbidity to influenza A strains with differing external cell surface proteins [174-178].

DIO mice were infected with PR8 influenza virus, and then 5 weeks later re-challenged with pH1N1 influenza virus. All lean mice had a 100% survival rate, whereas 95% of the obese mice survived secondary infection to pH1N1 [172]. Hemagluttination inhibition (HAI) and microneutralization assays were used to determine correlates of protection to influenza A virus [179-182], and are a standard measure to determine levels of cross reactive antibodies to influenza strains. Prior to rechallenge with pH1N1, PR8 infected lean mice had high HAI titers to PR8, where HAI titers were not detectable in obese mice, and as expected, neither group had no HAI titers to pH1N1 [172]. Microneutralization assays demonstrated similar results, with lean

mice having significantly higher microneutralization titers [172]. Furthermore, obese mice present a delayed response of antibody titer, at day 7 were significantly decreased compared to lean mice, but at 35 days post infection obese mice had no detectable levels demonstrating the inability to maintain antibody titers [172]. Nucleoprotein specific antibody titers were measured to determine cross reactive neutralization to pH1N1 infection, and obese mice has significantly lower levels at 5 weeks post infection, increased viral titer, and increased lung pathology [172]. Lungs of obese mice also had increased immune cell infiltration, and increased levels of albumin from bronchoalveolar lavage fluid (BAL), indicating damage to the lung epithelium. Evaluation of regulatory T cell populations (CD25+Foxp3+) demonstrated that obese mice had increased numbers in the lung, but these populations were less suppressive, indicating impaired Treg function [172]. Overall, these data suggest that obesity alters the cross protective response to pH1N1 influenza infection in mice which had not previously been demonstrated.

These experiments established that obesity impairs the humoral and cell mediated immune response to influenza infection. Diet-induced obese mouse models were used, but it was unclear whether obesity was driving altered immune responses, or whether it was diet related factors associated with feeding a high fat diet. Further experiments investigated mechanisms to provide evidence that obesity, and not diet, is driving the altered immune responses to influenza infection.

Chow diet (CD), 10% low fat diet (LFD), and 60% high fat diet (HFD) fed mice were maintained on respective diets for 14-16 weeks, and infected with influenza A/California/04/2009 virus (pH1N1). While the CD group did not have any deaths to infection, LFD and HFD had greater mortality to infection [183]. This was of particular importance as LFD had not been previously demonstrated to have increased mortality to pH1N1 infection compared to CD. There were no differences between the three groups in viral titer, or infiltrating immune cells to the lung, but HFD did have increased lung epithelial damage compared to CD and LFD. HFD decreased regulatory T cells (Treg) and macrophages in the bronchoalveolar lavage fluid compared to CD diet. Lung tissue was harvested and metabolically profiled to determine metabolite differences

between the three groups prior to and after pH1N1 influenza infection. CD and HFD had significantly different lung metabolic profiles, with HFD having altered lipid metabolites prior to infection and post infection. This suggests that influenza infection alters the lung metabolome in obesity. LFD consistently had intermediate results between CD and HFD, so to confirm that obesity is driving these differences and not diet, a genetic model of obesity was used to compare findings with HFD.

As previously described, the leptin receptor knock out mouse, *db/db*, is severely obese due to hyperphagia. However, because leptin receptors are present on immune cells, this model is not appropriate for obese immune studies. Therefore, a mouse model of leptin receptor deficiency only in the hypothalamus was used. Heterozygous breeding between $LepR^{H\ fl/fl}$ and $LepR^{H+/-}$ mice resulted in homozygous $LepR^{H-/-}$ that lack leptin receptor signaling in the hypothalamic neurons, leading to hyperphagia induced obesity [184]. These mice were fed CD, so their obesity was not induced by a high fat diet, but rather the same diet fed to lean, control mice. $LepR^{H-/-}$ mice gained significantly more weight than the $LepR^{H\ fl/fl}$ and $LepR^{H+/-}$ mice, and were more susceptible to pH1N1 influenza infection by increased mortality [183]. Viral titers between the three groups did not differ, however, $LepR^{H-/-}$ mice had significantly fewer inflammatory cells in the BAL, but increased total protein indicating epithelial damage 8 days post infection [183]. Lung immune cell populations were enumerated with $LepR^{H-/}$ mice having significantly decreased CD4+, CD8+, Tregs, activated Tregs, and macrophages at 8 days post infection [183]. Lung tissue at day 0 and 8 post pH1N1 infection was harvested and metabolically profiled for $LepR^{H\ fl/f}$ and $LepR^{H-/}$ mice by global liquid chromatography-mass spectrometry. Metabolic data demonstrated that metabolites were different in $LepR^{H-/}$ mice between day 0 and day 8 post infection suggesting that infection results in metabolic changes distinct from obesity alone. In addition, there were differences in metabolic profiles between $LepR^{H\ fl/f}$ and $LepR^{H-/}$ in nucleotide, fatty acid, and amino acid metabolism [183]. Urine from these mice at day 2 post pH1N1 infection was also metabolically profiled to determine pathways that are affected by influenza infection in the obese state. Similar to the lung metabolic profiling, differences in nucleotide, fatty acid, and amino acid pathways were altered. Thus, all of the results indicate that

obesity, not diet, impairs the immune response to influenza infection. Furthermore, the lung and urine metabolome of obese and lean influenza infected mice was different, suggesting that obesity and infection together alter the metabolism of the infected animals.

Taken together, it's clear that obesity is a global public health problem that contributes to a constellation of metabolic diseases that result in a decline in health. Obesity often results in metabolic syndrome and is an independent risk factor for increased morbidity and mortality from influenza infection. As fundamentally a metabolic disease, it is possible that obesity can alter immune cell metabolism, resulting in dysfunction. Furthermore, although a number of studies have documented impaired immunity to influenza infection and vaccination, what is lacking is a mechanistic explanation for these findings, and whether or not weight loss can restore immune cell metabolism and function.

CHAPTER III: OBESITY IMPAIRS T CELL METABOLISM AND FUNCTION, WHICH IS NOT CORRECTED BY WEIGHT LOSS

INTRODUCTION

The 2009 H1N1 influenza pandemic shed light on obese adults as a vulnerable population at risk for complications from infection with influenza. For the first time, obesity was recognized as an independent risk factor for increased morbidity and mortality to influenza infection [14]. This is concerning, as the rates of obesity have risen dramatically over the last several decades [1] and for many years, seasonal influenza infection and pneumonia have remained in the top ten leading causes of death in the United States [97, 98]. Currently, 36% of adults in the US and 10% of men and 14% of women worldwide are obese [2].

Diet-induced obese mouse models and human studies provided evidence that both the innate and adaptive immune responses were impaired following infection with influenza [79, 81, 169-173, 183, 185, 186]. Dendritic cells from obese mice fail to present antigen to CD8+ T cells in a primary influenza infection [173], and natural killer cells from obese mice display impaired cytotoxicity [169]. Obese mice have increased mortality to primary [169] and secondary [170] influenza infections, impaired maintenance of influenza-specific memory T cells [171], and decreased memory T cell function [170]. Compared with healthy weight adults, influenza stimulated-peripheral blood mononuclear cells (PBMCs) from both overweight and obese adults vaccinated with influenza trivalent inactivated influenza vaccine (TIV) have decreased CD4+ and CD8+ activation and functional markers [79, 81]. Together, these data demonstrate that obesity impairs the immune response to influenza infection and vaccination.

T cell metabolism is a dynamic process that changes to meet the energetic demands of the cell. As T cells become activated, metabolic demands switch from resting, naïve or memory T cells to effector T cells to fight infection [157]. Naïve T cells at rest utilize oxidative phosphorylation for metabolic needs to maintain homeostasis and immune surveillance, but upon activation, switch to glycolysis and glutaminolysis for rapid biosynthetic precursors for growth, proliferation and effector functions [73]. The conversion of effector T cells to long lived memory T cells requires fatty acid oxidation to sustain function [161, 162]. Although we and others have demonstrated impaired T cell function in the context of obesity, we do not know if obesity will impair T cell metabolism, thereby altering T cell function.

Obesity results in systemic alterations in metabolism, including insulin resistance, elevated glucose levels, and altered adipokines (e.g. increased leptin, decreased adiponectin), leading to what has classically been termed “metabolic syndrome” [15, 187]. At the cellular level, metabolic syndrome has traditionally been associated with adipocyte, hepatocyte, muscle and pancreatic beta cell impairments, particularly with respect to glucose usage and fatty acid oxidation and storage. However, the effect of obesity on T cell metabolism has not been addressed. Because it is well-understood that T cell metabolism drives T cell function, we hypothesized that the altered metabolic environment of obesity would impair T cell metabolism, leading to memory T cell dysfunction.

Although we and others have shown that obesity impairs memory T cell function, would weight lose restore their metabolism and therefore their function? Furthermore, if memory T cells were generated in a lean state, would these memory T cells now fail to function following weight gain? Our study addresses this question by infecting diet-induced lean and obese mice with influenza virus to generate memory T cells, followed by diet switching to induce either weight loss or weight gain. Once weight change was established (lean becoming obese and obese becoming lean), mice were reinfected with influenza.

Our results demonstrated that compared to always lean mice, always obese mice had impaired T cell memory subset generation, function and metabolism. Furthermore, we found that despite weight loss, memory T cell populations were decreased at re-infection, and effector CD8+ T cell functions were impaired at 3 days post infection. Of note, the timing of memory T cell generation appears to program the T cell metabolism with regard to the obese state. We found that the T cell metabolism of the weight loss group (formally obese) closely mirrored the metabolism of the always obese group, and conversely, the T cell metabolism of the weight gain group (formally lean), closely paralleled the always lean group. These results suggest that memory T cell metabolism is "set" by the metabolic conditions present during their generation, and that changing their current environment (lean to obese or obese to lean) could not overcome this reprogramming.

Taken together, our findings suggest that T cell metabolism and function are impacted by the metabolic environment, both at the time of generation and at the time the memory cells are activated.

RESULTS

Development of a model to study the effects of weight loss and weight gain on memory T cells

A number of studies, including our own, have documented the finding that obesity impairs both the primary and memory T cell response to infection with influenza virus [79-81, 170-173, 183, 185]. However, all of these studies were carried out in the obese state. The generation of the memory response and the functional response of the memory T cells occurred in mice that were always obese or always lean during both the primary and secondary infection. What we don't know is whether weight loss will restore memory T cell function, and conversely, whether obesity will impair T cell memory generated in a lean state. To address these questions, we utilized a

well-established mouse model for both influenza infection and obesity studies [84, 86, 188]. Male 6-week old C57BL/6J mice were randomly assigned to 2 groups: lean and obese (Figure 1A). The lean group was placed on a standard chow diet (n=60) and the obese group was placed on 60% high fat diet (HFD; n=60) for 18 weeks. As expected, mice fed the 60% HFD gained significantly more weight than chow fed mice (Figure 1B).

Following 18 weeks on their respective diets, mice were infected with influenza X-31,an H3N2 influenza virus containing the external surface proteins of A/Aichi/2/68 and the internal proteins of H1N1 A/Puerto Rico/8/34 (PR8) [189]. Following the primary infection, mice were kept on their respective diets for 4 weeks, allowing T cell memory to develop while mice were either lean or obese. Following the 4 week period of memory development, half of the mice from each diet group were switched to the opposing diet. Thus, 30 lean mice were switched to HFD, and 30 lean mice remained on the chow diet. Similarly, for obese mice, half (30 mice) were switched to chow diet and half remained on the HFD. This created four groups of mice: 1) always lean; 2) weight gain; 3) always obese and 4) weight loss (Figure 1A). Mice were maintained on the indicated diets for 18 weeks. As shown in Figure 1B, obese mice switched to the chow diet (weight loss group) lost significant amounts of weight, and, compared with the always obese group, were now significantly separated by weight. Conversely, the lean mice switched to HFD gained significant amounts of weight, and were now significantly separated from the always lean group. Thus, the diet switch protocol induced both weight loss and weight gain in the mice.

In addition to body weight, obesity is often associated with hyperglycemia. As shown in Figure 1C, fasting serum glucose levels of always lean and weight loss groups were statistically lower than the always obese and weight gain groups. Thus, mice that lost weight developed a metabolic phenotype similar to the always lean mice, and mice that gained weight developed a metabolic phenotype similar to the always obese mice in terms of glycemia.

Following 15 weeks post diet switch, mice were infected with PR8. All groups lost weight following PR8 infection (Fig 1D and E). At 0 (uninfected control), 3 and 7 days post infection, lung inflammation (Fig S1A), lung viral titers (S1B) and total protein levels of bronchoalveolar

lavage (BAL) fluid (Fig S1C) did not differ among the diet groups. Weight gain, obese, and weight loss statistically decreased titers from day 3 to day 7 post infection, while the lean group did not decrease. This was not surprising, as we purposely chose the secondary PR8 dose to induce a milder secondary infection. Previous studies in our lab have induced a significant difference in lung pathology between lean and obese mice by increasing the viral load, but this also resulted in significant mortality in obese mice [170-172, 183].

Memory T cell populations were impaired despite weight loss.

Resolution of primary influenza infection results in ~90-95% of effector T cell death, with the remaining T cells acquiring a long-lived memory phenotype [190]. These long-lived memory T cells are a heterogeneous population, consisting of central memory T cells (T_{CM}), effector memory T cells (T_{EM}) [150, 191, 192], and tissue resident memory T cells (T_{RM}) [193, 194]. All of these populations are important for prevention and recovery from infection. Circulating T_{CM} and T_{EM} differ by anatomical location: T_{CM} are abundant in secondary lymphoid organs, whereas T_{EM} are primarily found in peripheral tissues [150, 195, 196]. Following influenza infection, T_{RM} reside for extended periods of time within the lung parenchyma and are critical for protection from influenza re-infection [151, 154, 197].

Using flow cytometry, we identified CD4+ and CD8+ T cell subsets in the lungs of mice at 0, 3 and 7 days post infection in always lean, always obese and weight loss groups. Although the weight loss group generated memory T cell populations in an obese state, they were challenged with re-infection following weight loss and a return to the lean state. Although we found no differences in CD4+ T_{EM} comparing all groups at day 3, and at day 7 post infection (data not shown), mice from the always lean group increased percentage of CD4+ T_{EM} from day 3 to day 7 (Figure 2A). No such increases were seen in the always obese group nor in the weight loss group. We found no differences with CD4+ T_{CM}, either among groups or between days post infection (data not shown).

For CD4+ T_{RM} cells, we found that, compared to always lean mice, always obese mice had significantly fewer CD4+ T_{RM} in the lungs at day 3 post infection (Figure 2B,2C). Although the weight loss groups also had decreased numbers of T_{RM} in the lung compared with the always lean group, this group was not significantly different from either always lean or always obese group. Influenza-specific CD4+ T cells were measured using a Class II influenza tetramer, and found day 3 post infection, compared with always lean mice, obese and weight loss groups had significantly fewer influenza-specific CD4+ T cells in their lungs (Figure 2D,E).

T regulatory cells (Tregs) are also important to reduce the inflammatory response once the infection has been cleared [198]. We found that, compared with always lean mice, at days 0 prior to infection, CD4+ Treg populations in weight gain, obese and weight loss were significantly reduced (Figure 2F). At day 3 post infection obese and weight loss remained significantly reduced compared to the always lean mice (Figure 3G). However, at day 7, compared with lean mice, Treg populations were significantly increased in the always obese group (Figure 1H, 1I).

Next, we examined CD8+ T cells subsets as they have different functions that CD4+ helper T cells. Although no differences were seen in CD8+ T_{EM} populations among groups at any time, the weight loss group was the only group which did not have a significant increase in this population between day 3 and day 7 post infection (Figure 2J). For CD8+ T_{CM} cells, at day 3 post infection, compared with always lean mice, the always obese mice had a significant decrease (Figure 2K,L). At day 3 post infection, CD8+ T_{RM} cells, compared with always lean, were significantly lower in the always obese group (Figure 2M). At day 7 post infection, the always lean mice had a significantly higher increase in CD8+ T_{RM} populations compared with always obese and weight loss groups. (Figure 2N). When we examined expression of IFNγ and granzyme B as functional markers of T cell activation and function, at day 3 post infection, compared with always lean mice, CD8+ T cells expressing IFNγ and Granzyme B were significantly decreased in both weight loss and always obese groups (Figure 2P,Q). Next, we investigated whether weight gain, after generation of the memory T cell populations, would also affect these T cell populations.

Memory T cell populations generated in a lean state were impaired following weight gain.

Memory T cell populations were also impaired in the weight gain group. Although, compared with lean mice, numbers of CD4+ and CD8+ central and T_{EM} cells from the weight gain mice did not differ at any time point, at day 3 post infection, there were fewer CD8+ T cells expressing IFNγ and granzyme B (Fig 2P). At day 7 post infection, compared with lean mice, there were fewer CD8 T_{RM} in the lungs of the weight gain mice (Fig 2N). CD4+ Tregs, compared with lean mice, were also decreased in weight gain mice prior to infection (Fig 2F), but increased at day 7 (Fig 2H).

Metabolic phenotype at time of primary infection sets CD4+ and CD8+ T cell metabolism for subsequent infection, despite weight gain or weight loss.

Using extracellular flux analysis, the metabolism of CD4+ and CD8+ T cells isolated from spleens at day 0 (uninfected) (n=5) and day 7 post infection (n=5) was measured in all groups (n=40). For CD4+ T cells, prior to secondary infection (day 0), compared with always lean and weight gain mice, always obese mice had a higher oxygen consumption rate (OCR), a measure of oxidative phosphorylation (Fig 3A). This suggests that the basal OCR rate was set for the memory T cells at the time of primary infection, as there were no differences between the always lean and weight gain mice, which were lean when the memory T cells were generated.

This finding was even more pronounced following a secondary infection. At 7 days post infection, OCR was significantly lower in the always lean and weight gain mice compared with the always obese and weight loss mice (Fig 3B). The increase in OCR from day 0 to day 7 was highest in the weight loss group, which was obese at the time of primary infection, and in the always obese group (Fig 3C). The extracellular acidification rate (ECAR) was also determined, as a measurement of glycolytic activity (Supplementary 3A,B), and was used to calculate the OCR:ECAR ratio, which determines the preference for oxidative phosphorylation vs glycolysis (Fig 3D). Although the ratios among groups were not different at day 0 (Supplementary 3D), we

found that 7 days post secondary infection, compared to always lean and weight gain mice, the always obese and weight loss mice had significantly higher OCR:ECAR ratios. Thus, the metabolism of the CD4+ T cells was set during the metabolic state of the primary infection, which was not corrected by weight loss. Furthermore, the memory CD4 T cells generated in the lean state maintained a lean, metabolic phenotype, despite the secondary infection occurring in the obese state (weight gain mice).

Similar impairments in metabolism were found for CD8+ T cells. Prior to secondary infection, OCR was not different among the groups (Fig 3E). However, following secondary infection, compared with the always lean mice, CD8+ T cells from the always obese mice and weight loss mice had significantly elevated OCR (Fig 3F). As for CD4+ T cells, this finding suggests that the metabolism of CD8+ T cells was set at the time the primary infection occurred, resulting in similar OCR levels between always lean and weight gain mice (formally lean) and between always obese and weight loss (formally obese) mice. Again, similar to the CD4+ T cells, the highest increases in OCR from day 0 to day 7 occurred in the always obese and weight loss mice (Fig 3G). OCR:ECAR ratio was also significantly higher in the always obese and weight loss mice compared with the always lean and weight gain mice (Fig 3H).

Spare respiratory capacity impaired by obesity

The ability of the T cell to produce energy under activation conditions is a function of the mitochondrial capacity available for the increased workload. This is considered to be the spare respiratory capacity (or “extra” capacity) available to the mitochondria under stressed conditions. Although there were some differences in CD4+ and CD8+ T cells prior to the secondary infection (FigS3I and S3J), at seven days post infection, CD4+ T cells from the lean mice had higher SRC compared with weight loss mice. In addition, the weight gain mice had higher SRC compared with the weight loss mice (Fig 3I). For CD8+ T cells, the findings were even more striking, with always lean and weight gain mice demonstrating significantly higher SRC compared with obese and weight loss mice (Fig 3J). Thus, the always lean and weight gain (formerly lean) mice tracked

together and the always obese and weight loss (formerly obese) also tracked together, suggesting that the metabolic environment at the time of primary infection sets the metabolism that remains during the secondary infection.

Glut1 expression on CD4+ and CD8+ effector memory T cells is altered in mice that were obese at any time

Glut1 is the dominant glucose transporter in T cells that traffics to the cell surface upon activation to increase glucose influx and glycolytic activity [199]. When naïve or memory cells are activated and transition to effector T cells, increased glycolysis occurs, which is supported by the upregulation of Glut1. At day 0 and day 7 post secondary infection, we measured Glut1 expression in spleen CD4+ and CD8+ effector T cells by flow cytometry. Prior to secondary infection (day 0), compared with always lean and always obese mice, Glut1 expression on CD4+ cells in the weight loss group were significantly decreased (Fig 4A). After infection, compared with always lean mice, Glut1 expression was decreased in always obese, weight gain and weight loss groups (Fig 4B). When comparing day 0 to day 7 post infection, there were no differences among groups (Fig 4C).

For CD8+ T cells, although there were no differences among groups at day 0 (Fig 4D), we found that, following infection, compared with always lean mice, both weight gain and weight loss groups had decreased Glut1 expression (Fig 4E). Interestingly, a significant reduction in Glut1 expression at day 7 compared with day 0 occurred in the always lean, always obese and weight gain groups. The weight loss group was not significantly different between these two time points.

Together these data suggests that obesity at any time alters expression of Glut1 on both CD4+ and CD8+ effector T cells.

Cytochrome C expression in CD4+ and CD8+ effector T cells is altered in mice that were obese at any time

Cytochrome c is an indicator of mitochondrial electron transport and therefore mitochondrial function. Although we did not detect any differences in CD4+ cytochrome c expression among groups prior to infection (Figure 4G), at day 7 post infection, compared with all other weight groups (always obese, weight gain and weight loss) always lean mice have significantly increased CD4+ cytochrome c expression (Figure 4H). Along with weight gain and weight loss mice, always lean mice had significant increase in cytochrome c expression from day 0 to day 7 (Figure 4I). For CD8+ T effector cells, similar to the CD4+ T cells, there were no differences in cytochrome c expression in any of the groups at day 0 (Figure 4J). However, at day 7 post secondary infection always lean was significantly increased compared to weight gain, always obese, and weight loss mice (Figure 4K). This finding was the result of significantly decreased CD8+ expression of cytochrome c in weight gain, weight loss and always obese groups, whereas the cytochrome c expression in always lean mice did not vary from day 0 to day 7 (Fig 4L).

DISCUSSION

Obesity continues to remain a global public health problem, contributing to an impaired immune response to influenza infection and vaccination [79-81, 170-173, 183, 200, 201]. Our lab has reported that, despite vaccination, obese adults are twice as likely to contract influenza or influenza like illness [200], likely due to impaired memory T cell function. In addition, mouse models of obesity, from our lab and others, have demonstrated a dysfunctional T cell response to both primary and secondary influenza infections. The human studies and animal models all point to impaired memory T cell generation and function as the primary explanation for the increased morbidity in mortality from influenza infection in obese humans and obese mice.

Although these studies have established immune impairment in an obese state [79-81, 170-173, 183, 200, 201], they have failed to determine if weight loss can restore the immune response to influenza infection. Understanding these mechanisms is key to developing strategies to restore immunity. Several studies have shown that weight loss through intervention or surgical means restores metabolic health, decreasing insulin resistance, onset of type II diabetes and impaired glucose intolerance, and decreased hypertension in adults [21-27]. Because these studies reported metabolic improvements with weight loss, we hypothesized that weight loss would also improve memory T cell metabolism and function. Surprisingly, however, we found that weight loss does not fully restore the memory T cell response nor metabolism upon re-infection.

In addition to weight loss, we also investigated the effect of weight gain on memory T cells generated in a lean state. We hypothesized that lean memory T cells would be able to function in an obese environment. However, we found that obesity at the time of re-challenge negatively impacted the generation and function of both CD4 and CD8+ memory T cells. Together, these findings indicate that obesity at any time during a primary or secondary influenza infection significantly impairs memory T cells.

For a memory T cell to be effective in participation against reinfection, it must be present at the site of the secondary infection. Following influenza infection, long-lived influenza-specific CD4+ and $CD8^+$ memory cells can be found in the lung airways, spleen, lymphatic system, liver and circulation [202]. In addition to anatomical location, subsets of memory T cells can be distinguished by phenotype and function. Central memory T cells (T_{CM}) are found in secondary lymphoid organs and express CD62L and CCR7. Effector memory T cells (T_{EM}) lack the expression of CD62L and CCR7 and have a wider distribution in the peripheral organs and tissues [150, 203, 204].

In addition to T_{CM} and T_{EM} populations, primary influenza infection generates a unique population of resident memory (T_{RM}) T cells that persist in both the airways and parenchyma of the lungs following the resolution of the infection [205-207] Unlike T_{CM} and T_{EM}, T_{RM} do not recirculate throughout the body and they provide a high degree high degree of heterosubtypic immunity [177].

Specific to the lung airway environment, $CD8^+$ T_{RM} cells in the airway downregulate the T cell migration factor LFA-1 (CD11a) which is thought to trap them in the lung and prevent them from reentering circulation[208, 209]. T_{RM} cells help to keep influenza reinfection from spreading to the lower airways and production of IFNγ by these cells promotes lung cell antiviral mechanisms and induces production of chemokines to recruit other immune cells. Lung T_{RM} , which are generally marked by high CD103 expression, have been shown to be an important for greatly increasing the protective capacity of influenza vaccination in mice given live attenuated influenza vaccine (LAIV)[210]. Indeed, they have been proposed to be responsible for viral clearance and protection from morbidity from influenza infection following vaccination.

When examining memory T cell subsets in the lungs of mice reinfected with influenza virus, we found that obesity at any time could reduce both their numbers and their function. Although, compared with always lean mice, weight loss and weight gain mice did not significantly differ in CD4+ or CD8+ T_{CM} and T_{EM} populations, always obese mice did have significantly lower CD8 T_{CM}. This was interesting, as the formation of CD8+ T_{CM} cell populations can be driven by decreased activation of the metabolic pathway mTOR in acute viral infections [161, 163]. It is possible that the impaired metabolism of the obese phenotype failed to properly decrease activation of the mTORC1 pathway, leading to reduced generation of CD8+ T_{CM}.

In addition to T_{CM} and T_{EM} populations, we also identified T_{RM} populations in the lungs from mice following a secondary influenza infection. We did not detect any significant differences in this population in our four weight groups at day 0 (uninfected), but at day 3 post PR8 infection, we found that numbers of CD4+ T_{RM} from always lean mice were significantly higher than the always obese group. Numbers of CD4+ T_{RM} in the weight loss and weight gain groups were intermediate between always lean and always obese mice. Our previous studies have demonstrated significantly increased morbidity and mortality in obese mice following a secondary influenza infection, and decreased lung CD4+ T_{RM} in obese mice have contributed to this outcome.

Larger numbers of CD8+ T_{RM} were found in the always lean group compared with the always obese group at days 0 and 3 post re-infection. By day 7 post infection, CD8+ T_{RM} cells were significantly higher in the lungs of the always lean mice compared with weight loss, weight gain and always obese mice. These results suggest that obesity at any time impairs the either the generation of CD8+ T_{RM} memory T cells in the lung.

In addition to finding decreased populations of memory T cells in the lungs of mice obese at any time, we also examined the expression of the functional markers INFγ and granzyme B. Compared with always lean mice, at day 3 post infection, weight loss (formerly obese) and always obese mice had significantly lower IFNγ and granzyme B expressing T cells. Thus, weight loss did not restore function in CD8+ memory T cells to re-infection with influenza infection, suggesting that memory T cells generated in an obese state retained an obese phenotype, despite weight loss. These results suggest that the epigenome of the memory T cells generated in an obese environment was altered and carried into the lean environment.

Although the exact requirements to establish and maintain memory T cells is not completely understood, it is becoming increasingly clear that chromatin remodeling programs immune response genes toward a primed state, prior to terminal differentiation, and is associated with histone acetylation and DNA methylation[211, 212]. Komori et al. [213] demonstrated that a lower level of DNA methylation in memory T cells was associated with a higher level of induction upon stimulation, attributed to loss of repressor binding. Thus, changes in methylation status would be expected to affect memory T cell generation and function. Mitchell *et al*. [214] compared methylation patterns of naïve CD4+ T cells with memory CD4+ T cells and identified specific molecular changes in methylation involved in the transition from naïve to resting memory CD4 T cells. This finding has also been observed by others[215]. Recently, Rodriquez et al., [216] used whole genome methylation profiling on CD8+ T cell subsets (naïve, resting effector memory (EM) and terminally differentiated EM (TEMRA) cells isolated from healthy adults. Based on methylation patterns by using principal component analysis, they found distinct populations between naïve cells

and EM and TEMRA, which clustered together. Furthermore, they were able to correlate DNA methylation and gene-expression changes during CD8+ T cell differentiation.

With particular relevance to our findings that CD8+ T cells from obese mice have significantly lower levels of IFNγ, several studies have found that the methylation status of the IFNγ promoter is related to its function and its transition from naïve to memory to effector cell [217]. We suggest that the obese environment can influence the methylation pattern of T cells, altering their metabolism and thereby affecting the ability of memory T cells to function. For example, cellular metabolism and metabolite availability is an important determinant of epigenetic enzyme activity and has been shown to influence the epigenetic mechanisms that control macrophage activity [218-220]. It is also well-recognized that epigenetic modifications can contribute to disease (56). For example, epigenetic changes can occur in diabetic patients which result in micro and macrovascular complications that occur despite normalization of blood sugar. Of particular importance to our study, Leung *et al.* [221] reported that epigenetic changes in the livers of formerly obese mice persisted well after weight loss.

In addition to the weight loss mice, mice that gained weight and became obese also had impaired memory T cell generation and function, even though they were generated in a lean state. These results suggest a metabolically unhealthy environment at the time of secondary infection can also impair T cell function. Together, these data demonstrate that the metabolic environment at the time of memory T cell generation and at the time of re-infection both impact the function of T memory cells.

In addition to the activation of memory and naïve cells to functional effective cells upon reinfection with influenza virus, Treg populations are also induced to prevent an excessive immune response that could result in increased lung pathology [222]. Additionally, memory Tregs can regulate influenza specific CD8+ T cell numbers and IFNγ production to reinfection, controlling pulmonary inflammation [222]. Previous studies in our lab have demonstrated that lung Treg numbers peak at day 5 post secondary infection in obese mice, with lean mice maintaining consistent numbers from day 5 to 8 post infection [172]. Consistent with these

results, we found that lean mice have significantly greater Treg populations before infection, and then maintain these populations throughout the course of infection. However, weight loss and always obese groups have significantly decreased Tregs at day 0 and 3 post infection, and increased numbers of Tregs at 7 days post infection. Again, mice obese at any time have dysregulated T cell subsets. Obesity delayed Treg infiltration, suggesting that these cells are not available to regulate the CD8+ T cell recall response.

As mentioned previously, obesity is primarily a metabolic disease, and T cell function is tied unquestionably to T cell metabolism. Reports that metabolic fuels such as glucose and fatty acids elicit distinct metabolic profiles depending on the cell state (naïve, effector or memory) has led to a revolution in the understanding of T cell driven immunity. Research in immunometabolism highlights the plasticity of T cells to respond to the energetic and biosynthetic demands required to fight influenza infection. Obesity is associated with hyperglycemia, insulin resistance and elevated leptin and triglycerides. Therefore, it was important to determine the metabolic state of memory T cells under conditions of obesity. Although T cells respond to antigenic challenge by altering their metabolic state, how might the metabolic condition of obesity impair the metabolic programming of the T cell during infection? In addition, could weight loss (formally obese) or weight gain (formally lean) re-program T cell metabolism?

T cell metabolism is a dynamic process, with metabolic needs changing with the environment and stress signals. The function of T cells is completely dependent on their cellular metabolic state. Activation of T cells from a resting, quiescent state to an active effector state requires metabolic reprogramming from fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) to aerobic glycolysis [223]. This metabolic switch from fatty acids to glucose reflects the fuel and substrate needs for both proliferation and effector functions, e.g. the production of interferon gamma (IFNγ). The high glucose demand of activated T cells is met by increasing glucose transporter proteins and their location on the plasma membrane [168]. Activating the T cell receptor with the co-receptor CD28 leads to the phosphorylation of Akt and upregulation of Glut1 mRNA and protein [199]. Glut1-deficient CD4 T effector cells have reduced production of IFNγ

[224]. Although activated T cells use glycolysis and oxidative phosphorylation of glutamine to support cell proliferation and function, inhibition of glycolysis limits effector function and cytokine production decreases [225]. Compared to always lean mice, we found that Glut1 expression in both CD4+ and CD8+ T effector T cells was decreased in weight loss, weight gain and always obese mice. This finding suggest that the metabolic state of the infected animal is critical for metabolic reprogramming of T cells. Reduced Glut1 expression would likely lead to reduced glucose uptake, thereby altering T cell metabolism and consequently function.

In addition to Glut1 expression, we also measured cytochrome C expression in lung CD8+ and CD4+ T cells as a measure of mitochondrial activity. We found that, at day 7 post reinfection, cytochrome C expression was significantly higher in the always lean mice compared with weight loss, weight gain and always obese mice. Again, these results suggest that obesity at any time alters the metabolism of the T cell.

After an infection resolves, most (>90-95%) T effector cells are removed through apoptosis and the remaining T cells transition into memory T cells, capable of rapidly responding to reinfection. For the effective transition from active glycolytic effector cells into a resting, memory population, glucose uptake and glycolysis must be reduced [226]. Memory CD8+ T cells require FAO to function and rather than taking up free fatty acids (FFA) to fuel their basal functions while quiescent, work from Pearce's group suggests that these cells synthesize fatty acids from glucose, which they then feed into the mitochondrial beta oxidation pathway to generate ATP[166]. Although this appears to be a futile cycle (cells use ATP in order to synthesize fatty acids that they then use to generate ATP), this cycle keeps the memory cell metabolism in a state that prevents mitochondria loss during prolonged (possibly years) quiescence. Fatty acids are also important for memory CD4+ T cells as well, because they are a key regulator of de novo fatty acid synthesis by inhibiting acetyl-CoA carboxylase 1 (ACC1).

In addition to utilizing FAO and OXPHOS for their metabolic needs, tumor necrosis factor receptor-associated factor 6 (TRAF6)[160] and IL-15[162, 164] are needed for long-term maintenance of memory CD8+ T cells. Unlike naïve T cells, memory T cells need to be

metabolically primed to initiate a faster immune response to re-encounter with antigen. IL-15 promotes mitochondrial biogenesis and increases the expression of CPT1a which transports long-chain fatty acids into the mitochondria to be used for FAO[162]. Enhanced mitochondrial biogenesis and increased CPT1a expression contribute to the spare respiratory capacity (SRC) of memory T cells, where SRC is the difference of the ATP produced at the basal level by oxidative phosphorylation compared to maximal potential [165].

Van der Windt, *et al.* proposed a model of how SRC regulates memory T cell survival to infection, where increased mitochondrial mass allows increased use of fatty acids for OXPHOS, allowing memory cells to quickly switch their metabolism for pathogen clearance. We found that SRC was higher in CD8+ T cells at 7 days post infection in the lean and weight gain (formally lean) mice compared with the obese and weight loss (formally obese) mice. Thus, the metabolism of the T cells was set at the time of the primary infection, and did not reprogram following reinfection. In other words, the SRC of the weight gain mice tracked with the always lean mice, and the SRC of the weight loss mice tracked with the always obese mice.

To further characterize T cell metabolism in each of our groups, we analyzed respiration through oxygen consumption (OCR) and glycolysis via extracellular acidification rate (ECAR). We found that CD8+ T cells did not differ in OCR, an indicator of OXPHOS, in any of the groups at day 0. However, at day 7 post infection, we found significantly lower OCR in always lean mice compared to always obese and weight loss mice. Lower OCR in always lean mice suggests lower respiration at day 7 while the always obese and weight loss (formally obese) are significantly more metabolically active. Again, the always lean and weight gain mice tracked together with similar OCR metabolic profiles, while always obese and weight loss mice tracked together. This again recapitulates our other findings that the time of memory T cell generation locks in the metabolic profile and drives function, as demonstrated in the differences induced with infection.

Altogether, our data demonstrate that memory T cell metabolism is set at the time of primary infection and T cell subsets and function are influenced by both the metabolic

environment at the initial generation, and at the time of immune challenge. We found that weight loss in obese mice did not restore immune function to reinfection, likely linked to the metabolic reprogramming of the T cells in the obese state, which was still present in the weight loss state. Although weight loss alone is not sufficient to restore function, metabolic drugs such as metformin may be therapeutic by improving the dysfunctional metabolism of the T cells, thereby restoring their ability to respond to infection. Metformin inhibits complex I of the electron transport chain, thereby decreasing energy output [227]. As increased metabolic activity and reduced SRC was found in the always obese mice and weight loss mice (formally obese) compared to always lean mice, metformin use may provide a method to decrease the excessive metabolic activity, increase SRC and restore immune function.

The findings from this data have significant public health importance and impact. Compared with lean adults, influenza vaccination of obese adults is less protective against influenza and influenza-like illness and our data suggest that weight loss alone may not be sufficient to restore vaccine efficacy. Further interventions and revisions of vaccine strategies need to be considered. More studies are needed to determine if giving additional booster vaccinations while on a regime of metabolic restoring drugs might provide an effective strategy for obese individuals and those who have lost weight to restore immune health.

Experimental Model and Subject Detail

Mice and Diet

Experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. C57BL/6J six week old, male mice were obtained from The Jackson Laboratory and allowed one week of acclimation. Thirty mice were placed on a chow diet (Harlan Laboratories, 2920X) and the remaining 30 were placed on a 60% high fat diet (Research Diets, D12492), for 22 weeks. Fifteen mice from the chow diet were then placed on the

60% high fat diet designated as weight gain experimental group, and 15 mice from the 60% high fat fed diet were then placed on a chow diet and designated at the weight loss group. Mice were maintained on the weight switched diets for 15 weeks. Mice were provided with food and water ad libitum and housed 3-5 per cage.

Method Details

Influenza Infection

For primary influenza infection, mice were lightly anesthetized with isoflurane and infected intranasally with 400 egg infectious dose 50% (EID_{50}) in 30ul of sterile PBS of live X-31 mouse-adapted recombinant influenza virus strain that consists of external hemagglutinin and neuraminidase proteins of A/Aichi/2/68 (H3N2) at 18 weeks on the diet. Mice were weighed every day for 14 days to monitor weight loss. For secondary infection, mice were lightly anesthetized and infected intranasally with 0.5 hemagglutination units (HAU) in 30ul sterile PBS of A/Puerto Rico/8/34 (PR8, American Type Culture Collection, Manassas, VA), a H1N1 influenza virus. Mice were then sacrificed at day 0, 3 and 7 post secondary infection with PR8.

Quantitation of viral titers

Lung viral titers were determined using a modified $TCID_{50}$ protocol using hemagluttination as an endpoint. Half of the lung was removed at harvest, and immediately frozen in liquid nitrogen. Madin Darby canine kidney (MDCK) tissue culture cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% glutamine and penicillin/streptomycin overnight in a 96 well plate in 37°C at 5% CO_2. Lung tissue was weighed, and homogenized in 0.2 ml minimal essential medium (MEM) and centrifuged at 9000 x g for 20 minutes. Supernatant was serially diluted starting at 1:10 dilution in MEM containing 20 mg/l trypsin. Media was

removed from the MDCK cultured plate, and serial dilutions (0.1ml) were added to the 80% confluent MDCK plate in replicates of six in the 96-well plate and cultured for 5 days at 37°C at 5% CO_2. A suspension of 0.5% of turkey red blood cells was added to each well and incubated at room temperature. $TCID_{50}$ was determined by the Reed and Muench calculations [228]. Values were normalized to the weight of the lung tissue.

Bronchoalveolar lavage total protein measurements

Tracheas of sacrificed mice were exposed and cannulated with a 22-guage angiocath, and the lungs were lavaged four times with HBSS and 3.75 ml was collected (first wash was 0.75 ml, other washes 1 ml). Washes were centrifuged, and the cell pellet was combined with lung cells for flow cytometry. The supernatant was used for the measurement of total protein. The first wash was diluted 1:10 and total protein was measured using bicinchoninic acid kit (Sigma-Aldrich, St. Louis, MO).

Histopathology

Lungs were removed and inflated with 4% paraformaldehyde fixative in 0.1M sodium phosphate buffer pH 7.2, for 72 hours, and then placed in 70% ethanol and submitted to the UNC Lineberger Comprehensive Cancer Center Animal Histopathology Core lab for lung tissue paraffin embedding and H&E staining. The presence of mononuclear infiltrate was scored blindly based on a numerical scale from 0 to 4: 0 with no presence of inflammation; 1 with mild influx of inflammatory cells; 2, inflammation of ~25-50% of the total lung; 3, inflammation of 50-75% of the total lung; and 4 with the majority of the lung consisting of inflammatory cells.

Antibodies and Flow Cytometry

Lungs were placed in collagenase II solution (Worthington) at 7mg/5mL of HBSS with Calcium and Magnesium and homogenized with Stomacher (Seward) until single cell suspension. Cells were filtered with Falcon 40 um cell strainer (BD Biosciences), and centrifuged for 5 minutes at 1500 RPM, pellet was treated with Ack lysis buffer at room temperature. Cells were washed with HBSS without Calcium and Magnesium and resuspended in PBS with 2% FBS. For the identification of TRM and Treg the following antibodies were used: Alexa Fluor 700 anti-mouse CD3 (17A2, eBioscience), APC-Cy7 rat anti-mouse CD4 (GK1.5, BD Pharmingen), PacBlue rat anti-mouse CD8a (53-6.7, BD Pharmingen), PerCP-Cy5.5 rat anti-mouse CD11a (2D7, BD Pharmingen), FITC rat anti-mouse CD25 (3C7, BD Pharmingen), APC anti-mouse Foxp3 (FJK-16s, eBioscience), BUV395 hamster anti-mouse CD69 (H1.2F3, BD Biosciences), BV480 rat anti-mouse CD103 (M290, BD Biosciences) and Zombie Yellow Fixable Viable dye (BioLegend). For the identification of effector T cells and function the following antibodies were used: Alexa Fluor 700 anti-mouse CD3 (17A2, Biolegend), Alexa Fluor anti-mouse 594 CD4 (GK1.5, Biolegend), BUV395 rat anti-mouse CD8 (53-6.7, BD Biosciences), FITC rat anti-mouse CD62L (MEL-14, BD Biosciences), APC anti-mouse Granzyme B (GzA-3G8.5, eBioscience), PE-Cy7 anti-mouse Interferon gamma (XMG 1.2, eBioscience), BV421 hamster anti-mouse PD-1 (J43, BD Biosciences), and Zombie NIR (Biolegend). The following antibodies were used for TEM and TCM flow staining panel: Alexa Fluor 700 anti-mouse CD3 (17A2, Biolegend), BUV395 Rat anti-mouse CD4 (GK1.5, BD Biosciences), BV650 rat anti-mouse CD8a (53-6.7, BD Biosciences), PerCP-Cy5.5 Rat anti-mouse CD62L (MEL-14, BD Biosciences), PE-Cy7 anti-mouse CCR7 (4B12, eBioscience), PE-CF594 hamster anti-mouse KLRG1 (1F1, BD Biosciences), BV421 rat anti-mouse CD127 (SB/199, BD Biosciences), BB515 rat anti-mouse CD44 (IM7, BD Biosciences), APC hamster anti-mouse (J43, BD Biosciences) and Zombie Yellow Fixable Viable dye (BioLegend). One million cells were plated in a 96 well plate in PBS without added protein and stained at room temperature in the dark with Zombie viability dye according to manufacturer's protocol. Cells were stained at room temperature in the dark with class II tetramer I-A(b) Influenza A NP 311-325 (QVYSLIRPNENPAHK, courtesy of NIH Tetramer Core Facility) in PBS

with 2% FBS for one hour. Cells were then incubated with rat anti-mouse CD16/32 (BD Biosciences) for 5 minutes on ice, and then the remaining cell surface antibody master mix was added and incubated for 30 minutes on ice and kept dark. Cells stained for the effector and function panel were incubated with Golgi Plug (BD Biosciences) and permeabilized and fixed with Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. Cells stained for TRM and Treg panel were stained for cell surface markers and then fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer kit (eBioscience) for intracellular transcription factor Foxp3. Cells stained for the TEM and TCM were incubated with Zombie viability dye, tetramer, and rat anti-mouse CD16/32 as described above, then with antibody master mix for cell surface markers and then fixed with fixation bugger (life technologies). All samples were acquired on a BD LSR II flow cytometer, and data was analyzed using FlowJo (Treestar).

Extracellular Acidification Rate and Oxygen Consumption Rate

T cells were isolated from mouse splenocytes following euthanasia on day 0 and day 7 of influenza virus infection. Cells were isolated using magnetic bead negative selection (Miltenyi) for CD4+ (Cat# 130-104-454) and CD8+ (Cat# 130-104-075) T cells in MACS buffer (PBS + 0.5% FBS + 2mM EDTA). Isolated T cells were counted using Bio-Rad TC20 with trypan blue exclusion for viability. XF96 cell culture microplates were treated with Cell-Tak^{TM} (Corning, Cat#354240) in 0.1M sodium bicarbonate to allow for cell adherence and washed twice with sterile water. CD4+ and CD8+ T cells were plated in non-buffered RPMI-1640 with freshly added 10mM glucose and 2mM glutamine at 150,000 cells per well. Extracellular acidification (ECAR) and oxygen consumption rates (OCR) were determined using the Seahorse XFe96 Flux analyzer (Agilent) at 37^{o}C in response to the mitochondrial stress test with injections of 1.0μM oligomycin (Sigma, Cat#O4876), 1.5μM FCCP (Sigma, Cat#C2920), antimycin-A (Sigma, Cat#A8674) and rotenone (Sigma, Cat# R8875).

Values for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) from technical replicates were averaged and reported for each well. Values ≤ 0 were excluded. Basal respiration rate was determined as the last OCR value before the administration of the first injection of oligomycin. Basal ECAR was taken as the last value of ECAR before the injection of oligomycin. OCR to ECAR ratios were calculated by taking the basal respiration rate divided by the basal ECAR. Maximal respiration was used for the calculation of spare respiratory capacity (SRC) and determined as the peak OCR (pmoles/min) following injection of FCCP. SRC was calculated by subtracting basal respiration (pmoles/min) from the maximal respiration (pmoles/min).

Statistical analysis

All statistical analysis was performed using Prism 7 for Mac OSX, version 7.0c (GraphPad Software, Inc., La Jolla, CA). Data comparing values for glucose, T cell populations by flow cytometry, and metabolic data of the four weight loss groups was tested for differences by one way ANOVA, follow by Tukey's multiple comparison's test. Data comparing metabolic data and flow cytometry data day 0 to day 7 in each weight loss group was tested for significance by Student's t test. All data was determined as significant by $p<0.05$.

Figure 1. Development of a model to study the effects of weight loss and weight gain on memory T cells. (A) Male, 6 week old C57BL/6J mice were randomly assigned to two groups, chow (n=60) or high fat diet (n=60). Mice were fed their respective diets for 18 weeks. Mice were infected with X-31 influenza virus, after generation of memory T cells (4 weeks), diets were switched and half of the mice receiving chow diet (n=30) were then placed on high fat diet, half of the mice being fed high fat diet were placed on chow diet (n=30) and the remaining mice maintained their original diet of chow diet (n=30), or high fat diet (n=30). Mice were then maintained on switched diet for 15 weeks and then infected with PR8. (B) High fat diet fed mice gained significantly more weight than chow diet, and when switched diets, obese mice lost weight (weight loss) and lean mice gained weight to obese weight (weight gain). Mice that did not switch diets maintained lean (always lean) or obese (always obese) weights. (C) Fasting glucose for mice 1 week prior to secondary infection (n=60). (D) Percent weight loss to PR8 infection (n=120). (E) Total weight loss to PR8 infection (n=120).

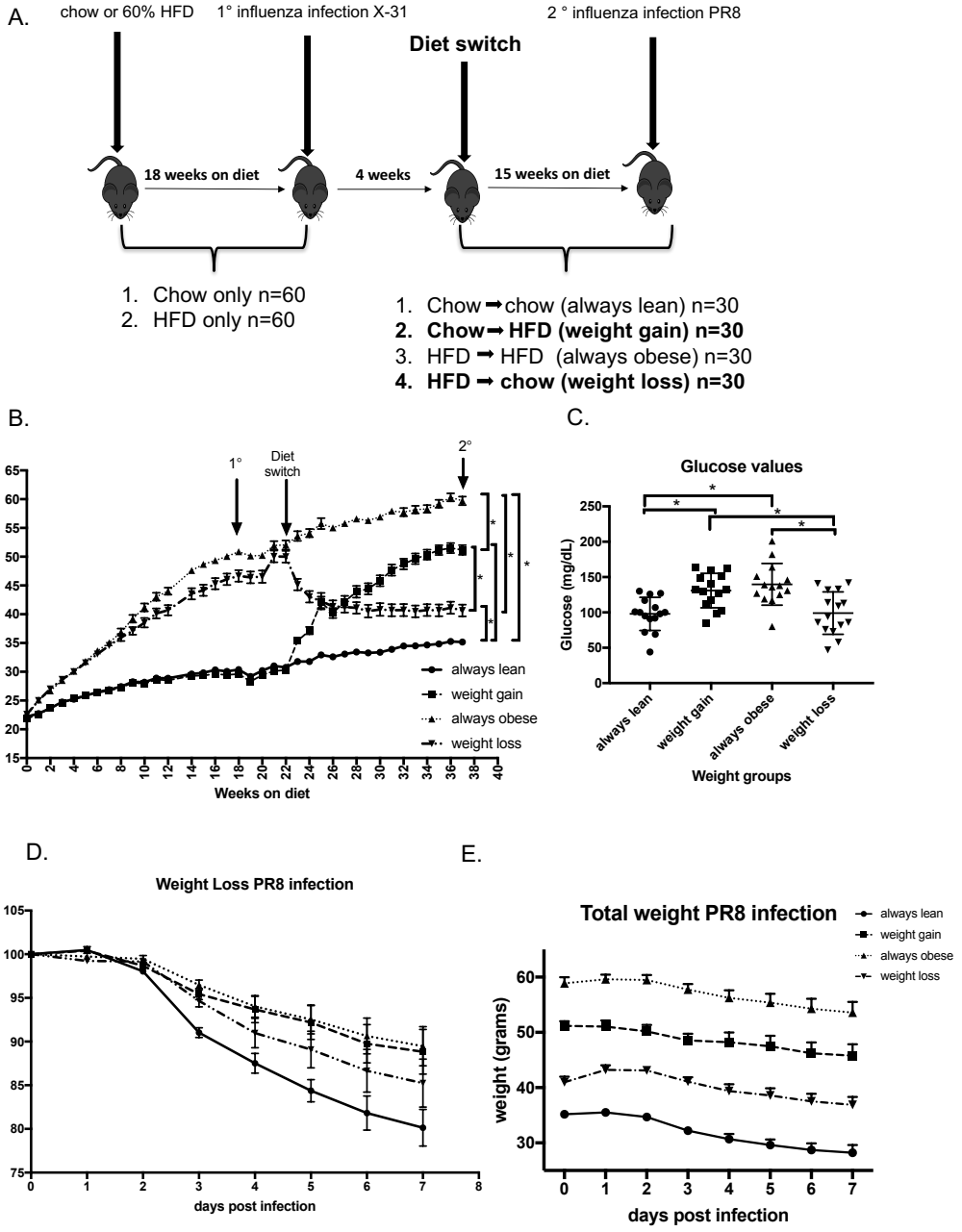

A.
chow or 60% HFD
1° influenza infection X-31
2 ° influenza infection PR8
Diet switch
18 weeks on diet
4 weeks
15 weeks on diet
1. Chow only n=60
2. HFD only n=60
1. Chow ➡ chow (always lean) n=30
2. Chow ➡ HFD (weight gain) n=30
3. HFD ➡ HFD (always obese) n=30
4. HFD ➡ chow (weight loss) n=30
B.
1°
Diet switch
2°
weight (grams)
Weeks on diet
always lean
weight gain
always obese
weight loss
C.
Glucose values
Glucose (mg/dL)
always lean
weight gain
always obese
weight loss
Weight groups
D.
Weight Loss PR8 infection
% original weight
days post infection
E.
Total weight PR8 infection
always lean
weight gain
always obese
weight loss
weight (grams)
days post infection

Figure 2. Obesity at any time results in dysregulated generation of memory T cell populations and function to influenza infection. Lungs were harvested, digested with collagenase, and homogenized into single cell suspensions and $1x10^6$ cells were stained for memory T cell populations and function by flow cytometry. (A) Percent of CD4+ T cell effector memory populations at day 3 and day 7 post infection to PR8 (n=4-5). (B) Percent of CD4+ tissue resident memory T cells at day 3 post PR8 infection (n=4-5). (C) Percent of CD4+ tissue resident memory T cells at days 0, 3, and 7 post PR8 infection. (D) Percent of Class II tetramer I-A(b) Influenza A NP 311-325 day 3 post PR8 infection (n=4-5). (E) Percent of Class II tetramer I-A(b) Influenza A NP 311-325 at days 3 and 7 post PR8 infection (n=4-5). Percent of CD4+ CD25+ Foxp3+ Tregs at day 0 (F), day 3 (G) and day 7 (H) post PR8 infection (n=4-5). (I) Percent of CD4+ CD25+ Foxp3+ Tregs at days 0, 3 and 7 post infection for lean, weight gain, obese and weight loss groups (n=4-5). (J) Percent of CD8+ T cell effector memory populations at day 3 and day 7 post infection to PR8 (n=4-5). (K) Percent of CD8+ central memory T cells at day 3 post PR8 infection (n=4-5). (L) Percent of CD8+ central memory T cells comparing day 3 and 7 post PR8 infection (n=4-5). (M) Percent of CD8+ tissue resident memory T cell populations at day 3 (M) and day 7 (N) post infection to PR8 (n=4-5). (O) Percent of CD8+ tissue resident memory T cell populations at comparing days 0, 3 and 7 post PR8 infection (n=4-5). (P) Measurement of intracellular functional markers granzyme B and IFNγ at day 3 post PR8 infection (n=4-5). (Q) Comparison of functional markers intracellular functional markers granzyme B and IFNγ at days 0,3 and 7 post PR8 infection (n=4-5). Each bar represents the mean $\pm$ SEM. Data was defined as significant $p<0.05$.

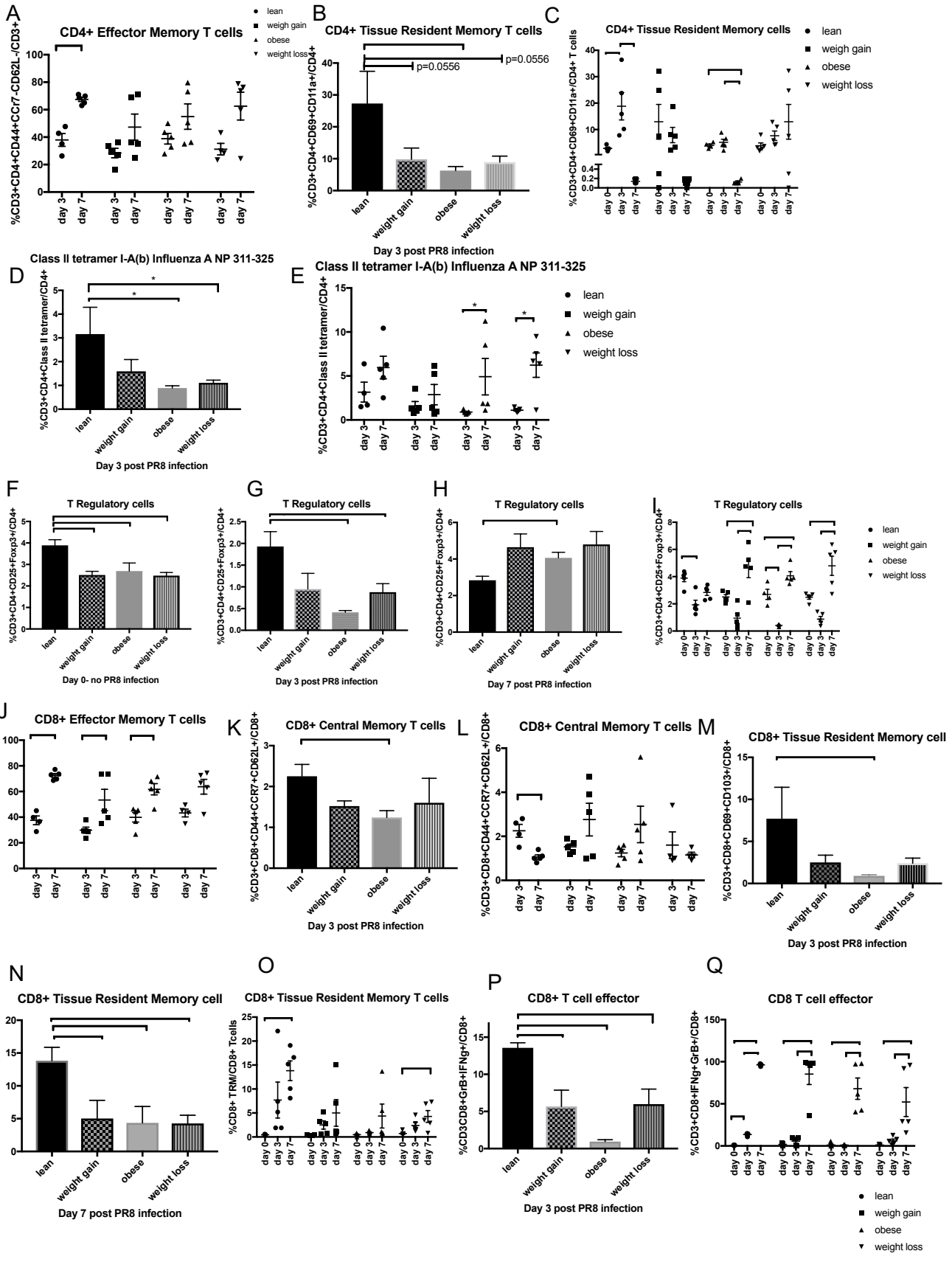

A
CD4+ Effector Memory T cells
%CD3+CD4+CD44+CCr7-CD62L-/CD3+
lean
weigh gain
obese
weight loss
day 3
day 7
B
CD4+ Tissue Resident Memory T cells
%CD3+CD4+CD69+CD11a+/CD4+
p=0.0556
p=0.0556
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
C
CD4+ Tissue Resident Memory cells
%CD3+CD4+CD69+CD11a+/CD4+ T cells
lean
weigh gain
obese
weight loss
day 0
day 3
day 7
D
Class II tetramer I-A(b) Influenza A NP 311-325
%CD3+CD4+Class II tetramer/CD4+
*
*
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
E
Class II tetramer I-A(b) Influenza A NP 311-325
%CD3+CD4+Class II tetramer/CD4+
*
*
lean
weigh gain
obese
weight loss
day 3
day 7
F
T Regulatory cells
%CD3+CD4+CD25+Foxp3+/CD4+
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
G
T Regulatory cells
%CD3+CD4+CD25+Foxp3+/CD4+
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
H
T Regulatory cells
%CD3+CD4+CD25+Foxp3+/CD4+
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
I
T Regulatory cells
%CD3+CD4+CD25+Foxp3+/CD4+
lean
weight gain
obese
weight loss
day 0
day 3
day 7
J
CD8+ Effector Memory T cells
%CD8+CD44+CCR7-CD62L-/CD8+
day 3
day 7
K
CD8+ Central Memory T cells
%CD3+CD8+CD44+CCR7+CD62L+/CD8+
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
L
CD8+ Central Memory T cells
%CD3+CD8+CD44+CCR7+CD62L+/CD8+
day 3
day 7
M
CD8+ Tissue Resident Memory cell
%CD3+CD8+CD69+CD103+/CD8+
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
N
CD8+ Tissue Resident Memory cell
%CD3+CD8+CD69+CD103+/CD8+
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
O
CD8+ Tissue Resident Memory T cells
%CD8+ TRM/CD8+ Tcells
day 0
day 3
day 7
P
CD8+ T cell effector
%CD3CD8+GrB+IFNg+/CD8+
lean
weight gain
obese
weight loss
Day 3 post PR8 infection
Q
CD8 T cell effector
%CD3+CD8+IFNg+GrB+/CD8+
day 0
day 3
day 7
lean
weigh gain
obese
weight loss

Figure 3. Metabolic profile is programmed at time of memory T cell generation. Spleens were harvested from mice, and CD4+ and CD8+ T cells were isolated by negative magnetic bead separation. Cells were plated at 150,000 cells per well, and extracellular flux analysis was performed using the Seahorse XFe96 Flux analyzer at 37^{o}C in response to the mitochondrial stress test with injections of 1.0μM oligomycin, 1.5μM FCCP, antimycin-A and rotenone. (A) Basal respiration rate was measured in CD4+ isolated T cells as the last measurement of OCR before the injection of oligomycin at day 0 (A) and day 7 (B) and the comparison of both days (C) post PR8 infection (n=4-5). (D) OCR:ECAR ratio was calculated as the last measurement of OCR before oligomycin divided by the last measurement of ECAR before oligomycin (n=4-5). CD8+ T cell basal respiration was measured as the last measurement of OCR before oligomycin at day 0 (E), day 7 (F), and the comparison of days 0 and 7 (G) post PR8 infection (n=4-5). (H) OCR:ECAR ratio was calculated as described above for CD8+ isolated T cells (n=4-5).

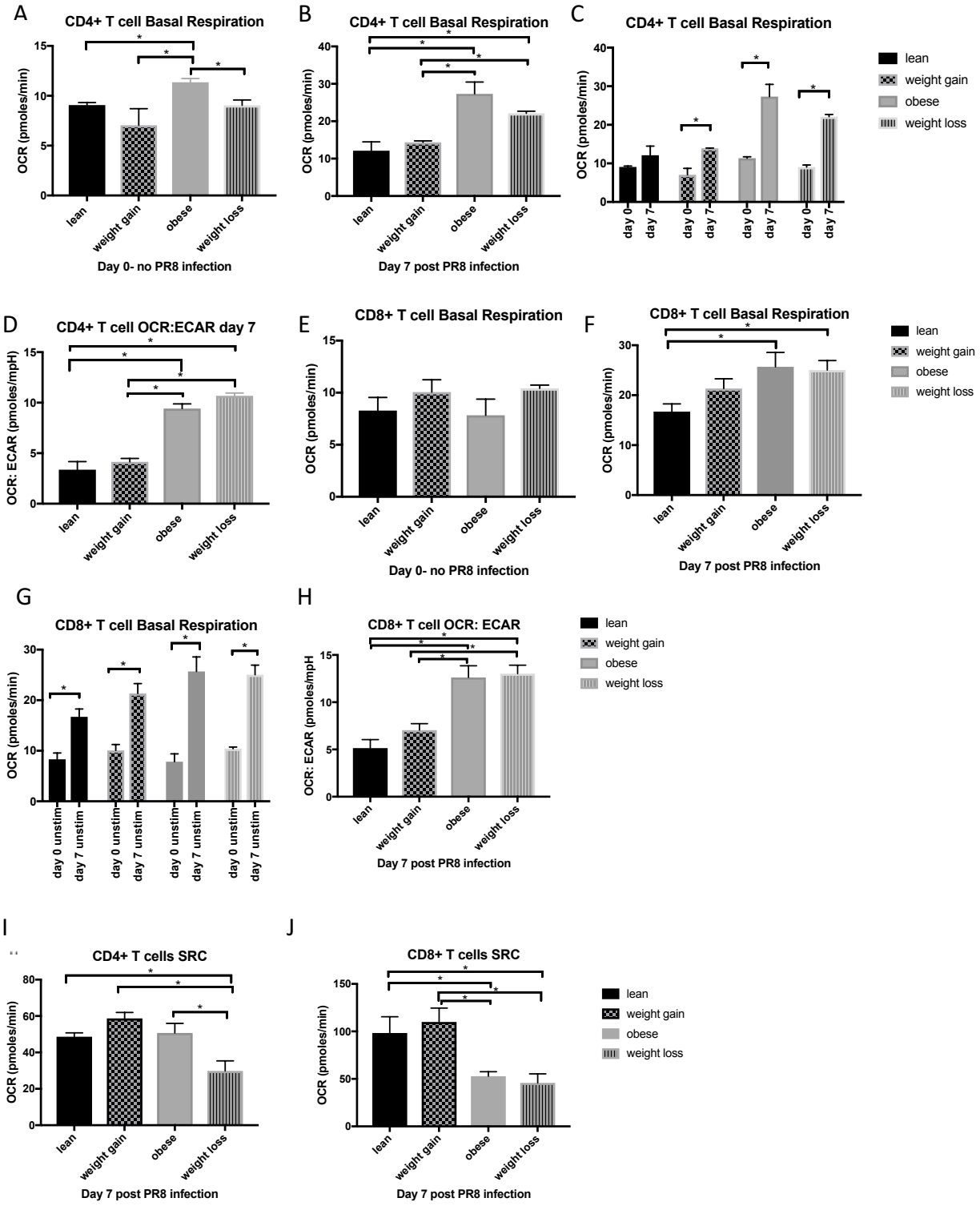
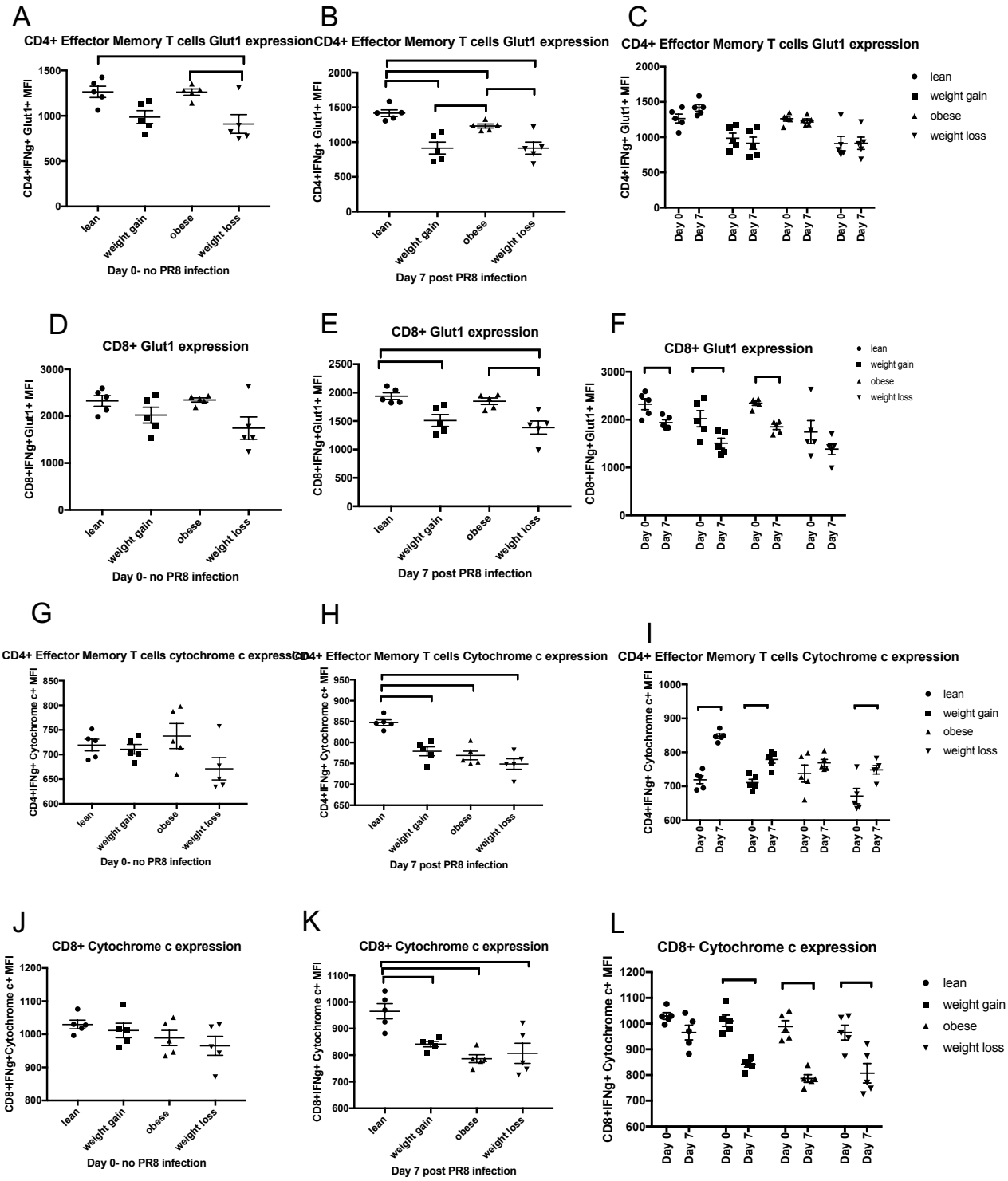
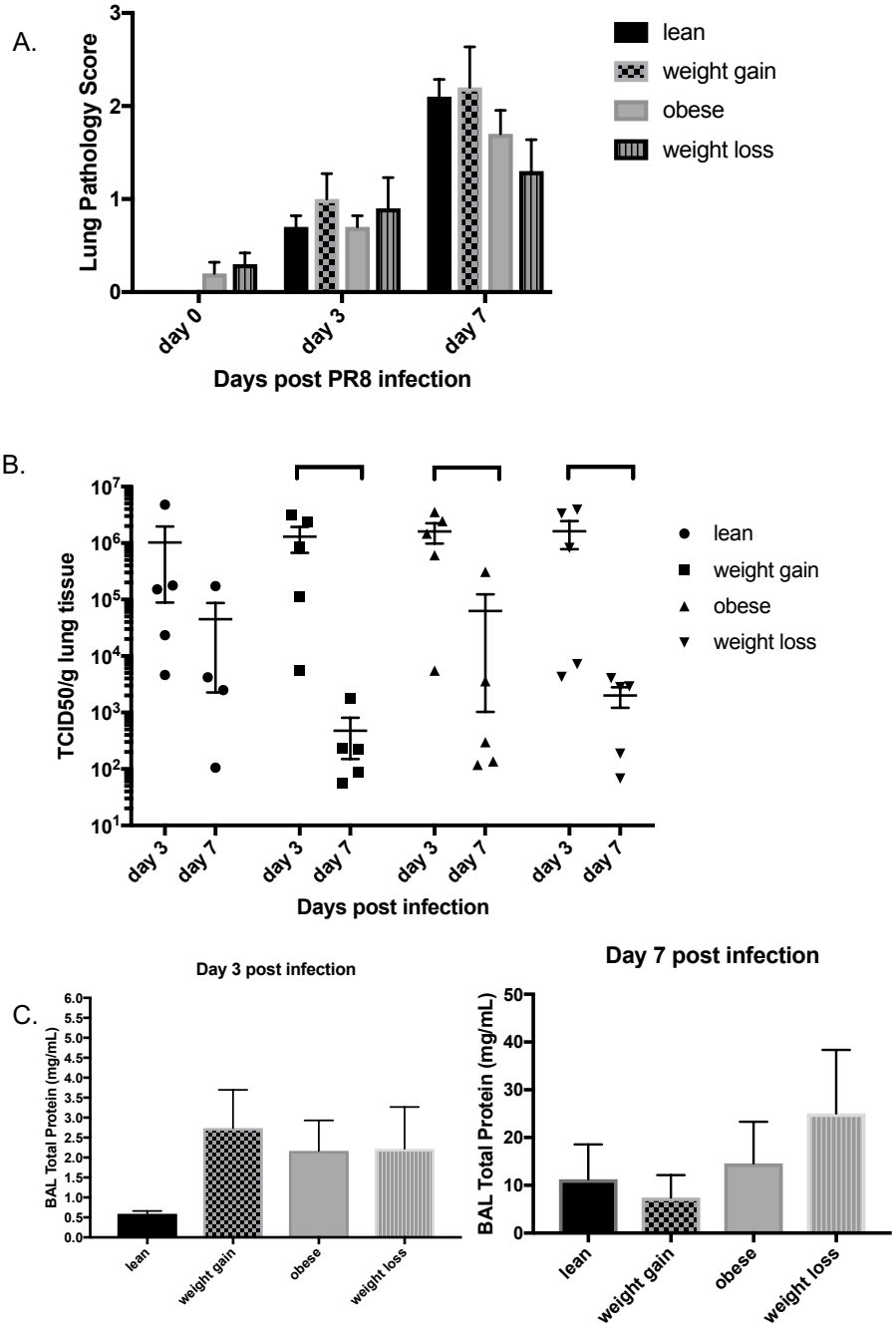

A
CD4+ T cell Basal Respiration
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
B
CD4+ T cell Basal Respiration
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
C
CD4+ T cell Basal Respiration
OCR (pmoles/min)
day 0
day 7
lean
weight gain
obese
weight loss
D
CD4+ T cell OCR:ECAR day 7
OCR: ECAR (pmoles/mpH)
lean
weight gain
obese
weight loss
E
CD8+ T cell Basal Respiration
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
F
CD8+ T cell Basal Respiration
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
G
CD8+ T cell Basal Respiration
OCR (pmoles/min)
day 0 unstim
day 7 unstim
H
CD8+ T cell OCR: ECAR
OCR: ECAR (pmoles/mpH
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
I
CD4+ T cells SRC
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
J
CD8+ T cells SRC
OCR (pmoles/min)
lean
weight gain
obese
weight loss
Day 7 post PR8 infection

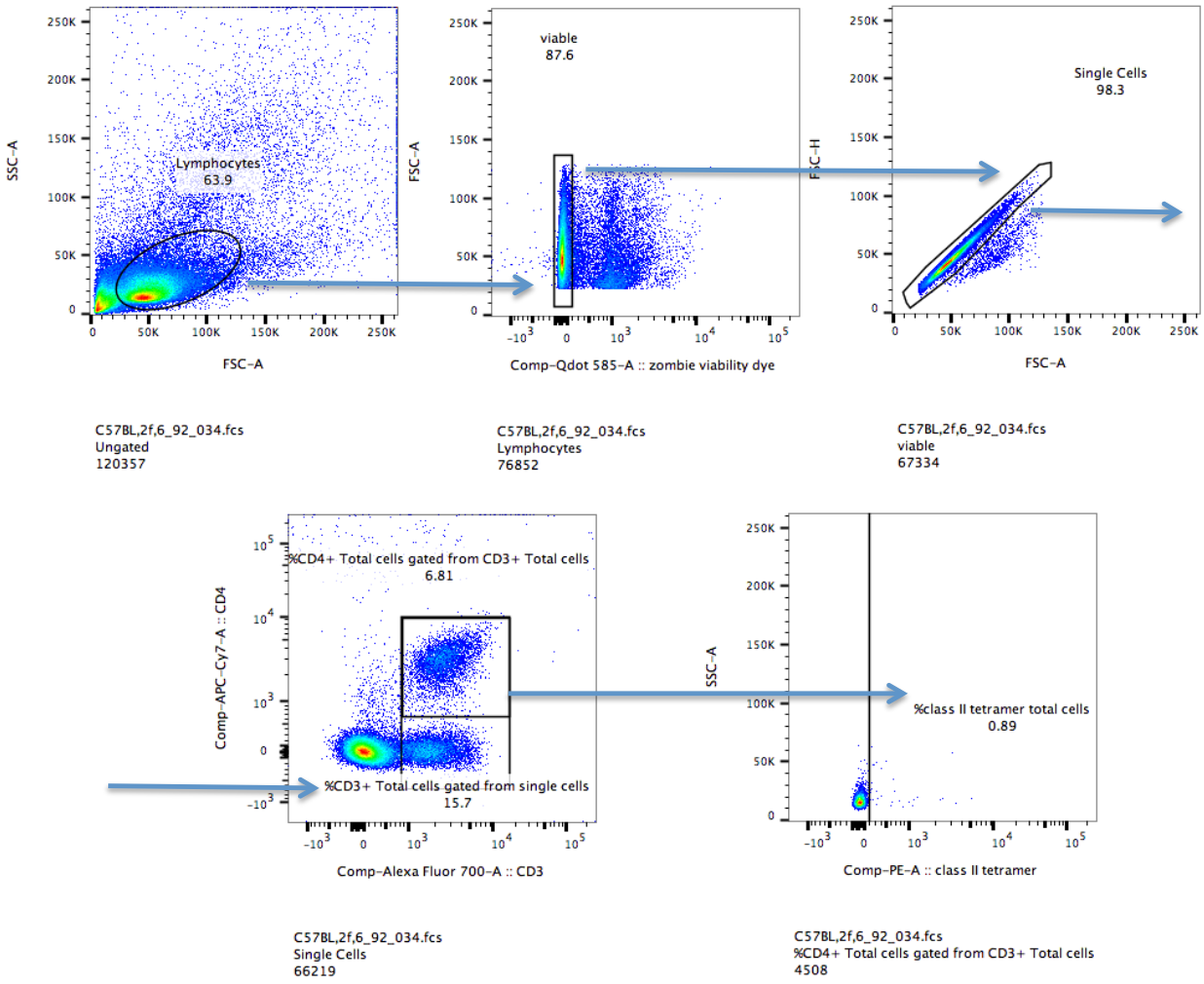
Figure 4. Obese mice at any point have impaired Glut1 and cytochrome c expression. Cells were stained for intracellular proteins Glut1 and cytochrome c and measured by flow cytometry. Glut1 and cytochrome c expression were measured as the median fluorescence intensity (MFI). CD4+ IFNγ+ cells were measured for Glut1 expression at day 0 (A) day 7 (B) and the comparison of both days (C) post PR8 infection (n=5). CD8+ T cells were measured for Glut 1 expression at day 0 (D) day 7 (E) and the comparison of both days (F) post PR8 infection (n=5). Cytochrome c expression was measured in CD4+ IFNγ+ T cells at day 0 (G), day 7 (H) and the comparison of both days (I) post PR8 infection. CD8+ T cells were measured for cytochrome c expression day 0 (J), day 7 (K) and the comparison of both days (L) post PR8 infection (n=5).


A
CD4+ Effector Memory T cells Glut1 expression
CD4+IFNg+ Glut1+ MFI
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
B
CD4+ Effector Memory T cells Glut1 expression
CD4+IFNg+ Glut1+ MFI
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
C
CD4+ Effector Memory T cells Glut1 expression
CD4+IFNg+ Glut1+ MFI
Day 0
Day 7
lean
weight gain
obese
weight loss
D
CD8+ Glut1 expression
CD8+IFNg+Glut1+ MFI
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
E
CD8+ Glut1 expression
CD8+IFNg+Glut1+ MFI
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
F
CD8+ Glut1 expression
CD8+IFNg+Glut1+ MFI
Day 0
Day 7
lean
weight gain
obese
weight loss
G
CD4+ Effector Memory T cells cytochrome c expression
CD4+IFNg+ Cytochrome c+ MFI
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
H
CD4+ Effector Memory T cells Cytochrome c expression
CD4+IFNg+ Cytochrome c+ MFI
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
I
CD4+ Effector Memory T cells Cytochrome c expression
CD4+IFNg+ Cytochrome c+ MFI
Day 0
Day 7
lean
weight gain
obese
weight loss
J
CD8+ Cytochrome c expression
CD8+IFNg+Cytochrome c+ MFI
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
K
CD8+ Cytochrome c expression
CD8+IFNg+ Cytochrome c+ MFI
lean
weight gain
obese
weight loss
Day 7 post PR8 infection
L
CD8+ Cytochrome c expression
CD8+IFNg+ Cytochrome c+ MFI
Day 0
Day 7
lean
weight gain
obese
weight loss

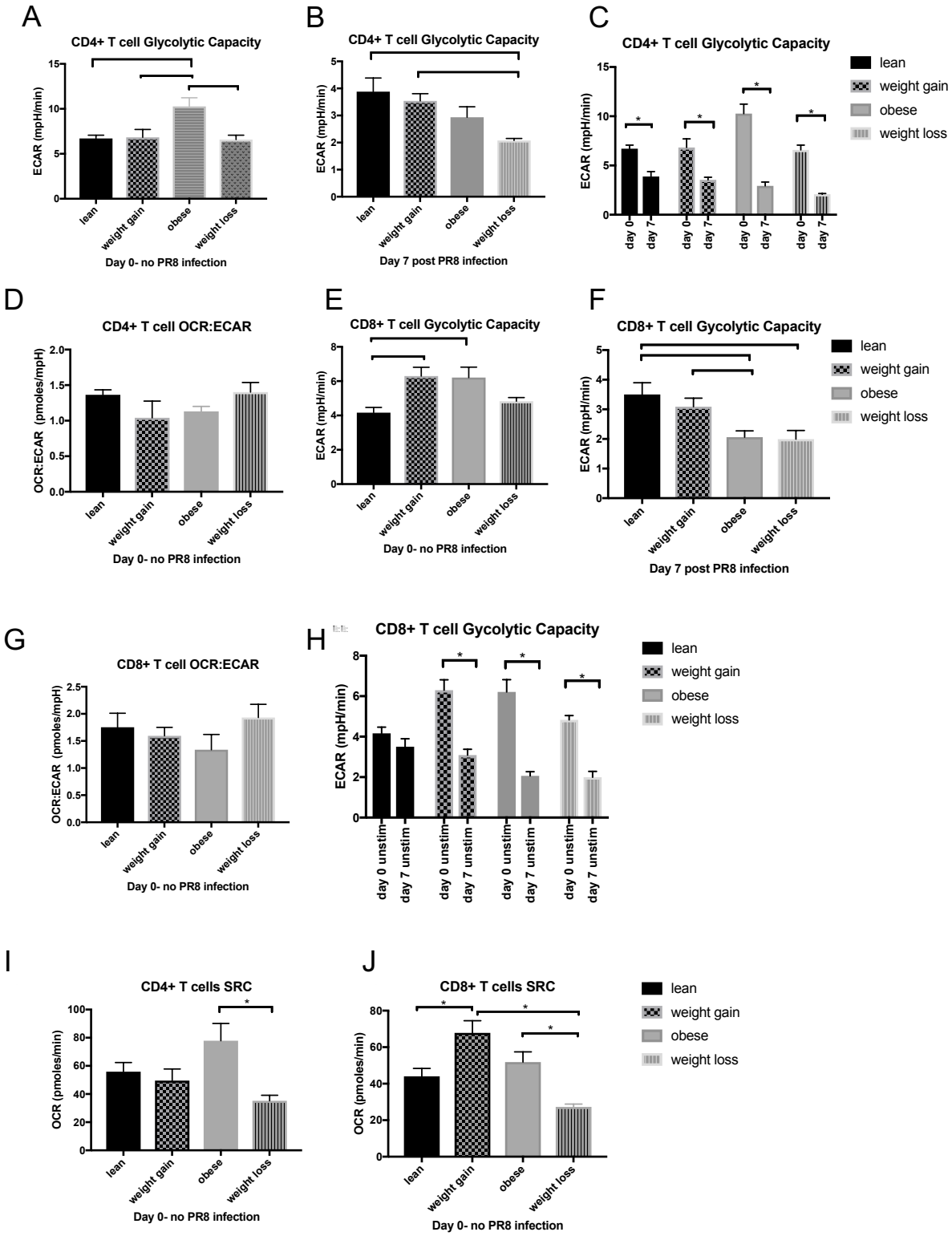
Supplementary Figure 1. Lung infection titers, total protein, and pathology. No differences in lung pathology (A) viral titers (B) or albumin protein (C) between any of the diet groups to secondary influenza infection to PR8 (n=60).



Supplementary Figure 2. Representative diagram of flow cytometry analysis for memory T cell populations and functional markers. Cells were acquired on a BD LSRII instrument and 150,000-200,000 cells were collected. Cells were gated with a primary gate to exclude debris, then gated on negative population of the viability dye for viable cells. Using area verses height to exclude doublet cells, then cells were gated off CD3, and then CD4 or CD8. Cells were then analyzed for populations of interest from the CD3+CD4+ or CD3+CD8+ populations.



Supplementary Figure 3. Metabolic profile is programmed at time of memory T cell generation. Spleens were harvested from mice, and CD4+ and CD8+ T cells were isolated by negative magnetic bead separation. Cells were plated at 150,000 cells per well, and extracellular flux analysis was performed using the Seahorse XFe96 Flux analyzer at 37^{o}C in response to the mitochondrial stress test with injections of 1.0μM oligomycin, 1.5μM FCCP, antimycin-A and rotenone. (A) Basal glycolytic rate was measured in CD4+ isolated T cells as the last measurement of ECAR before the injection of oligomycin at day 0 (A) and day 7 (B) and the comparison of both days (C) post PR8 infection (n=4-5). (D) OCR:ECAR ratio for day 0 was calculated for CD4+ T cells as the last measurement of OCR before oligomycin divided by the last measurement of ECAR before oligomycin (n=4-5). CD8+ T cell glycolytic rate was measured as the last measurement of ECAR before oligomycin at day 0 (E), day 7 (F), and the comparison of days 0 and 7 (G) post PR8 infection (n=4-5). (H) OCR:ECAR ratio was calculated as described above for CD8+ isolated T cells (n=4-5).


A
CD4+ T cell Glycolytic Capacity
ECAR (mpH/min)
lean
weight gain
obese
weight loss
Day 0- no PR8 infection
B
CD4+ T cell Glycolytic Capacity
ECAR (mpH/min)
Day 7 post PR8 infection
C
CD4+ T cell Glycolytic Capacity
ECAR (mpH/min)
day 0
day 7
D
CD4+ T cell OCR:ECAR
OCR:ECAR (pmoles/mpH)
E
CD8+ T cell Gycolytic Capacity
F
CD8+ T cell Gycolytic Capacity
G
CD8+ T cell OCR:ECAR
H
CD8+ T cell Gycolytic Capacity
day 0 unstim
day 7 unstim
I
CD4+ T cells SRC
OCR (pmoles/min)
J
CD8+ T cells SRC

CHAPTER IV: CORRELATION OF METABOLIC PROFILE TO T CELL FUNCTION IN ADULTS VACCINATED WITH INFLUENZA QUADRAVALENT INACTIVATED VACCINE

Introduction

Seasonal influenza infects millions of people worldwide, causing up to half a million deaths in a non-pandemic year. Influenza virus is a highly transmissible virus that infects infection nose, throat and lungs. It can cause mild or severe illness, or can result in life threatening complications and death. In 2009, a novel swine-origin H1N1 reassorted virus emerged resulting in the first human influenza pandemic of the twenty-first century [229]. Notably, for the first time, obesity was found to be a risk factor for increased severity from influenza infection. The most effective protection to decrease the incidence or severity of influenza infection is vaccination, however, our lab has shown that despite vaccination, obese adults are twice as likely to develop influenza or an influenza like illness [200]. Furthermore, we have reported that CD4+ and CD8+ T cells from vaccinated obese adults express decreased activation and functional markers to pandemic H1N1(pH1N1) influenza virus [81]. We reported impaired humoral immunity, as obese individuals have impaired antibody maintenance to influenza vaccination over time [79]. Additionally, overweight children have decreased antibody response to tetanus vaccination [82], and obese individuals have impaired seroprotection after hepatitis vaccination [230]. It is evident that obesity impairs the immune response to various infections and decreases vaccine immunogenicity, but it is also critical to understand these mechanisms to develop better public health strategies and vaccines for this population.

Recent studies have shown that immune cell function is tied to their metabolism. Resting T cells primarily utilize oxidative phosphorylation with low levels of glycolysis to meet metabolic needs. When naïve T cells are activated by immune challenge, they increase glycolysis and glutamine metabolism to support effector functions. The ability to metabolically switch will dictate

a functional immune response to influenza challenge. This study was designed to determine if the metabolic status of T cells from vaccinated obese adults was responsible for their impaired response to influenza antigen.

Materials and Methods

Study population

Subjects were enrolled between March 2016 to March 2017 from the University of North Carolina at Chapel Hill Family Medicine Center as part of an ongoing, observational study to investigate the effects of obesity on the immune response to influenza infection conducted by the Beck lab. The study population consisted of adult white females ages 35 to 65 that were vaccinated with either the 2015-2016 or 2016-2017 quadravalent influenza vaccine. The influenza vaccine for the 2015-2016 influenza season consisted of A/California/07/2009, A/Switzerland/9715293/2013, B/Phuket/3073/2013, and B/Brisbane/60/2008, while the 2017-2017 vaccine consisted of A/Christchurch/16/2010 (H1N1) NIB-74XP (A/California/7/2009 (H1N1) pdm09-like virus), A/Hong Kong/4801/2014 (H3N2) NYMC X-263B, and B/Phuket/3073/2013 inactivated influenza strains. At time of enrollment, height and weight measurements were taken to calculate body mass index, and fasting serum and blood samples were obtained. Exclusion criteria for enrollment of subjects were immunosuppression, use of immunolodulator or immunosuppressive drugs, acute febrile illness, hypersensitivity to vaccine components, Guillian-Barre syndrome, use of theophylline preparations or warfarin, or immunotherapy for cancer. Protocols and written consent procedures were approved by the University of North Carolina Biomedical Institutional review board.

Cell isolations and metabolic assays

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples for each subject by Ficoll gradient centrifugation as per the manufacturers protocol for Sigma Aldrich Ficoll histopaque 1077. CD4+ and CD8+ T cell populations were isolated from PBMCs by magnetic negative selection using StemCell technologies (StemCell Technologies, Vancouver, Canada) exclusion beads and magnet. CD4+ and CD8+ T cells were measured for metabolic parameters freshly isolated, and with stimulation beads to activate and expand T cell populations to test function. CD4+ and CD8+ isolated T cells were stimulated with anti-CD3/anti-CD28 magnetic beads with added 30 IU IL-2 (ThermoFisher Scientific, Waltham, MA) in RPMI-1640 with supplemented 10mM glucose, 2mM glutamine, 1% penicillin/streptomycin, and 5% autologous plasma for 72 hours at 37°C with 5% CO_2. CD4+ and CD8+ purified populations were plated for extracellular flux analysis at $2x10^5$ cells per well in non-buffered RPMI-1640 with 10mM glucose, 2mM glutamine and 1 mM pyruvate for unstimulated conditions.

Extracellular flux analysis was performed using an Agilent XFe96 flux analyzer using the mitochondrial stress test kit. The mitochondrial stress test kit consists of the sequential administration of three inhibitors oligomycin, carbonyl cyanide-4 trigluoromethoxy phenylhydrazone (FCCP), and combination of retenone and antimycin A, to inhibit ATP synthase (Sigma-Aldrich, St. Louis, MO), uncouples the proton gradient, and complex I and III, respectively. Inhibitor concentrations are as follows: 1uM oligomycin, 1.5uM FCCP, 0.1 uM retenone and 1.0 uM antimycin A. Basal respiration rate measurement was determined as the third OCR measurement before oligomycin, and basal glycolytic rate was determined as the third ECAR measurement before oligomycin injection. The OCR to ECAR ratio was determined by dividing the basal respiration rate by the basal glycolytic rate.

Flow cytometry

PBMC's were cultured in a 96 well tissue culture plate at $1.0x10^6$ cells per well in RPMI-1640 with added 1% penicillin/streptomycin, 10% fetal bovine serum at 37°C in 5% CO_2. Cells were stimulated with pandemic H1N1 (pH1N1) multiplicity of infection ~1 for 72 hours. At 66 hours, culture media was removed and fresh media was replaced in the presence of GolgiPlug to inhibit intracellular cytokine export for staining for flow cytometry intracellular antibodies. Cells were stained with the following antibodies: CD3 AmCyan, CD4 V450, CD8 APC-Cy7, CD28 BUV395, CD40L Per-CP Cy5.5, CD69 BV605, IL-12R APC, PD-1 PE-Cy7, IFNg FITC, granzyme B PE, and Zombie Yellow viability dye. Cells were stained with Zombie Yellow viability dye in 1X PBS at room temperature for 15 minutes protected from light. Cells were washed, and incubated with Human BD Fc Block for 10 minutes at room temperature protected from light in PBS with 2% FBS. Extracellular antibody master mix was added and incubated for 30 minutes at 4°C, protected from light. Cells were washed, and Cytofix/Cytoperm (BD Biosciences) was added according to manufacturer's protocol. Cells were washed with permeabilization buffer, and intracellular antibody master mix was added in permeabilization buffer and incubated for 20 minutes at 4°C, protected from light. Cells were washed, and resuspended in 400ul of PBS with 2% FBS. Cells were acquired on the BD LSR II flow cytometer equipped with FACS Diva acquisition software (BD Biosciences) and data analysis was conducted with FlowJo software (FLowJo, LLC). Cells were acquired by exclusion of debris, gated on negative selection by viability staining, doublet exclusion, and then the selection of populations of interest.

Results

Subject demographics for flow cytometry and metabolic assays.

Three groups of participants were recruited from a subgroup of white woman ages 35 to 65: healthy weight non-diabetics (BMI 21.5 to 23.8; 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). PBMC's isolated from participants for flow cytometry assays are found in Table 1. The mean age for the healthy weight women was 49±5.2, 50±8.5 for the obese non-diabetic, and 49±6.3 for the obese metformin-treated diabetic adults. Patients were monitored on a weekly basis for confirmed influenza, or influenza like illness. Confirmed influenza was defined as laboratory reported positive test, and influenza like illness was defined as fever over 100^{o}F, and a cough in the absence of any other medically reported illness. Healthy weight, and obese non-diabetic adults did not report any illness, while there was one obese metformin-treated diabetic adult who reported confirmed influenza or influenza like illness.

Subject demographics for the cells used for metabolic assays can be found in Table 2. Healthy weight non-diabetics (BMI 20.4 to 24.1 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) were recruited as part of the subgroup of women. There was one confirmed influenza or influenza like illness in the healthy weight group, none in the obese non-diabetic group, and one in the obese metformin-treated diabetic group.

No significant differences in percent of populations of CD4+ or CD8+ T cells in unstimulated and pH1N1 stimulated PBMCs isolated from healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

There were no statistical differences of unstimulated or pH1N1 stimulated conditions in CD4+ or CD8+ T cell populations isolated from PBMC's from healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults (Figure 1A, D). CD4+ and CD8+ T cells did not statistically differ between groups at unstimulated conditions (Figure 1B, E) and at pH1N1 stimulated conditions (Figure 1C,F).

No significant differences in CD28 or CD69+ expression in unstimulated and pH1N1 stimulated CD4+ and CD8+ T cells from healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

CD4+ T cells from healthy weight, obese healthy, and obese metformin treated diabetic women within each group increased CD28 signaling marker and early activation marker CD69 from unstimulated to pH1N1 stimulation conditions (Figure 2A). While each group increased expression, these were not significant. Additionally, comparing the groups to each other at unstimulated and stimulated, there were no significant differences (Figure 2B,C). CD8+ T cells from healthy weight had increased expression from unstimulated to pH1N1 stimulated (Figure 2D) compared to obese healthy and obese diabetic, but this was not significant. Each group increased CD28 and CD69 markers to pH1N1 stimulation, but these increases were not significant. Comparisons with the three groups at unstimulated and stimulated did not provide any significant results (Figure 2E,F).

No significant differences in expression of functional markers granzyme B and IFNγ in influenza stimulated PBMC's from healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

CD4+ T cells from healthy weight and obese healthy PBMC's had higher functional markers granzyme B and IFNγ from unstimulated to pH1N1 influenza stimulated conditions, however, the obese diabetic population did not increase (Figure 3A), and these differences were not significant. At unstimulated conditions, there were no differences between the three groups (Figure 3B), and no differences at stimulated conditions (Figure 3C). CD8+ T cells isolated from PBMC's from the healthy weight group slightly increased from unstimulated to pH1N1 stimulated conditions, where the obese healthy did not increase, however these differences were not significant (Figure 3D). CD8+ T cells from obese diabetic PBMC's were lower, and did not increase functional markers from unstimulated to stimulated conditions, however, these differences were not significant (Figure 3D). There were no statistical differences comparing the

three groups expressing functional markers at unstimulated conditions (Figure 3E), and pH1N1 stimulated conditions (Figure 3F).

No significant differences in expression of PD-1 in unstimulated and pH1N1 stimulated CD4+ and CD8+ T cells from healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

PD-1 expression was measured as a cell surface inhibitory marker associated with T cell exhaustion [231]. Within each group in healthy weight, obese healthy and obese metformin treated diabetic subjects there were no significant differences in expression between unstimulated and pH1N1 stimulated CD4+ and CD8+ T cells (Figure 4A,D). Additionally, there were no differences comparing the groups of CD4+ and CD8+ T cells at unstimulated (Figure 4B,E) and pH1N1 stimulated (Figure 4C,F) conditions.

No significant differences in metabolic profile of resting, quiescent CD4+ T cells in healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

There were no statistical differences in OCR and ECAR values between healthy weight, obese non-diabetic, or obese metformin-treated diabetic CD4+ T cells at rest (Figure 5C, D). Additionally, there were no statistical difference in CD4+ T cell OCR to ECAR ratios between healthy weight, obese non-diabetic, or obese metformin-treated diabetic groups, nor showed any trends (Figure 5E).

No significant differences in metabolic profile of activated CD4+ T cells in healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

CD4+ T cells stimulated with anti-CD3/anti-CD28 beads from obese healthy adults had slightly increased OCR (Figure 5C) and ECAR (Figure 5D) compared to healthy weight and

metformin-treated diabetic adults. Stimulated CD4+ T cells from obese metformin-treated displayed slightly lower OCR and ECAR compared to stimulated CD4+ T cells from healthy weight adults (Figure 5C,D). Stimulated healthy weight CD4+ T cells had slightly lower OCR to ECAR ratio compared to obese healthy and metformin-treated diabetic adults, however, these were not statistically different (Figure 5E).

No significant differences in metabolic profile of resting, quiescent CD8+ T cells in healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

Obese healthy, resting CD8+ T cells have slightly increased OCR (Figure 7C) and decreased ECAR compared to healthy weight CD8+ T cells (Figure 7D). Obese metformin-treated diabetic adults have slightly increased OCR compared to CD8+ T cells from healthy weight adults, but decreased OCR compared to obese healthy adults (Figure 7C). CD8+ T cells from obese metformin-treated adults had decreased ECAR compared to the other two groups, however, these results were not statistically significant (Figure 7D). Resting CD8+ T cells from healthy weight and obese healthy adults had decreased OCR to ECAR ratios compared to obese diabetic which was higher (Figure 7E), yet these were not statistically different.

No significant differences in activated CD8+ T cells in healthy weight, obese non-diabetic, and obese metformin-treated diabetic adults

Stimulated CD8+ T cells from obese healthy adults and obese metformin-treated obese adults displayed increased OCR compared to healthy weight adults (Figure 8A). ECAR from stimulated CD8+ T cells from obese metformin-treated adults was slightly higher than healthy weight and obese healthy adults (Figure 8D), however these results were not statistically different. There was no statistical differences in stimulated CD8+ T cells from healthy weight, obese healthy, and metformin-treated diabetic adults in the OCR to ECAR ratios (Figure 8E).

Discussion

Although this study was underpowered in order to achieve statistical significance, we can gleam some insights for future studies. Our results suggest these differences are also cell type specific, and the function of CD4+ vs. CD8+ in adults is differentially affected with obese adults taking metformin. With flow cytometry we did not detect any differences in percent population of CD4+ or CD8+ T cells from the three groups suggesting that impairments in the immune response are function driven, and not a result of decreased cell populations. Studies in other groups showed mice administered metformin improved CD8+ memory T cell function by a TRAF dependent mechanism [161]. This suggests that the diabetic drug metformin taken by obese adults may have cell type specific effects on T cells that may improve response to influenza vaccination and infection. We used anti-CD3/anti-CD8 bead stimulation as a method to polyclonally activate and expand T cell populations for metabolism studies, but also used antigen-specific activation by stimulating with the pandemic H1N1, for the flow studies.

We did not detect any statistically significant results in our studies due to small sample size, but did observe slight trends between the obese healthy and metformin-treated obese adults compared to healthy weight adults. Overall, the metformin-treated obese adults had similar trends to the healthy weight adults. T cells at rest primarily utilize oxidative phosphorylation with low levels of glycolysis, but upon activation upregulate glycolytic activity while decreasing oxidative phosphorylation. CD4+ T cells at rest from metformin-treated obese adults displayed levels of OCR and ECAR that were closer to the healthy weight adults than the obese healthy adults. Upon activation, metformin-treated obese adults did have higher OCR and lower ECAR values than both groups, but the overall OCR to ECAR ratio was more similar to healthy weight, suggesting that metformin-treated obese adults may have improved metabolic health driving better CD4+ T cell functional response compared to the non-treated obese adults. CD8+ T cells from metformin-treated obese adults did display improved metabolism compared to the obese healthy adults. At rest we see increased OCR and decreased ECAR which is expected

metabolism for resting cells. With bead stimulated activation, we see higher ECAR in the metformin-treated obese adult group, which activation should upregulate glycolytic activity and effector function.

Functional data measuring markers granzyme B and IFNγ had lower expression compared to healthy weight and obese healthy adults, yet these differences were not statistically different. We did not detect differences in the activation markers CD28+ and CD69+ in CD4+ or CD8+ T cells, but the healthy weight adults showed increased expression from rest to influenza stimulation conditions. In our previous studies we have seen differences between healthy and obese adults, so adding more sample may help to detect differences between all three populations. With such a small sample size, and the nature of variability of humans it is difficult to determine if these are true differences. It is interesting to note that metformin-treated adults did have increased PD-1 expression, which is typically associated with cell exhaustion [232]. Increased expression of PD-1 would affect the function and signaling mechanisms of T cell to provide an efficient effector response to infection.

Further experiments are needed to determine if these preliminary results hold true with a bigger sample size. Additionally, experiments in mice are needed to determine the specific mechanisms and pathways that are being altered in obesity and metformin treated obesity. As mentioned before, studies in mice have shown improved CD8+ T cell memory response to infection, however, these studies were not performed in the context of obesity. As metformin was administered to already healthy mice, it is possible that metformin enhanced the already functioning memory T cell response. Using metformin treated mice to examine the mTOR pathway, which regulates effector activation and memory T cell differentiation in CD4+ and CD8+ T cells, would shed light on metformin effect on immune cells. Additionally, CD4+ T cells should be metabolically phenotyped by subsets Th1, Th2, Th17 and regulatory T cell populations as these subsets have different metabolism profiles which we may not be detecting differences by looking at overall CD4+ T cells.

Overall, trends in metabolic profiling and flow cytometry data demonstrated that metabolic-treated obese adults may have different immune responses to influenza infection which has not been previously established. Furthermore, determining the mechanisms of impairment is needed to see if metabolism altering drugs may improve immune response in other contexts of disease or infection in obese populations.

Table 1: Flow cytometry subject demographics

	Healthy Weight	Obese Non-Diabetic	Obese Metformin-treated Diabetic	Total
Participants	3	2	3	8
BMI	22.7 ± 1.1	37.6 ± 3.7	37.1 ± 4.5	
BMI Range	21.5-23.8	34.9-40.2	32.5-41.6	
Age (years)	49 ± 5.2	50 ± 8.5	49 ± 6.3	
Age Range (years)	40-58	44-56	37-58	
Race	White	White	White	
Sex	Female	Female	Female	
Diabetes	No	No	Yes	
Metformin	No	No	Yes	
Mean Fasting Glucose (mg/dL)	87 ± 7.2	103 ± 18.4	160 ± 62.4	
Fasting Glucose Range (mg/dL)	81-95	90-116	123-232	
Menopausal (%)				
yes	1 (33.33%)	1 (50%)	1 (33.33%)	3 (37.5%)
no	2 (66.67%)	1 (50%)	2 (66.67%)	5 (62.5%)
Smoking History (%)				
yes	0 (0%)	0 (0%)	2 (66.67%)	2 (25%)
no	3 (100%)	2 (100%)	1 (33.33%)	6 (75%)
Confirmed Flu or ILI (%)				
yes	0 (0%)	0 (0%)	1 (33.33%)	1 (12.5%)
no	3 (100%)	2 (100%)	2 (66.67%)	7 (87.5%)

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

	Healthy Weight	Obese Non-Diabetic	ıse Metformin-treated Diab	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	White	White	White	
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%)

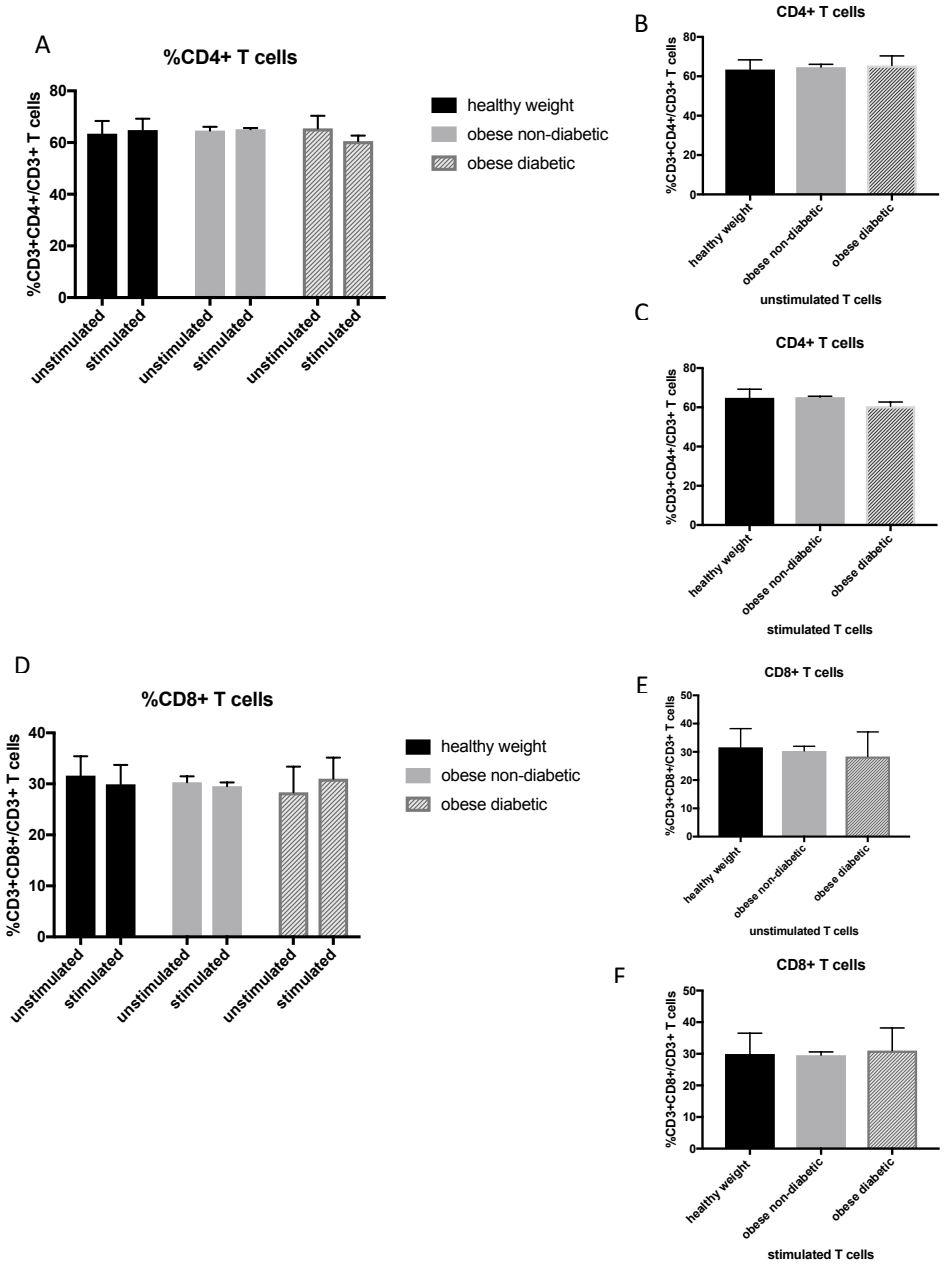


Figure 1. Frequency of CD4+ and CD8+ T cell populations from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. PBMC's were isolated from blood, and cells were characterized by flow cytometry. $1.0x10^6$ freshly isolated cells were stained for CD4+ and CD8+ T cell populations in healthy weight (n=3), non-diabetic obese (n=2), and metformin-treated obese diabetic adults (n=3). Stimulated cells were plated at $1.0x10^6$ cells per well in a 96 well plate, and stimulated with pH1N1 for 72 hours and analyzed by flow cytometry. There were no differences in CD4+ T cell populations comparing unstimulated to stimulated CD4+ T cells in healthy weight, non-diabetic obese, or metformin-treated obese diabetic adults (A). No significant differences of CD4+ T cell populations between the three groups at rest (B) and pH1N1 stimulation (C). No significant differences in CD8+ T cell populations comparing unstimulated to stimulated conditions in healthy weight, non-diabetic obese, and metformin-treated obese adults (D). No significant differences in CD8+ T cell populations at rest (E) and to stimulation (F) between the three groups. Results are displayed as the mean ± SEM.

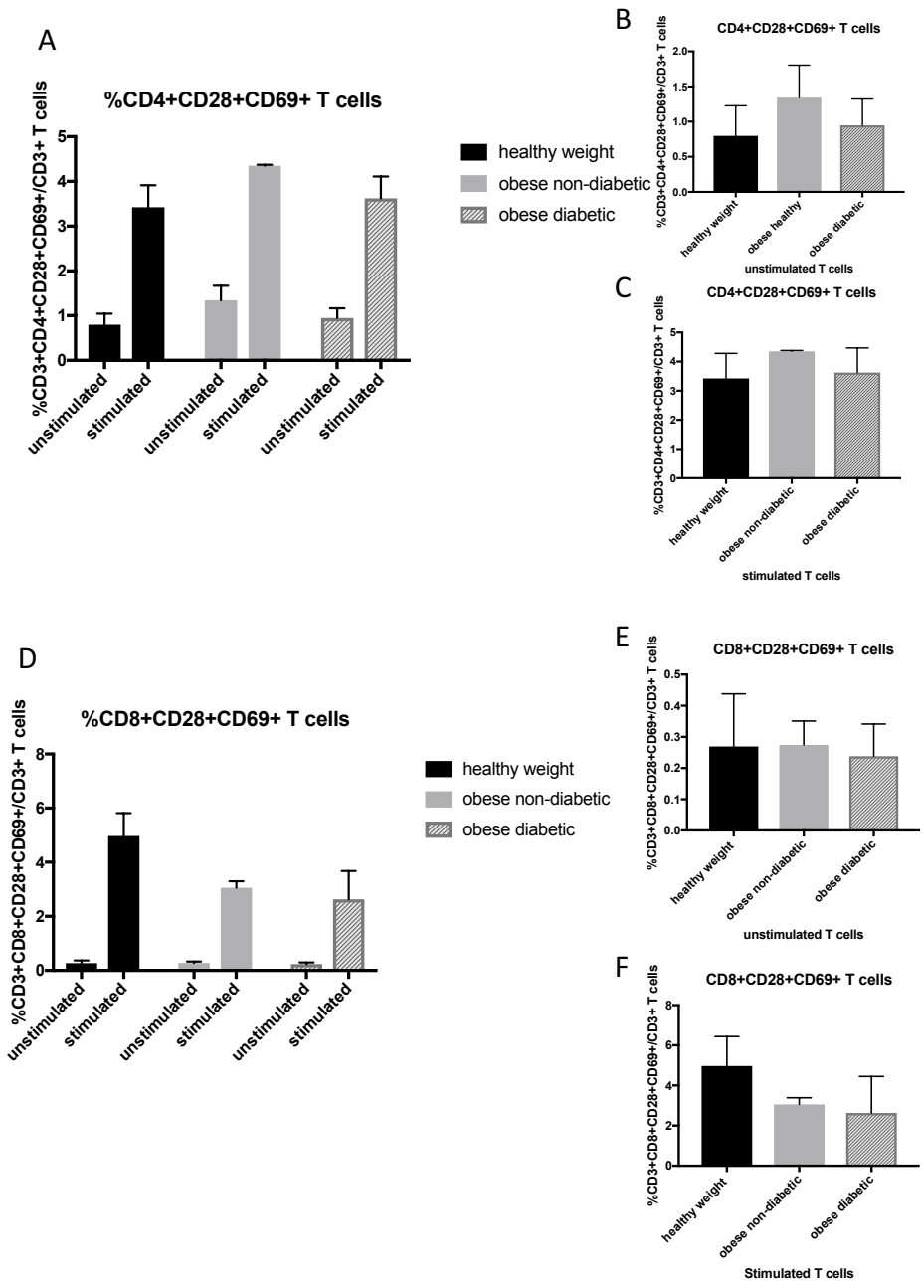


Figure 2. Frequency of CD4+ and CD8+ T activation markers CD28 and CD69 from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. PBMC's were isolated from blood, and cells were characterized by flow cytometry. $1.0x10^6$ freshly isolated cells were stained for CD28 and CD69 activation markers in CD4+ and CD8+ T cell populations in healthy weight (n=3), non-diabetic obese (n=2), and metformin-treated obese diabetic adults (n=3). Stimulated cells were plated at $1.0x10^6$ cells per well in a 96 well plate, and stimulated with pH1N1 for 72 hours and analyzed by flow cytometry. There were no differences in CD4+ CD28+CD69+ T cell populations comparing unstimulated to stimulated CD28+CD69+ T cells in healthy weight, non-diabetic obese, or metformin-treated obese diabetic adults (A). No significant differences of CD28+CD69+ T cell populations between the three groups at rest (B) and pH1N1 stimulation (C). No significant differences in CD8+CD28+CD69+ T cell populations comparing unstimulated to stimulated conditions in healthy weight, non-diabetic obese, and metformin-treated obese adults (D). No significant differences in CD8+CD28+CD69+ T cell populations at rest (E) and to stimulation (F) between the three groups. Results are displayed as the mean ± SEM.

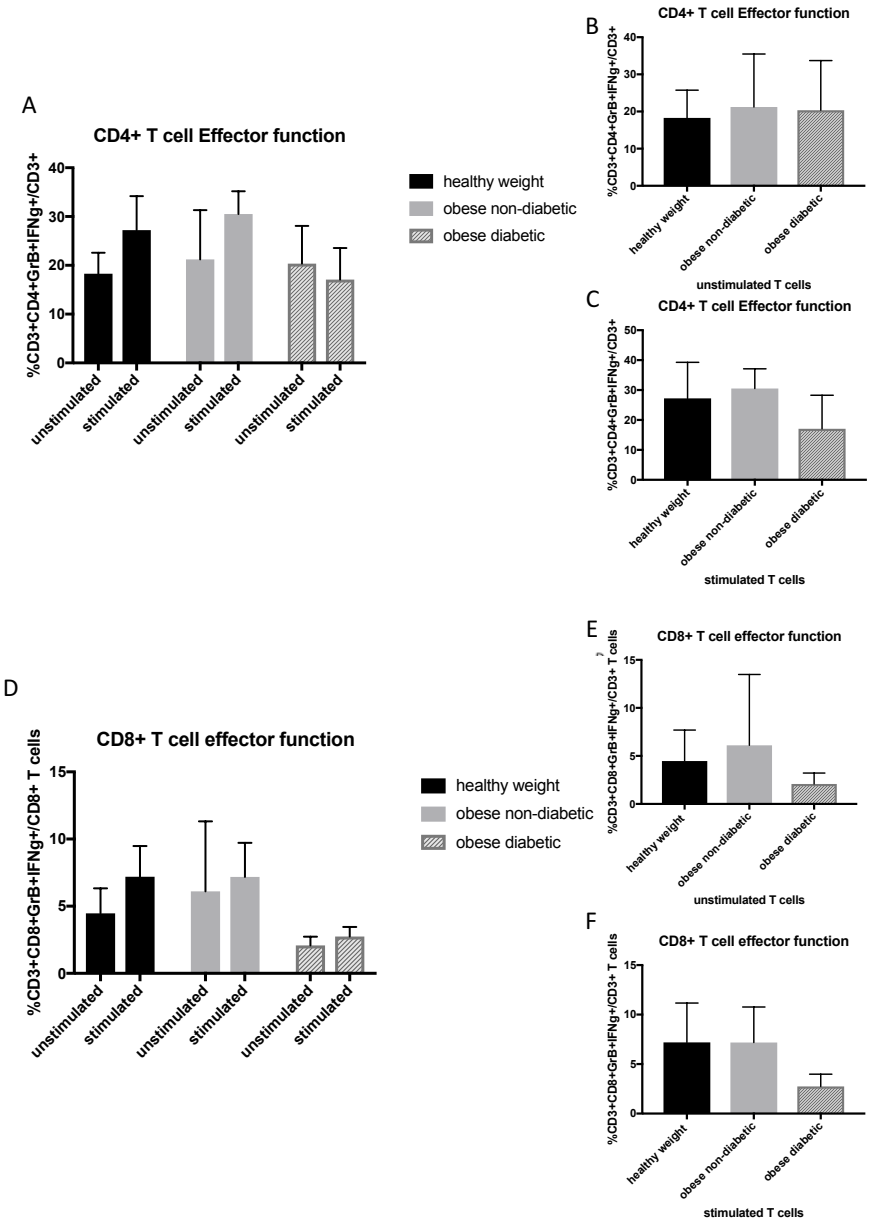


Figure 3. Frequency of CD4+ and CD8+ T functional markers granzyme B (GrB) and IFNγ from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. PBMC's were isolated from blood, and cells were characterized by flow cytometry. $1.0x10^6$ freshly isolated cells were stained for GrB and IFNγ functional markers in CD4+ and CD8+ T cell populations in healthy weight (n=3), non-diabetic obese (n=2), and metformin-treated obese diabetic adults (n=3). Stimulated cells were plated at $1.0x10^6$ cells per well in a 96 well plate, and stimulated with pH1N1 for 72 hours and analyzed by flow cytometry. There were no differences in CD4+GrB+IFNγ+ T cell populations comparing unstimulated to stimulated CD4+GrB+IFNγ+ T cells in healthy weight, non-diabetic obese, or metformin-treated obese diabetic adults (A). No significant differences of CD4+GrB+IFNγ+ T cell populations between the three groups at rest (B) and pH1N1 stimulation (C). No significant differences in CD8+ GrB+IFNγ+ T cell populations comparing unstimulated to stimulated conditions in healthy weight, non-diabetic obese, and metformin-treated obese adults (D). No significant differences in CD8+GrB+IFNγ+ T cell populations at rest (E) and to stimulation (F) between the three groups. Results are displayed as the mean ± SEM.

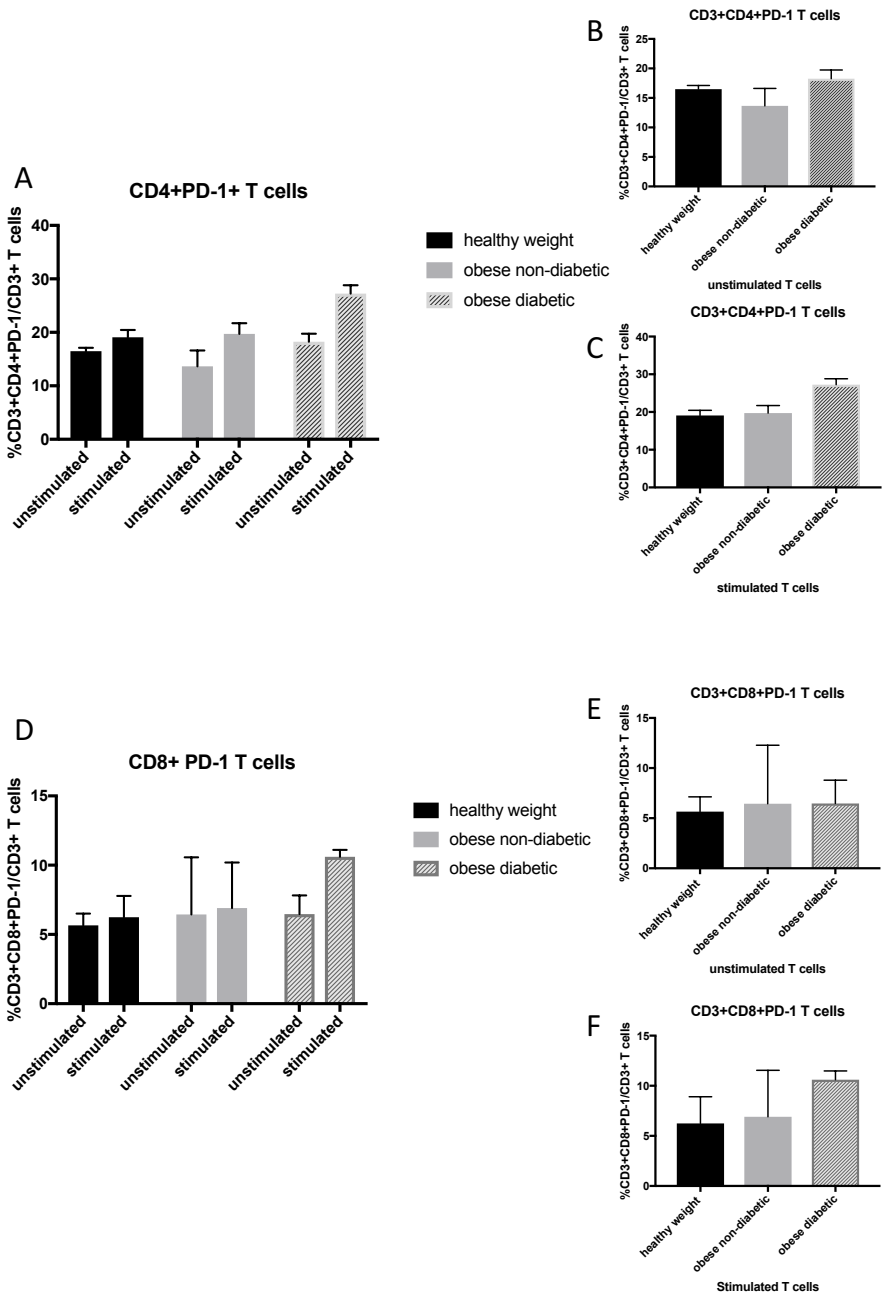


Figure 4. Frequency of PD-1 expression on CD4+ and CD8+ T cells from healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. PBMC's were isolated from blood, and cells were characterized by flow cytometry. $1.0x10^6$ freshly isolated cells were stained for PD-1 cell surface marker in CD4+ and CD8+ T cell populations in healthy weight (n=3), non-diabetic obese (n=2), and metformin-treated obese diabetic adults (n=3). Stimulated cells were plated at $1.0x10^6$ cells per well in a 96 well plate, and stimulated with pH1N1 for 72 hours and analyzed by flow cytometry. There were no differences in CD4+PD-1+ T cell populations comparing unstimulated to stimulated CD4+PD-1+ T cells in healthy weight, non-diabetic obese, or metformin-treated obese diabetic adults (A). No significant differences of CD4+PD-1+ T cell populations between the three groups at rest (B) and pH1N1 stimulation (C). No significant differences in CD8+ PD-1+ T cell populations comparing unstimulated to stimulated conditions in healthy weight, non-diabetic obese, and metformin-treated obese adults (D). No significant differences in CD8+ PD-1+ T cell populations at rest (E) and to stimulation (F) between the three groups. Results are displayed as the mean ± SEM.

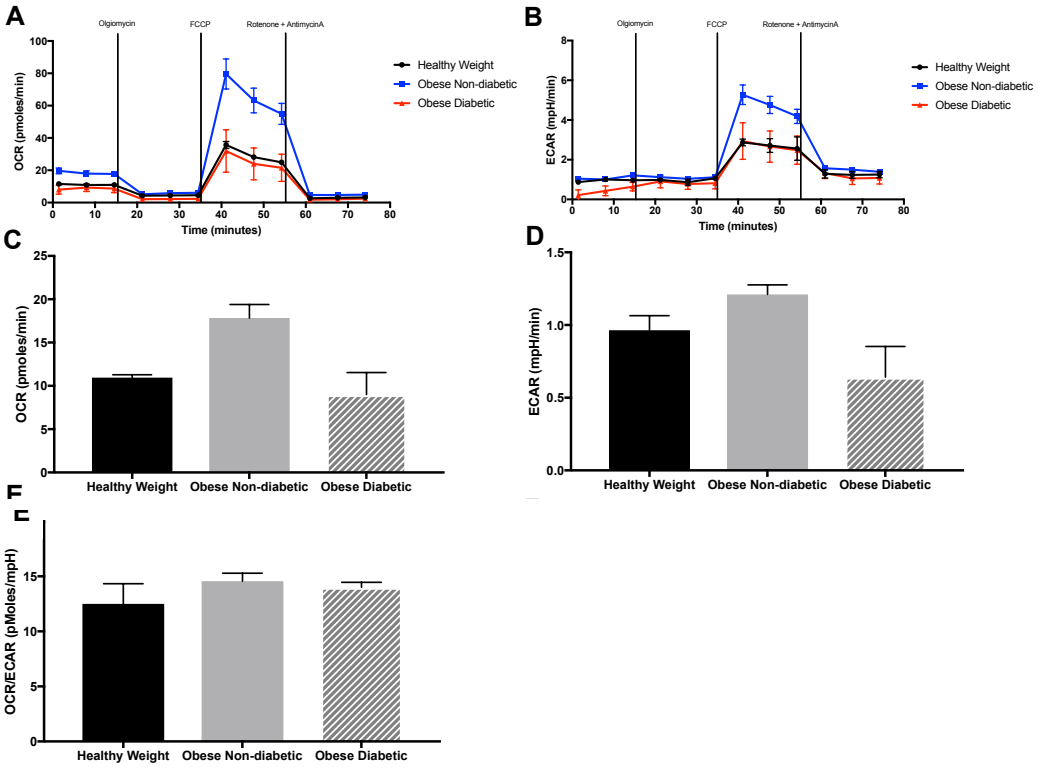


Figure 5. Metabolic profile of quiescent CD4+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of freshly isolated CD4+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects exhibits 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). No differences in trends were seen for OCR:ECAR (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.

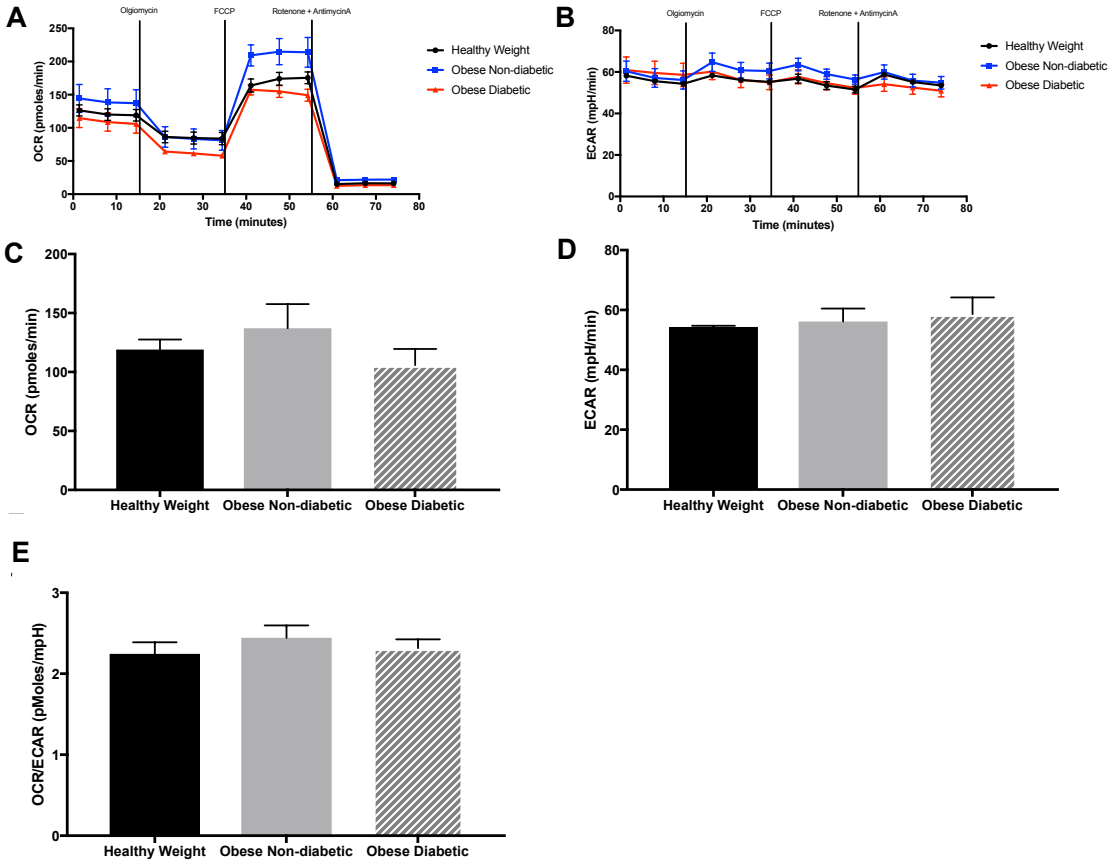


Figure 6. Metabolic profile of activated CD4+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of anti-CD3/anti-CD28 + IL-2 stimulated CD4+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (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 (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 taken from William Green Master Thesis.

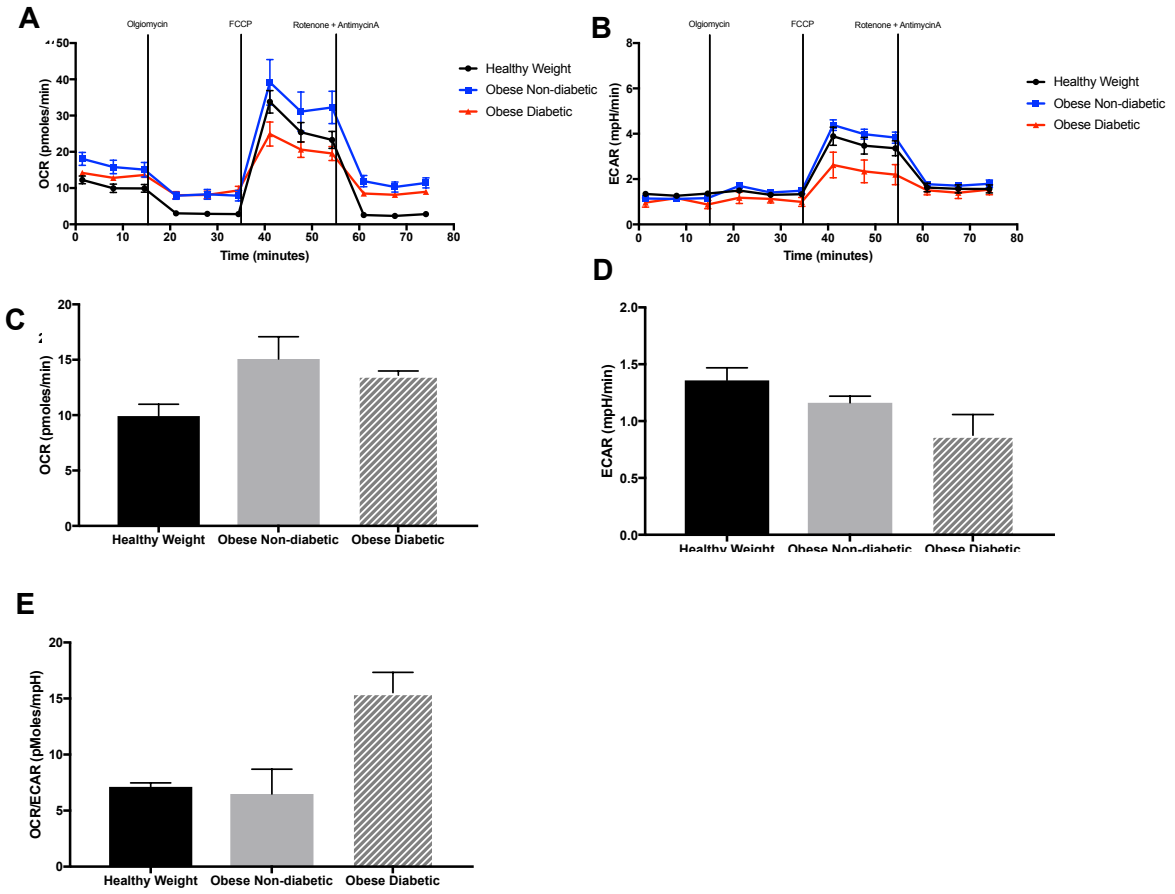


Figure 7. Metabolic profile of quiescent CD8+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of fresh CD8+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (n=3) subjects exhibit changes 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 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 taken from William Green's Master's Thesis.

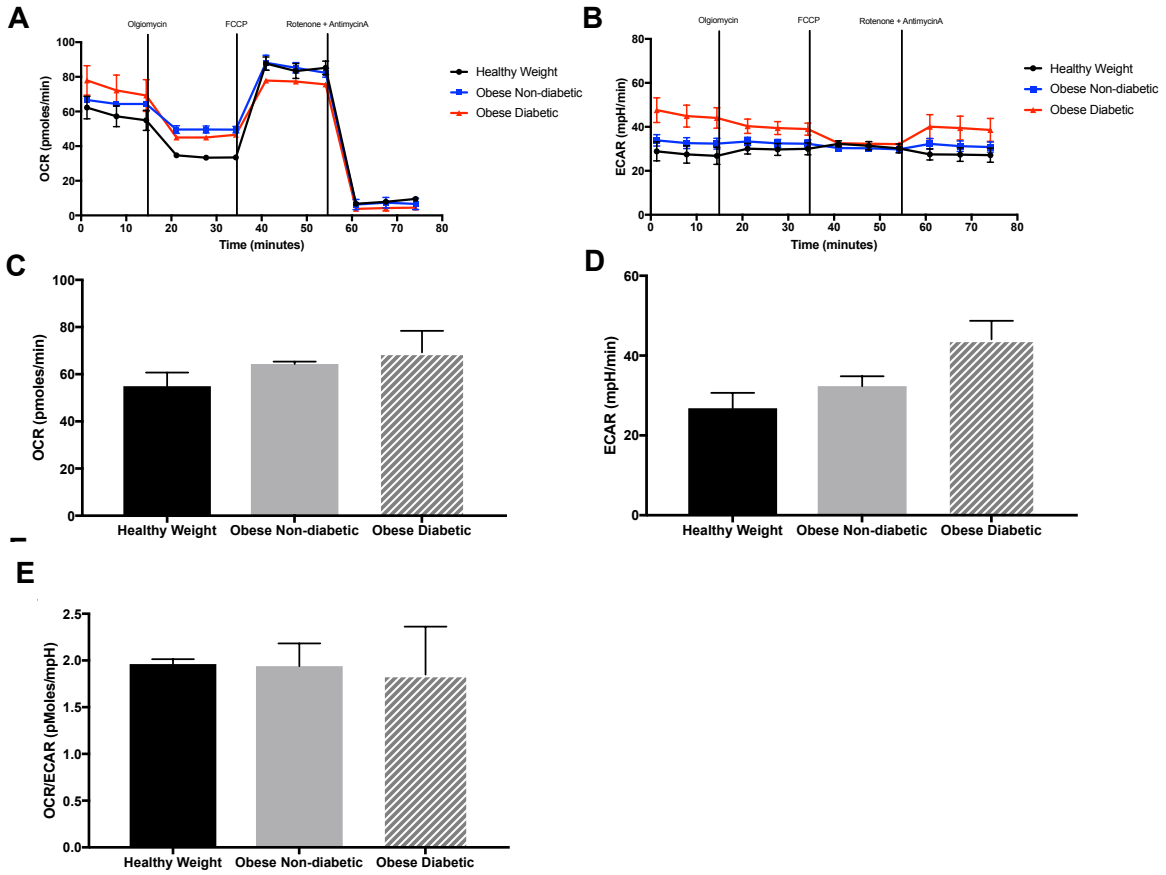


Figure 8. Metabolic profile of activated CD8+ T cells in healthy weight, obese non-diabetic and obese metformin-treated diabetic adults. Mitochondrial stress test of anti-CD3/anti-CD28 + IL-2 stimulated CD8+ T cells from healthy weight (n=3), obese non-diabetic (n=3) and obese metformin-treated diabetic (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 (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 taken from William Green Master Thesis.

CHAPTER V: INCREASED RISK OF INFLUENZA AMONG VACCINATED ADULTS WHO ARE OBESE[1]

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Authors declare no conflicts of interest.

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INTRODUCTION

Influenza is a serious worldwide public health problem. Seasonally, 5-10% of adults and 20-30% of children contract influenza virus, resulting in up to 500,000 deaths [233] and influenza pandemics greatly increase the number of infections and deaths. Indeed, the 1918 influenza

[1] This chapter previous appeared as an article in the *Journal of Obesity*. The original citation is as follows: Neidich SD, Green WD, Rebeles J, Karlsson EA, Schultz-Cherry S, Noah TL, Chakladar S, Hudgens MG, Weir SS, Beck MA. "Increased risk of influenza among vaccinated adults who are obese," *International Journal of Obesity*, no. 10 (June 2012):1038.

pandemic was estimated to have infected 20-40% of the world's population, causing approximately 50 million deaths [234]. Historically, the highest risk groups for increased morbidity and mortality from influenza infection include the elderly [235], the very young [236], individuals with chronic diseases such as diabetes [237] or congestive heart failure [238], and pregnant women [239]. During the 2009 H1N1 pandemic, obesity was recognized as an independent risk factor for complications from influenza [240] and continues to be a risk factor for seasonal influenza strains [241] as well as for emerging influenza virus strains such as A(H7N9) [242]. Obesity is not only a concern in the US, with 37% of adults obese [243], but also affects 14% of the worldwide adult population [5]. Therefore, with a growing obesity epidemic, complications from influenza infection would be expected to increase.

Influenza vaccine remains the primary method currently available for prevention of influenza infection. Each year, vaccines are formulated based on evaluations of previously circulating influenza strains. Typically, the vaccine contains two influenza A strains and one, or more recently two, influenza B strains. Vaccine-generated antibodies against the viral surface protein hemagglutinin (HA) are considered to be protective, therefore vaccines are standardized to the quantity of HA, generally 15 μg of HA per strain [244]. A serum hemagglutination inhibition (HAI) titer of 40 or greater has historically been considered an immunological correlate of protection from influenza infection, corresponding to 50% protection [245]. Protection against influenza infection increases up to an HAI titer of 160, beyond which further protective capacity is minimal [180]. High risk groups for influenza infection, including the elderly and children under 6 years of age, may need to reach titers greater than 40 to achieve protection [246]. To determine if obesity altered the risk of developing influenza or ILI in a vaccinated adult population, we report the incidence of influenza infection and influenza-like illness (ILI) in vaccinated obese and healthy weight adults as well as the extent to which participants with influenza infection and ILI produced influenza specific antibodies.

SUBJECTS AND METHODS

Study Design

Participants were recruited as a part of a prospective observational study carried out at the University of North Carolina at Chapel Hill Family Medicine Center, an academic outpatient primary care facility in Chapel Hill, North Carolina. All procedures were approved by the Biomedical Institutional Review Board at the University of North Carolina. At enrollment, informed written consent was received.

Participants

Recruitment criteria for this study were adults 18 years of age and older receiving the seasonal trivalent inactivated influenza vaccine (IIV3) for the years 2013-2014 and 2014-2015. Exclusion criteria were immunosuppressive diseases including HIV, use of immunomodulatory or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillain-Barre syndrome, use of theophylline preparations, or use of warfarin. Height and weight were measured and a baseline serum sample drawn. BMI for each participant was calculated as weight (kg)/height(m) [234]. Healthy weight was defined as a BMI of 18.5-24.9, overweight as a BMI of 25-29.9 and obese as a BMI of ≥30.

Vaccines and Sample Collection

One dose of 2013-2014 trivalent inactivated influenza vaccine (0.5 mL Fluzone; Sanofi Pasteur, Swiftwater PA, USA) containing A/California/07/2009 H1N1, A/Texas/50/2012 H3N2, and B/Massachusetts/02/2012 or 2014-2015 trivalent inactivated influenza vaccine (0.5 mL Fluvirin; Novartis Vaccines and Diagnostics Limited, Basel, Switzerland) containing A/California/07/2009 H1N1, A/Texas/50/2012 H3N2, and B/Massachusetts/02/2012 was administered in the deltoid muscle, using an inch and half needle, at baseline. Participants

returned 26-35 days later for a post-vaccination blood draw. In the 2013-2014 vaccine year, vaccination of participants started on September 16, 2013 and the last vaccination was given on November 4, 2013. Influenza was first detected in NC on November 30, 2013, and cases peaked on January 11, 2014, with influenza levels back to baseline on May 17, 2014. In the 2014-2015 vaccine year, vaccination of participants started on September 15, 2014 and were completed on October 28, 2014. Influenza was first detected in NC on November 29, 2014 and cases peaked on December 27, 2014. Influenza levels were back to baseline on April 25, 2015. Serum samples were stored at -80^{o} C until analyzed. During the 2013-2014 season in North Carolina, influenza 2009 A/H1/N1 was the predominate circulating strain, and during the 2014-2015 season, influenza A/H3N2/Switzerland was the predominate circulating strain.

Surveillance and diagnosis of influenza and/or influenza-like illness (ILI)

Participants were contacted weekly beginning with the first report of influenza activity in the community and contact was discontinued when influenza was no longer active. Participants were contacted by phone or email and asked to report any symptoms of fever, cough, runny nose, sore throat, muscle aches, headaches and fatigue to assess for influenza symptoms. Medical records of all study participants, whether they reported ILI or not, were reviewed at the end of each season for medically reported influenza-like illness or laboratory confirmed influenza. Participants were also instructed to contact the study nurse if they developed ILI. Laboratory confirmed influenza infection was determined from the medical records which reported a positive influenza specimen using the FDA cleared Cepheid Xpert Flu assay (Cepheid, Sunnyvale, CA). This assay distinguishes between influenza A and influenza B strains, but does not subtype the strains. All participants who tested positive for influenza were diagnosed with influenza A. ILI was defined using the CDC guidelines [247] as fever greater than 100^{o} F with a cough and in the absence of any other medical diagnosis. All subjects with laboratory confirmed influenza also met our criteria for ILI.

Immunogenicity

Hemaglutination Inhibition (HAI) Assay

The HAI titer was blindly determined in accordance with World Health Organization guidelines [248] for all patients reporting laboratory confirmed influenza or ILI, as well as matched non-illness reporting participants.

Microneutralization (MN) Assay

Standard microneutralization (MN) were blindly performed according to WHO guidelines [248]. Luminescent MN assays were blindly performed as previously described using a reverse genetics A/California/04/2009 virus or A/Texas/50/2012 containing an NLuc on its polymerase segment [78]. MN were conducted for participants with laboratory confirmed influenza and matched controls who did not report any ILI during influenza season.

Matched non-illness reporting participants

For every participant who either had laboratory confirmed influenza or reported ILI, we matched them with a non-illness reporting participant. Matching of the non-illness reporting participants was done on a one-to-one basis with the 74 participants with either confirmed influenza or reporting ILI based on the following criteria in the order provided: 1) same vaccine year; 2) sex; 3) Race; 4) weight category; 5) diabetes status; 6) statin use; 7) smoking status; 8) age (within 10 years). All samples were uniquely matched.

Statistics

Individuals were categorized as underweight (BMI < 18.5), healthy weight (BMI 18.5 - 24.9), overweight (BMI 25.0 - 29.9), or obese (BMI ≥ 30). The Jonckheere–Terpstra test was employed to assess associations between baseline covariates and the ordinal weight category. Risk ratios for laboratory confirmed influenza and influenza-like Illness (ILI) were estimated by

fitting a log-binomial model using generalized estimating equations (GEE) with an exchangeable working correlation structure to account for repeated observations per individual. Logistic regression models fit using GEE were utilized to examine associations of diabetes and statin use with obesity and risk of influenza/ILI. Microneutralization and HAI results were analyzed via 2-way repeated measures analysis of variance (ANOVA). The Wilcoxon signed-rank test was used for comparisons between matched pairs. Seroconversion and seroprotection were analyzed by the chi-square test of independence. P-values less than 0.05 were considered statistically significant. All analyses were conducted in Graphpad Prism, Stata, and/or R.

RESULTS

Demographics of Participants

During the 2013-2014 vaccine year, we enrolled 587 participants and 575 (98.0%) completed the study. During the 2014-2015 vaccine year, we enrolled 455 participants and 447 (98.2%) completed the study. As shown in Table 1, overall, our participants were 27% healthy weight, 28% overweight and 44% obese. In both years of the study, approximately 60% of the participants were Caucasian and 30% African-American. Female participants represented approximately 63% of the total participants. As has been reported for other studies, African-Americans [187, 249] and diabetics [250] were more likely to be obese, and statin use was associated with higher BMIs. However, statin use and diabetes was not independently associated with influenza or influenza-like illness. Most of the participants were either non-smokers, or had never smoked, with approximately 17% current smokers. There were 184 study subjects who participated in both years of the study, fifteen of which reported ILI in one or both years of the study.

Relative Risk for Influenza and ILI

In the total vaccinated adult participants for both vaccine years, there were 10 laboratory confirmed cases of influenza A and 64 cases of ILI (Table 2). Of the 74 participants with either confirmed influenza or ILI, 19% were healthy weight, 22% were overweight and 59% were obese. Relative to influenza incidence in vaccinated healthy weight adults, vaccinated obese adults had double the risk for laboratory confirmed influenza considered together with ILI (estimated risk ratio 2.06 with 95% confidence interval 1.14, 3.71). Risk ratio estimates were similar when adjusting for age, sex, and smoking status using log-binomial regression (results not shown). Diabetes and statin use were not associated with influenza or influenza-like illness, however, as expected, BMI category was a significant predictor of diabetes and statin use.

Seroprotection and Seroconversion

Among the 74 cases of confirmed influenza or ILI during 2013-2014 and 2014-2015, 30 (41%) seroconverted (four-fold increase from pre- to post-vaccination HAI titer) to vaccine strain A/H1N1/California/pdm2009 and 34 (46%) seroconverted to vaccine strain A/H3N2/Texas/50/2012. For these same 74 participants, 70% reached a seroprotective titer (26-35 day post vaccination HAI titer $\geq$ 40) for A/H1N1/California/pdm2009 and 80% reached a seroprotective titer for A/H3N2/Texas/50/2012. However, no differences were observed in seroprotective or seroconversion rates based on BMI (Table 3 and Figures 1a-b).

During the 2014-2015 influenza season, the H3N2 vaccine strain was a poor match for the circulating strain [187]. Therefore, for the 43 cases of laboratory confirmed influenza or ILI during 2014-2015, we measured HAI titers pre and post vaccination against the circulating influenza A/H3N2/Switzerland/9715293/2013 strain. Seroconversion for this strain occurred in 19 (44%) participants, and seroprotection was present in 30 (70%) participants. Again, no differences were observed based on BMI (Table 3 and Figures 1c). Higher, alternative cutoffs for seroprotection levels of HAIs at 80, 160 and 320 were also determined, and no differences were observed based on BMI (Table 3).

Laboratory confirmed Influenza and ILI compared to non-illness reporting matched controls

All 74 participants with either laboratory confirmed or ILI were matched with non-illness reporting participants and their demographics are shown in Supplemental Table 1. There were no differences in pre or post HAI titers for the vaccine strains A/California/H1N1/pdm2009 (Figure 1d) and A/Texas/H3N2/50/2012 (Figure 1e) or for the circulating 2015 influenza strain A/H3N2/Switzerland/9715293/2013 (Figure 1f) between participants reporting ILI and their non-reporting matched controls. Similarly, there was no difference in HAI (Figures 1g-h) or MN influenza titers (Figures 1 j-k) between participants with laboratory confirmed influenza and their matched, uninfected controls.

DISCUSSION

The first influenza pandemic of the 21st century resulted in identifying obesity as an independent risk factor for increased severity from infection with Influenza A/pH1H1/2009[8]. Since that time, obesity has also been identified as a risk factor for seasonal and emerging influenza strains. This is highly significant, in that obesity levels in the US population are at epidemic proportions, with 37% of adults overall obese [243] and even higher rates in non-Hispanic blacks (48%) [249]. Obesity rates worldwide have doubled since 1980 and currently 13% of the world's adult population is obese [5] leaving a large number of obese adults in the US and worldwide at significant risk for infection with influenza virus.

Influenza vaccination represents the best method of protection from infection with influenza virus. Several studies have suggested that overweight and obesity impairs vaccine response to several pathogens. For example, non-responders to hepatitis B vaccination are overrepresented in obese adults [76] while tetanus toxoid response in overweight children is

similarly impaired [251]. A recent review on the association of obesity with vaccine responses points to a number of studies that demonstrate diminished vaccine-induced immune responses in both obese adults and children [252]. We have also documented impaired vaccine-specific T cell responses in influenza vaccinated obese adults [81] and a waning serological response one year post vaccination [79]. Despite the growing number of studies implicating obesity in poor responses to vaccination, and specifically influenza vaccination, a key question remains unanswered: in obesity and healthy weight, does vaccination offer the same protection from influenza and ILI?

Here, for the first time, we demonstrate that obese adult recipients of IIV3 have two times greater incidence of influenza and/or ILI despite being vaccinated. One obvious hypothesis for the increase in influenza and ILI in obese adult participants is a failure to seroconvert or reach seroprotective levels of antibody. Serological responses to influenza vaccination are typically assessed as seroprotection, defined as an HAI titer of 40 or greater post vaccination, or seroconversion, defined as a 4 fold or greater increase in HAI from prevaccination titer to post vaccination titer. However, we found that the increased susceptibility to influenza and ILI in the obese adults was not associated with a failure to reach a seroprotective titer or to seroconvert. Indeed, we found no statistical differences in serological responses to vaccine between healthy weight and obese vaccinated adults. For the H1N1 strain, 36% of healthy weight adults seroconverted compared with 43% of obese adults. Similarly, seroconversion rates to the H3N2 vaccine strain were 43% of healthy weight adults and 50% of obese adults. When using the commonly defined seroprotective HAI titer of ≥40, more than 70% of the healthy weight and obese participants reached this HAI level.

The presence of a “seroprotective” level of antibody against influenza A strains demonstrates that, despite the vaccine inducing this this correlate of protection, the obese adults were still 2X more likely to develop influenza and ILI. This lack of protection, even with a seroprotective antibody titer, has also been observed in elderly and children [246], where a higher HAI definition as a correlate of protection has been proposed. Our data, however, do not suggest

an elevated definition is protective for obese adults. Raising the seroprotective cutoff level to 80, 160 and 320 still failed to differentiate healthy weight adults from obese adults.

The 2014-2015 influenza vaccine effectiveness overall was reduced (13% vs 61% in 2013-2014) due to the circulating Influenza A H3N2 strain having drifted from the H3N2 vaccine strain [76]. Therefore, for all participants who had influenza or ILI during the 2014-2015 vaccine season, we measured HAI antibody titer against the circulating A/Switzerland/9715293/2013 strain. Despite the mismatch with the vaccine strain, IIV3 induced seroconversion among 67% of the healthy weight and 32% of the obese participants. For a seroprotective level of ≥40 HAI, 67% of healthy weight and 60% of obese participants achieved this level. There were no statistical differences in seroprotection or seroconversion rates between healthy weight and obese adults.

We found no differences in HAI titers between non-illness reporting participants and participants reporting ILI. In addition to HAI, virus microneutralization (MN) titers are a highly sensitive and specific method for detecting antibodies that inhibit viral entry or exit out of the cell. Cheng *et al*. [253] reported that, compared to HAI titers, MN titers demonstrated a greater seroconversion rate and fold increase and suggested that neutralizing antibody titers may be a better correlate of protection for understanding influenza vaccine effectiveness. However, as was found for HAI titers, there were no differences in MN titers between uninfected controls and infected participants.

Our study has several limitations. Although we used the CDC's stringent definition for ILI and ILI is widely used for influenza surveillance reporting, we do not have nasal swabs from subjects with ILI. Therefore, we could be over-reporting, as some of the ILI subjects may be positive for a respiratory virus other than influenza, or under-reporting, as the more stringent CDC criteria may miss some milder ILI symptoms that are influenza positive. By only collecting ILI data during times of influenza circulating in the community, this helps to reduce over-reporting, but it doesn't eliminate this possibility. In addition, our study does not address the possibility that obese adults may be more exposed to influenza compared with healthy weight adults. Under this possibility, the influenza vaccine may equally protect healthy weight and obese adults, however

an increased rate of infection exposure in obese adults could lead to an increased rate of infection in vaccinated obese adults compared with healthy weight adults. However, Murphy et al. [254] used data from the 2010 Health Survey for England, which asked in a survey question administered during the year following the 2009 H1N1 influenza pandemic whether participants had experienced "flu-like illness where [respondents] felt feverish and had a cough or sore throat," and considered cases between May and December 2009 to be flu-like illness in that study. The investigators found no relationship between ILI (including laboratory confirmed influenza) and obesity. This finding may suggest that influenza infection rates in healthy weight and obese adults are similar, and therefore our findings are related to a failure of the vaccine to protect obese adults to the same extent as healthy weight adults. Indeed, in an animal model, Karlsson *et al.* [78] reported that although lean mice were protected from influenza infection following vaccination, diet-induced obese mice were still susceptible to influenza infection despite vaccination. This contrasts with school aged children, where live attenuated influenza vaccination was shown to reduce risk for laboratory confirmed influenza similarly for healthy weight and obese [255]. These contrasting findings may be driven by differences in vaccine preparation (live versus inactivated), or by differences between obese adults and children.

The findings reported here demonstrate that, compared to vaccinated healthy weight adults, vaccinated obese adults were 2X more likely to develop influenza infection and ILI. Notably, HAI antibody titers, widely viewed as correlates of protection against influenza, were unreliable as predictive of disease protection in obese adults. Previously, we [79] and others [256-258] have reported that HAI antibody titers 30 days post vaccination in obese adults or children are either slightly higher or no different from vaccinated healthy weight individuals. The present study confirmed these earlier reports on vaccine-induced antibody titers. However, here we found that an HAI antibody titer of 40 or higher was not a serological correlate for vaccine-induced protection and did not prevent laboratory confirmed influenza and ILI in obese adults. Additionally, MN titers in obese adults were also inadequate predictors of protection and these studies directly correlate with studies conducted in obese mice [78]. Although our study does not compare vaccinated obese adults with unvaccinated obese adults, it is clear that vaccinated

obese adults are at a higher risk for influenza and ILI compared to vaccinated healthy weight adults.

The mechanism for increased risk of influenza and ILI in the obese population may be due to poor T cell function. As we have reported previously, compared with T cells from vaccinated healthy weight adults, T cells from influenza vaccinated obese adults are less activated and less functional when stimulated with vaccine strains of influenza [79, 81]. As T cells are necessary for protection and recovery from influenza, impaired T cell function, despite a robust serological response, may render vaccinated obese adults more susceptible to influenza infection. Indeed, vaccinated elderly adults are also less protected from influenza infection despite having an adequate serological response, which was attributed to poor T cell responses [259].

Taken together, these results suggest that the effectiveness of influenza vaccines, and perhaps other vaccines as well, should be fully assessed in obese adults. Alternative approaches may be needed to protect obese adults. For example, use of adjuvanted influenza vaccines such as MF59 (FLUAD, Seqirus) or high-dose vaccine preparations recommended for vaccinating adults over 65 may be warranted for use in an obese population.

ACKNOWLEDGEMENTS

We thank our study coordinator, Kim Bartholomew, RN, MPH and all the study participants for their contribution. We thank Savanna Ledford and Qing Shi for technical assistance. This work was funded by NIH grants R01AI078090 and P30DK056350 to MAB and ALSAC to SSC.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Table 1: 2013-2014 Influenza Season Enrollment

		Underweight	*Healthy weight*	*Overweight*	*Obese*	*Total*
	Enrolled	6 (1.0)	143 (24.9)	170 (29.6)	256 (44.5)	575(100)
	Age*	53.0 ± (19.9)	55.8 ± 18.3	53.5 ± 16.2	54.1 ± 12.2	
Gender						
	Male	3 (0.5)	53 (9.2)	73 (12.7)	75 (13.0)	204 (35.5)
	Female	3 (0.5)	90 (15.7)	97 (16.9)	181 (31.5)	371 (64.5)
Race						
	Caucasian	6 (1.0)	103 (17.9)	115 (20.0)	140 (24.3)	364 (63.3)
	African American		24 (4.2)	43 (7.5)	108 (18.78)	175 (30.4)
	Other		16 (2.8)	12 (2.1)	8 (1.4)	36 (6.3)
Diabetes						
	Yes- Type 1		3 (0.5)	1 (0.2)	1 (0.2)	5 (0.9)
	Yes- Type 2		11 (1.9)	30 (5.2)	86 (15.0)	127 (22.1)
	Pre-Diabetes	1 (0.2)	7 (1.2)	10 (1.7)	29 (5.0)	47 (8.2)
	No	5 (0.9)	122 (21.2)	129 (22.4)	140 (26.3)	396 (68.9)

*Table 1: Demographics for the 2013-2014 seasonal enrollment. *Age is reported as average as average at day of years (standard deviation). All other values are number of participants (percent makeup of total).*

Table 2: 2014-2015 Influenza Season Enrollment		*Underweight*	*Healthy weight*	*Overweight*	*Obese*	*Total*
	Enrolled	6 (1.3)	134 (30.0)	112 (25.1)	195 (43.6)	447 (100)
	Age*	54.4 ± 20.9	54.1 ± 18.6	59.4 ± 16.7	54.4 ± 13.6	
Gender						
	Male	2 (0.4)	45 (10.1)	54 (12.1)	56 (12.5)	157 (35.1)
	Female	4 (0.9)	89 (19.9)	58 (13.0)	139 (31.1)	290 (64.9)
Race						
	Caucasian	3 (0.7)	98 (21.9)	74 (16.6)	112 (25.1)	287 (64.2)
	African American		20 (4.5)	33 (7.4)	74 (16.6)	127 (28.4)
	Other	3 (0.7)	16 (3.6)	5 (0.9)	9 (2.0)	33 (7.4)
Diabetes						
	Yes- Type 1			1 (0.2)	2 (0.4)	3 (0.7)
	Yes- Type 2		9 (2.0)	19 (4.3)	57 (12.8)	85 (19.0)
	Pre-Diabetes	1 (0.2)	2 (0.4)	7 (1.6)	18 (4.0)	28 (6.3)
	No	5 (1.1)	123 (27.5)	85 (19.0)	118 (26.4)	331 (74.0)

Table 2: *Demographics for the 2014-2015 seasonal enrollment. *Age is reported as average as average at day of years (standard deviation). All other values are number of participants (percent makeup of total).*

Table 3: Influenza-Like Illness during 2013-2015 Flu Seasons

		Underweight	*Healthy Weight*	*Overweight*	*Obese*	Total
2013-2014	Laboratory confirmed influenza	-	1	1	1	3
	Influenza-like Illness (excludes confirmed)	-	4	6	17	27
	No Flu-like Illness	6	138	163	238	545
2014-2015	Laboratory confirmed influenza	-	1	3	3	7
	Flu-like Illness (excludes confirmed)	-	8	6	22	36
	No Flu-like Illness	6	125	104	169	404
Total		12	277	282	451	1022
Odds Ratio vs Healthy Weight		1.40	1	1.13	1.98	
95% CI		(0.04-12.90)	(0.47-2.14)	(0.54-2.35)	(1.06-3.40)	
Significance		n.s.	n.s.	n.s.	*	

*Table 3: Summary of which participants acquired influenza during the subsequent season. Data incorporates participants from both 2013-2014 and 2014-2015. *Obese vaccinated participants were at a significantly greater risk of Flu-like illness (Odds ratio=2.03, 95% CI 1.02-4.08 p=0.0451). Note: Underweight participants are not included on this table.*

Table 4: Seroconversion among influenza-like Illness during 2013-2014 and 2014-2015

	A/California/09/H1N1					A/Texas/50/H3N2					A/Switzerland/H3N2				
	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N
Healthy Weight	41.67%	ns	66.67%	ns	12	33.33%	ns	66.67%	ns	12	75.00%	ns	62.50%	ns	8
Overweight	42.86%	ns	64.29%	ns	14	35.71%	ns	78.57%	ns	14	55.56%	ns	100.00%	ns	9
Obese	40.54%	ns	70.27%	ns	37	51.35%	ns	89.19%	ns	37	33.33%	ns[c]	58.33%	ns	24
Combined	41.27%		68.25%		63	44.44%		82.54%		63	46.34%		68.29%		41

Table 4: Seroconversion and Seroprotection rates in participants who presented with Influenza-like illness. Data are shown as percentage of data points. Only participants from 2014-2015 were assessed for A/Switzerland/H3N2. Data from 10 subjects were not analyzed for pH1N1 and a/Texas/50/H3N2, including one eligible for A/Switzerland/H3N2 analysis. a: Seroconversion is defined as 4-fold or greater increase in HAI. b: Seroprotection is defined as HAI≥40 at 30 days post vaccination. c: Seroconversion was insignificantly lower in obese subjects compared with healthy weight, p=0.0525.

Table 5: Demographics of Case-confirmed Influenza Participants	Age	BMI	Gender		Race		Study Year		Total Participants
			Female	Male	Caucasian	African American	2013-2014	2014-2015	
Confirmed Flu	64.31 ± 16.71	32.65 ± 6.71	7	3	5	5	3	7	10
Matched Controls	62.42 ± 16.02	33.16 ± 9.07	7	3	5	5	3	7	10

Table 4: Demographics of participants who acquired clinically verified influenza infection and matched controls. Age reported in years ± standard deviation, BMI in kg/m^2 ± standard deviation.

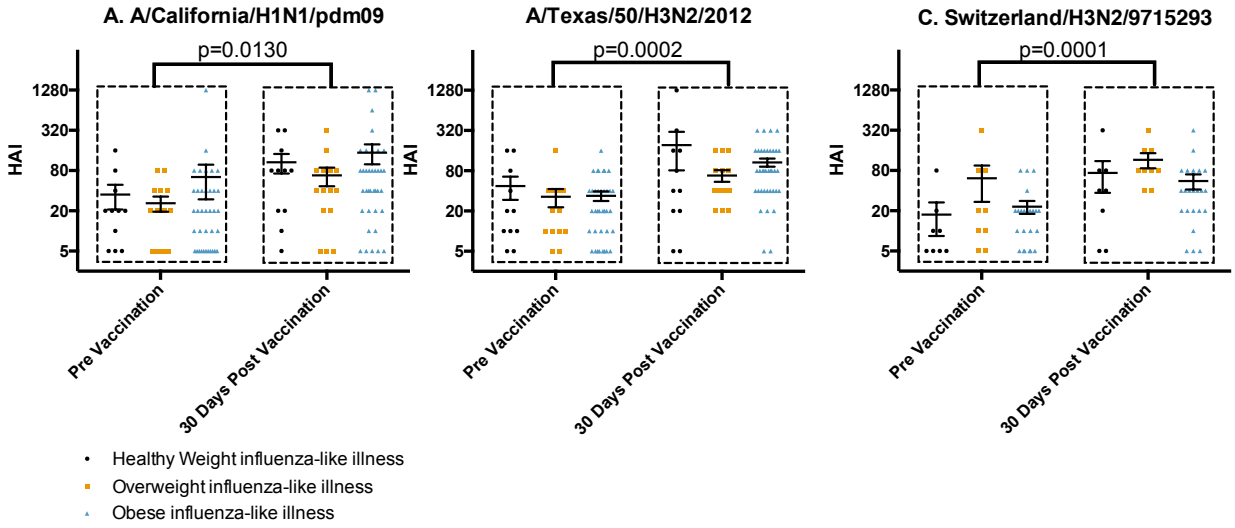

Figure 5.1. Weight does not impact Influenza HAI antibody generation. HAI for all subjects with flu/flu like illness displayed by weight status. HAI shown against vaccine-included strains A/California/H1N1pdm09 (A) and A/Texas/50/H3N2 (B), and major circulating strain A/Switzerland/9715293 (C). Comparison between weight and time post vaccination was assessed by ANOVA, $p<0.05$ is indicated.

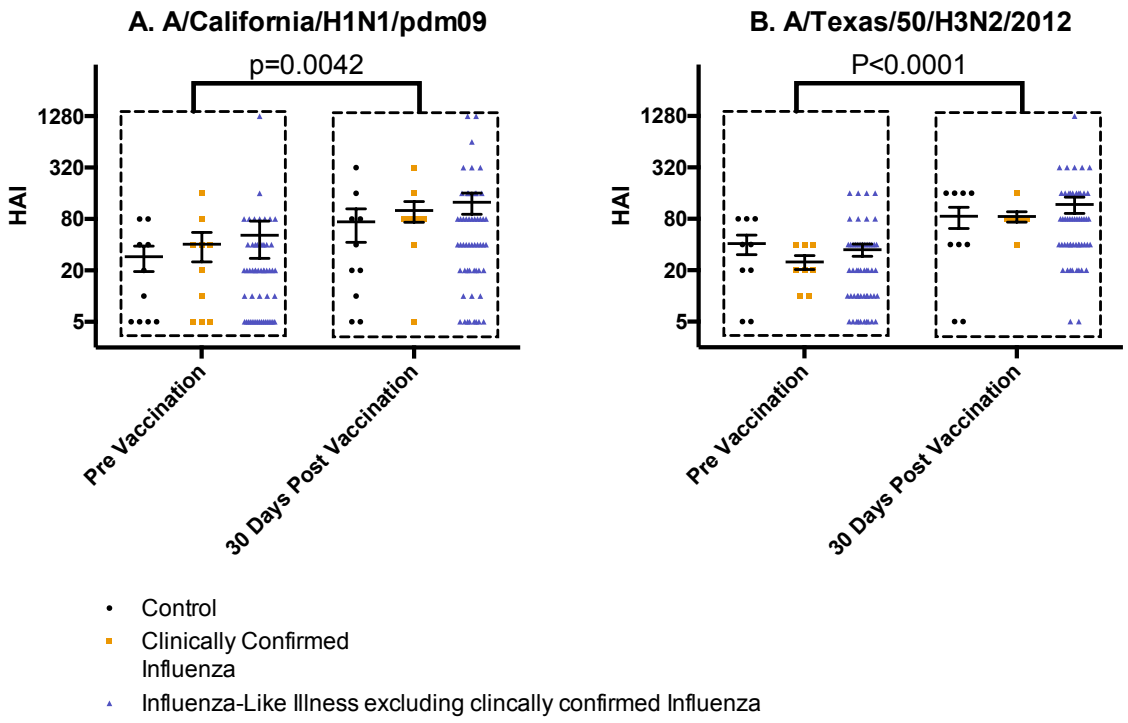

Figure 5.2. Confirmed Influenza was not predicted by HAI. Hemaglutination Inhibition against A/California/H1N1/pdm09 (A) and A/Texas/50/(H3N2) (B) broken down by influenza outcome. Data shown reflects sera from participants with laboratory-confirmed influenza infection (Yellow, n=10) with matched controls (black, n=10), and influenza-like-illness excluding confirmed influenza (purple, n=54). Statistical values reflect 2-way ANOVA pwith a statistical cutoff of p=0.05.

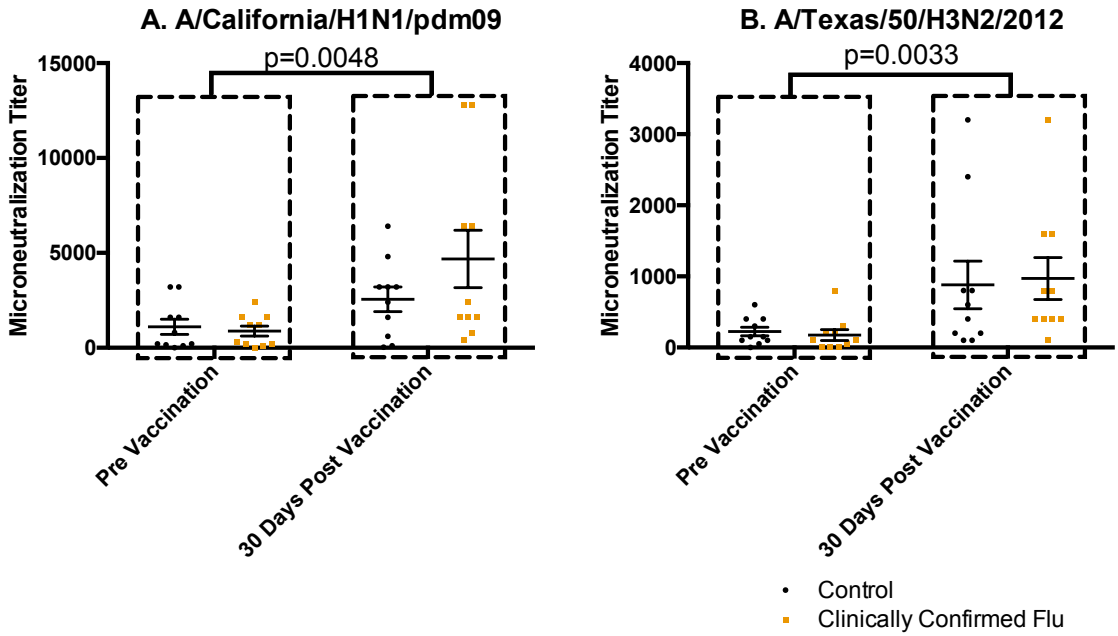

Figure 5.3. Confirmed Influenza was not predicted by Microneutralizing Antibodies. Microneutralization against A/California/H1N1/pdm09 (A) and A/Texas/50/H3N2 (B). Data shown reflects sera from participants with laboratory clinically influenza infection (red, n=10) with matched controls (black, n=10). Both subject groups responded to vaccination (indicated in A, C), but no differences or interaction between infected and uninfected were observed. Statistical values reflect 2-way ANOVA with a statistical cutoff of p=0.05.

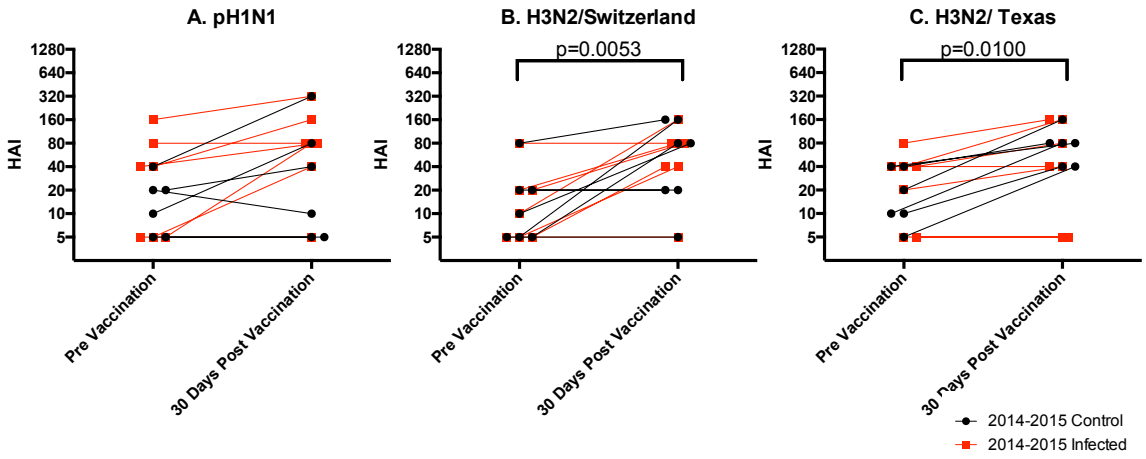

Figure 5.4. Subjects who later became infected had unimpaired HAI against Influenza Vaccine and Circulating Strains. *Subsequent Influenza outcome and Hemaglutination inhibition against vaccine-included viruses A/California/H1N1pdm09 (A), vaccine-included virus A/Texas/50/H3N2(C), and circulating strain A/H3N2/Switzerland. Statistical values reflect 2-way ANOVA with a cutoff of p=0.05.*

CHAPTER VI: SYNTHESIS

Overview of Research Findings

The data presented in this dissertation addresses the three aims proposed in this doctoral dissertation. The first aim of this dissertation was to determine if obesity impairs the metabolism of resting and activated memory T cells following a secondary influenza infection.

I hypothesized that compared to lean mice, the metabolism of obese mice would be more active at rest, limiting the metabolic potential to immune challenge. My hypothesis was found to be true. Memory CD4+ T cells from obese mice had substantially higher oxidative phosphorylation and glycolytic activity at rest, and at 7 days post infection increased oxidative phosphorylation significantly, but decreased glycolysis. CD8+ T cells from obese mice at rest, were highly glycolytic, and with infection significantly decreased glycolytic activity, and increased oxidative phosphorylation. The spare respiratory capacity (ability to produce energy in response to challenge or stress) of T cells from obese mice was significantly decreased with infection compared to lean mice, suggesting that the dysfunctional metabolism limits the capacity of T cells to respond to immune challenge. Overall, memory T cells from obesity display dysfunctional metabolic profiles in CD4+ and CD8+ T cells.

The second aim of this dissertation was to determine if weight loss restores T cell metabolism and function to a secondary influenza infection. I hypothesized that weight loss would restore T cell metabolism to a lean phenotype and improve the function of memory T cells. My hypothesis was incorrect, as our data shows continued impairments in the immune response to re-infection. We found that while weight loss in DIO mice after memory T cell generation restored glucose values similar to always lean mice, the metabolic profile of weight loss mice tracked with obese mice. Compared to always lean mice, weight loss had significantly higher

CD4+ and CD8+ T cell basal respiration rates at infection, in addition to significantly increased OCR to ECAR ratios. The expression of Glut1 and cytochrome c was significantly decreased compared to lean mice, further demonstrating altered T cell metabolism. The metabolic profile of weight loss mice paralleled the obese metabolic phenotype suggesting that time of memory T cell generations programs the metabolic profile which drives impaired function following reinfection. Weight loss mice had significantly fewer CD8+ T_{RM} populations in the lung, along with impaired effector function in CD8+ T cells. We found fewer influenza specific CD4+ T cells in the lung, and delayed infiltration of Tregs compared to always lean mice.

Lastly, aim three was to determine if weight gain would induce metabolic dysfunction and impair the function of memory T cells generated in a lean state. My hypothesis was that the obesogenic environment would alter the metabolism and function of the formally lean T cells. My hypothesis was partially correct, their metabolic profile that tracked with lean mice. Mice gained significant weight gain to induce obesity, and glucose values were significantly higher compared to always lean mice, establishing an obese phenotype. Weight gain mice mice had similar OCR and ECAR in CD4+ and CD8+ memory T cells compared to lean mice, demonstrating similar metabolic profiles at rest and to infection. However, at reinfection significantly fewer CD8+ T_{RM} populations in the lung 7 days post infection, impaired effector CD8+ T cells at day 3 post infection, and also delayed infiltration of Tregs.

Potential mechanisms and future directions

Our studies demonstrated that weight loss or weight gain following memory T cell generation can impact metabolic phenotypes and memory T cell subsets and function. These data suggest a combination of potential mechanisms that are metabolism and microenvironment driven. Weight loss did not restore metabolic function, even though at time of re-infection glucose levels were similar to always lean, but the T cell metabolic profile was similar to the always obese group. It is possible that the environmental conditions present at time of memory T cell generation induce epigenetic modifications that maintain an impaired metabolic phenotype at the

time of re-infection. Several studies have reported obesity altered epigenetic profiles in circulating PBMCs and lymphocytes [260-262]. Obese animals have increased global DNA methylation in lymphocytes, but not monocytes, indicating this may be cell type specific [260]. Some of the differences we see between CD4+ and CD8+ T cell metabolic profiles are cell type specific, and will have different epigenetic profiles that may be altered with obesity at the time of memory T cell generation. Epigenetic modifications such as DNA methylation, histone modifications, and non-coding RNA's have all been implicated in affecting immune function [263].

T cell differentiation following the interaction with an antigen presenting cell is driven by epigenetic regulation that initiates signaling cascades [264]. Previous experiments have demonstrated that Th1 or Th2 differentiated cells (after at least 4 cycles of cell division) will not revert to the opposing subset (Th1 to a Th2, and vice versa) if subjected to cytokines typically used to polarize to the opposing subset [265]. Many studies have characterized the epigenetic mechanisms that regulate T cell differentiation. For example, to silence the IFNγ gene in the Th2 subset, transcription factors GATA3 and STAT6 bind to the promoter region which initiates binding of specific DNA methyltransferases regions of the IFNγ gene, repressing IFNγ expression [266]. Our study found that weight loss did not restore IFNγ effector production following secondary infection, therefore it is possible that obesity is inducing dysregulated epigenetic modifications at time of differentiation to memory T cells.

Obesity is characterized as a state of low-grade inflammation, so it may be possible that memory T cell populations present in this environment may be subject to constant stimulation. Chronically activated lymphocytes in autoimmune disorders such as systemic lupus erythematosus (SLE), present altered metabolic phenotypes that upregulate oxidative metabolism rather than glycolytic activity for ATP synthesis [267] as opposed to aerobic glycolysis as seen in normal functioning lymphocytes. Additionally, T cells from SLE patients have increased mitochondrial mass and transmembrane potential, decreased intracellular ATP stores, and dysregulated cell death [268]. Constant stimulation and chronic activation of T cell signaling may be dysregulating mitochondrial function and altering cell metabolism. Our studies demonstrated

decreased expression of cytochrome c, a marker of mitochondrial health, in weight loss, weight gain and obese groups which may suggest impaired mitochondrial function and metabolism.

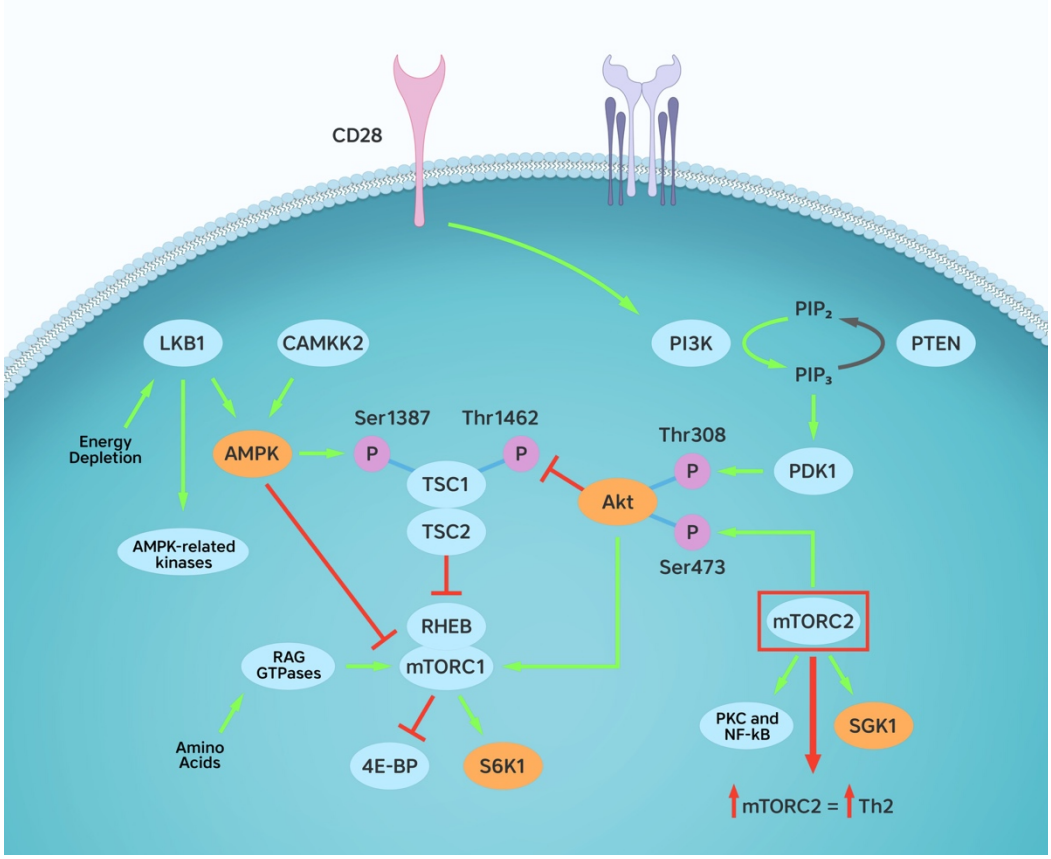

Figure 5.1. Proposed mechanism of Th2 skewing. Created by Jennifer Rebeles, illustration by Christopher Brothers Design.

The PI3K/Akt pathway in T cell signaling has downstream effects that control many T cell functions, including the activation from effector to memory T cell differentiation in T cells (Figure 1) [163]. Recent in vivo mouse studies and human studies have looked at the effects of high fat diet or obesity on effector CD4+ T cell differentiation by the PI3K/Akt pathway [269]. In both mouse and human studies they found increased Akt phosphorylation at serine 473 and no differences at threonine 308, indicating that mTORC2, as opposed to mTORC1, has increased activation with obesity [269].

In steady state conditions, mTOR is usually activated by a combination of factors that involve TCR and CD28 engagement and cytokines, environmental stimuli and nutrient availability [270]. The combination of these factors determines the fate of differentiation of T cells into specific subsets. CD4+ Th1 cells are differentiated by the activation of mTORC1 and mTORC2, while CD4+ Th2 cells are driven by increased expression of mTORC2. It is possible that obesity is driving this increased expression of mTORC2, skewing to Th2 cells rather than Th1 response.

Viral infections such as influenza infection differentiate CD4+ T cells predominantly to Th1 to promote activation of cytotoxic CD8+ T cells, macrophage activation and B cell activation [195]. If obesity increases CD4+ T cell differentiation to maintain a predominant Th2 subset, and not properly differentiate into Th1 to infection, this may contribute to impaired T cell function we see with obesity. Indeed, our previous human study showed an increased Th2 skewing in PBMC's from flu vaccinated obese adults stimulated with influenza [81]. Th2 T cells are not effective in viral clearance to influenza infection and recovery [271] and may contribute to influenza infection increases in morbidity and mortality with obesity.

Our studies addressed altered metabolic profiles in weight loss and weight gain mice at day 0 and 7 post infection, however, further time points need to be incorporated to help further determine a more comprehensive metabolic profile. As effector T cells become activated to stimulus, they should increase glycolytic activity and decrease oxidative phosphorylation. Incorporation of more timepoints such as day 3, and day 5 post infection may provide a better snapshot of when metabolic alterations in obesity is most affected. By limiting metabolic profiling to only 2 time points, we failed to see where the increase in glycolytic activity occurs in response to effector activity.

Additionally, we metabolically characterized CD4+ T cells and found statistical differences with OCR and ECAR between the diet switched groups, however we did not delineate the specific CD4+ T cell populations. Th1, Th2, Th17 and Tregs have very specific and different functions with varying metabolic profiles. Further characterizing the metabolic profiles of these cells will determine if all CD4+ T cells subsets are impaired, or if it is selective populations that

are impaired. As helper T cells have very different functions than Tregs we may be missing important metabolic differences between these populations. Our previous studies have established that compared with lean mice, Tregs from DIO obese mice have less suppressive activity, which may be driven by impaired metabolic profiles.

While few studies have addressed altered epigenetic profiles in immune cells in obesity, it is important to determine if epigenetic modifications are resulting in memory T cell impairment and if this altered immunity can be restored. Changes in global DNA methylation have been observed in autoimmune diseases of rheumatoid arthritis and SLE where CD4+ T cells are hypomethylated compared to healthy adults [272]. Weight loss can normalize DNA methylation patterns in genes associated with metabolism and mitochondrial function in obese people that have undergone gastric bypass surgery to levels similar to healthy weight controls in skeletal muscle [273]. Looking at global methylation in the different T cell populations between obese and lean conditions may suggest methylation inhibitors or other relevant pharmacological agents that could restore T cell effector function to influenza infection in obese individuals.

Conclusions

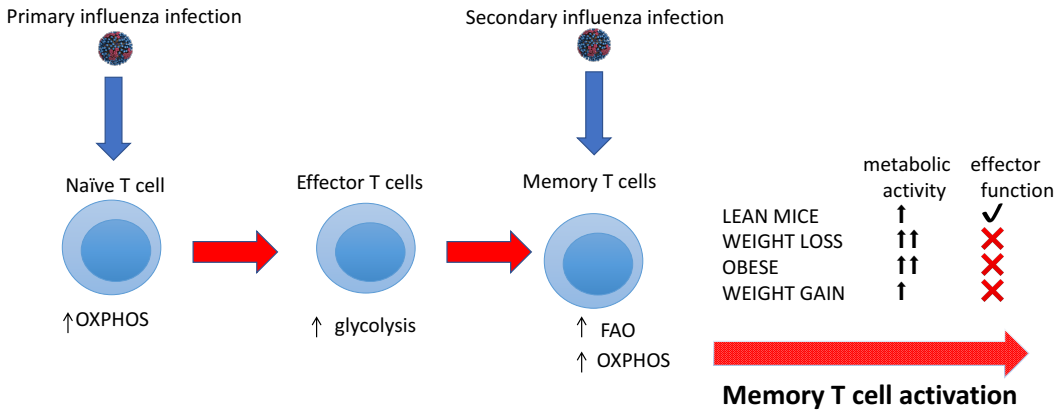

Figure 5.2. Conclusions model.

The data presented in this dissertation demonstrates that the metabolic environment programs the metabolism of memory T cells at time of generation, and dictates functional response to secondary infection. To summarize, weight loss mice maintained a metabolic phenotype compared to obese mice, but despite weight loss, had impaired function to secondary infection. Obese mice demonstrated dysregulated, higher metabolically active metabolism at memory T cell generation, and impaired function to secondary infection. Weight gain mice had similar metabolic profile to lean mice, but impaired function to secondary infection.

While there are many studies addressing restoration of health by behavioral and physical interventions in weight loss, what was lacking was whether this was improving immunity. Mouse models of weight loss and weight gain at time of memory T cell generation to influenza infection had not been previously characterized. The role of weight loss in obesity and weight gain at time of memory T cell generation was characterized by metabolic profiling, and functional measurements through flow cytometry. Additionally, T_{CM}, T_{EM}, T_{RM}, and effector functions of memory T cells were characterized in mice that lost weight and gained weight after the generation of memory T cells. Critical lung resident memory T cells were decreased in mice that were obese at any time, and effector function was impaired. To summarize, weight loss did not restore function to re-infection, and while weight gain maintained a metabolic profile similar to always lean mice, function was also impaired to re-infection. Although these findings suggest obesity at any time sets the stage for life-long T cell impairment, despite weight loss, there may be strategies to improve immune health. For example, treatment with the previous mentioned metformin may improve response. Revaccination once weight loss has occurred may also be successful.

These data have significant public health importance, as conventional thought of weight loss in obesity is assumed to restore metabolic health in public health interventions. While weight loss did restore glucose levels comparable to lean mice, it is important to recognize that there are other metabolic perturbations that are effecting immunity. We found that at time of memory T cell generation and the environment at time of re-infection BOTH affect the immunity to infection.

Future public health interventions need to stress the importance of obesity prevention to preserve immunity. Although we have characterized these results to influenza infection, it reasonable to speculate that these immunity impairments may affect the immunity to other infections or disease states. Furthermore, future studies investigating metabolic altering drugs may be needed to restore T cell metabolic health to improve effector function to influenza infection. Additionally, changes to vaccine strategies may provide additional considerations to boost the immune response of obese individuals to improve protection.

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