THE EFFECTS OF ADIPOSITY AND TYPE II DIABETES ON THE IMMUNE RESPONSE TO INFLUENZA VIRUS IN ADULTS

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A dissertation submitted to the faculty of 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, Gillings School of Global Public Health.

Chapel Hill
2013

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ABSTRACT

HEATHER ANN PAICH: The Effects of Adiposity and Type II Diabetes on the Immune Response to Influenza Virus in Adults
(Under the direction of Melinda A. Beck)

There are very limited data on mechanisms that mediate the obesity-associated and type II diabetes-associated impairments in immune function against viral infections, such as with the influenza virus. The purpose of this dissertation was to assess the humoral and cellular immune responses to the influenza virus, as well as to examine the effects of type II diabetes on T cell metabolism. This dissertation followed three aims. Aim 1 utilized a convenience sample to determine the antibody response to influenza vaccination in healthy weight, overweight, and obese adults at one and 11 months post vaccination. Higher body mass index (BMI) was associated with a greater decline in antibody titers to influenza strains at eleven months post vaccination, suggesting that overweight and obese individuals may not be as protected throughout the duration of the flu season compared to healthy weight individuals. Aim 2 consisted of a series of several studies comparing the cellular immune response to influenza virus in dendritic cells, cluster of differentiation (CD) 4^+ T cells, and $CD8^+$ T cells from healthy weight, overweight, and obese adults following *ex vivo* stimulation with live influenza virus.

Although markers of dendritic cell activation and function remained intact, markers of T cell activation and function were significantly impaired in overweight and obese, compared to healthy weight adults. Together these data suggest that there would be significant deficiencies in the activation and cytotoxic function of $CD8^+$ T cells, as well as in the activation and helper function of $CD4^+$ T cells resulting from overweight and obesity. Aim 3 was an exploratory aim designed to generate preliminary data towards answering the question of how obesity with or without type II diabetes will affect T cell glucose metabolism. The data suggests that there are differences in glucose metabolism in unstimulated T cells from obese individuals with and without type II diabetes. The results of this dissertation indicate that both overweight and obesity impair the humoral and cellular immune response to influenza virus and that type II diabetes may alter T cell metabolism.

ACKNOWLEDGEMENTS

I gratefully acknowledge the incredible support I have received from all the members of my dissertation committee: Melinda A. Back (chair), Jean Handy, Ilona Jaspers, Liza Makowski, and Patricia A. Sheridan. Dr. Beck has taught me a tremendous amount about how to be an effective scientist and her unwavering encouragement and guidance have allowed me the freedom to think creatively about approaching and solving scientific problems.

I am thankful for the support I received for the 2008-2009 and 2009-2010 years of my doctoral training from the NIH National Research Service Award Predoctoral Traineeship for the Department of Nutrition (T32-DK07686-16 and T32-DK07686-17), as well as the UNC Gillings School of Global Public Health Dissertation Award. Funding from NIH ROI AI078090 to Melinda A. Beck and from NIH DK056350 to the University of North Carolina Nutrition Obesity Research Center were also an integral part completing this dissertation research. I am also grateful for the supportive and helpful staff in the Department of Nutrition, in addition to the staff, graduate students, and undergraduate students in the Beck Lab.

I would also like to thank my entire family, including parents, grandparents, and sister, for always supporting my educational endeavors. I would like to thank my friends and fellow graduate students who have enhanced my doctoral training experience (Dori Steinberg, Carmina Valle, Amy Johnson, Trisha Grevengood, Amanda Mah, and Jenny Rebeles).

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AID	activation-induced deaminase
AP-1	activator protein-1
APRIL	a proliferation-inducing ligand
BAFF	B-cell activating factor
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
BCR	B cell receptor
Bid	BH3 interacting-domain
BMI	body mass index
CCR7	C-C-C chemokine receptor type 7
CD	cluster of differentiation
CD40L	CD40 ligand
CD62L	CD62 ligand
CDC	Centers for Disease Control and Prevention
CXCR5	chemokine (C-X-C motif) receptor 5
CXCL13	C-X-C motif chemokine 13
ERK	extracellular signal-regulated kinase
GLUT	glucose transporter
GM-CSF	granulocyte/macrophage-colony-stimulating factor
GrB	granzyme B

HA	hemagglutinin
HAI	hemagglutination inhibition
IFNα	interferon-α
IFNβ	interferon-β
IFNγ	interferon-γ
IgA	immunoglobulin
IL2	interleukin
IL7R	interluekin-7 receptor
IL12R	interluekin-12 receptor
IL15R	interluekin-15 receptor
IPS-I	IFNβ promoter stimulator-1
IRF-3	IFN response factor-3
JAK2	janus kinse-2
JNK	c-Jun NH(2)-terminal protein kinase
M1	matrix protein
M2	ion channel protein
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MH	major histocompatibility complex
MyD88	myeloid differentiation factor 88
NA	neuraminidase
NEP	non-structural protein-2

NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-B
NHANES	National Health and Nutrition Examination Survey
NP	nucleoprotein
NS1	non-structural protein-1
PBMC	peripheral blood mononuclear cell
pH1N1	2009-2010 pandemic influenza virus A H1N1
PI3K	phosphoinositol-3-kinase
PKCγ	protein kinase C-γ
RIG-I	RLR retinoic-acid-inducible gene I
RLR	retinoic-acid-inducible gene I-like receptor
RNP	ribonucleoprotein
Ser473	serine 473
STAT4	signal transducer and activator of transcription-4
TBK-I	tank-binding kinase-I
TCR	T cell receptor
TGF-β	transforming growth factor-β
Thr308	threonine 308
TIV	trivalent influenza vaccine
TLR	toll-like receptor
TNFα	tumor necrosis factor-α
TRAF	TNF receptor-associated factors

TRIF	toll/IL-I receptor domain-containing adaptor
US	United States
WHO	World Health Organization

CHAPTER I

OVERVIEW AND SPECIFIC AIMS

Overview

Obesity and type II diabetes are associated with immune system dysfunction, both in obese humans and obese mouse models. Reports indicate that obesity is an independent risk factor for morbidity and mortality from the 2009-2010 pandemic of influenza virus A H1N1 (pH1N1) in adults; data from our lab has demonstrated that obesity in humans is associated with a poorer antibody and cellular response to influenza vaccination and that diet-induced obese mice have impaired resistance to influenza virus infection. The specific obesity-associated mechanisms modulating the differential response of obese individuals to influenza are as of yet unknown. Influenza vaccination is the most effective method to decrease susceptibility to influenza infection and increase the ability to respond to the virus. Serum samples obtained from healthy weight, overweight, and obese individuals prior to and following vaccination allowed us to measure the immunoglobulin (Ig) G antibody levels specific to multiple strains of influenza, thereby elucidating the relationship between adiposity and the humoral immune response, while peripheral blood mononuclear cells (PBMCs) obtained from these individuals allowed us to conduct cell culture studies to

analyze differences in the cellular immune response to influenza vaccination. In addition, T cells isolated from the PBMCs obtained from obese individuals with and without type II diabetes were analyzed to determine differences in cellular metabolism under stimulated and unstimulated conditions. There are widespread outbreaks of influenza throughout the world each year and with the dramatic increases in obesity and type II diabetes this is an especially significant and urgent global health challenge. The data generated in this dissertation provides important information about the biochemical mechanisms underlying this phenomenon and will inform strategies to provide the most effective prevention and treatment for influenza for all individuals.

Specific Aims

Specific Aim 1: To determine if increasing body mass index (BMI) will alter the humoral immune response to influenza vaccination.

Hypothesis: Overweight and obesity will result in an impaired humoral immune response to influenza vaccination, characterized by lower levels of influenza-specific antibodies.

Specific Aim 2: To determine if increasing BMI will alter the cellular immune response to influenza vaccination.

Hypothesis: Overweight and obesity will result in an impaired cellular immune response to influenza vaccination, characterized by deficient activation and function of dendritic cells and cluster of differentiation (CD) 4^+ and $CD8^+$ effector T cells.

Exploratory Specific Aim 3: To generate preliminary data towards answering the question of if obesity in conjunction with type II diabetes will impair T cell glucose metabolism, compared to obesity without the presence of type II diabetes.

Hypothesis: Obesity in conjunction with type II diabetes will impair T cell glucose metabolism, including glycolytic capacity, glycolysis rate, and glycolytic reserve, as well as a suboptimal response to insulin, compared to T cells from obese humans without type II diabetes.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Introduction

Obesity is a serious health concern of throughout the world (1). Obesity is associated with numerous health conditions, including insulin resistance, type II diabetes, cardiovascular disease, and hyperlipidemia (2). In addition to these co-morbidities, obesity itself is considered an immunosuppressive condition (3-5). However, only recently has a correlation between obesity and influenza been suggested. During the pandemic of influenza A H1N1 (pH1N1), obesity was recognized for the first time as an independent risk factor for increased influenza morbidity and mortality (6) and a recently published large epidemiology study examining records over 12 flu seasons found that obese individuals were more likely to have a respiratory hospitalization during the influenza season than healthy weight individuals (7).

There are widespread outbreaks of influenza virus infection throughout the world each year. The WHO estimates that worldwide, influenza epidemics result in three to five

million cases of severe illness and 250,000 to 500,000 deaths each year, even in the absence of a major pandemic (8).

Although annual vaccination is the primary strategy available for decreasing the impact of influenza infection, no studies have examined how obesity may affect the response to influenza vaccination in humans. Obesity is associated with decreased antibody response to hepatitis B vaccine (9) and to tetanus toxoid (10). Diet-induced obese mice were found to have greater mortality following influenza infection and impaired innate immune responses (11), as well as an impaired $CD8^+$ T cell memory response that increased morbidity and mortality from a secondary influenza challenge (12). However, the effects of obesity on the humoral and cellular immune responses to influenza vaccine have not been characterized in humans. In addition, it is not known how the obesity-associated type II diabetes may affect circulating immune cells in humans,

It is well established that the type and amount of food that we eat can affect our body weight, in addition to other factors such as age and physical activity level; however the idea that obesity itself could alter the humoral and cellular immune responses to influenza is novel. The proposed study will contribute to knowledge in nutrition by determining, for the first time, how adiposity and type II diabetes affect circulating immune cells and their response to influenza vaccination in a human population.

The Obesity Epidemic

Obesity is a significant public health problem in many countries (1), as the WHO estimates that there are now over 1.4 billion overweight adults and approximately 500 million adults that are obese worldwide (1). Other estimates indicate that nearly 10% of men and 14% of women throughout the world are obese (13). Overweight and obesity are serious public health problems within the United States (US) as well, as more than two-thirds of the US adult population is overweight or obese (14). Furthermore, obesity and overweight are predicted to continue to rise significantly throughout the world and within the US in the future. WHO data predict that worldwide almost 2.3 billion adults will be overweight and 700 million of these adults will be considered obese by 2015 (1). Previously, overweight and obesity were generally considered to be problems affecting adults in high-income countries, such as in the US and Europe; however, overweight and obesity are quickly increasing rates are also rising in low- to middle-income countries as well. For example, a survey of South Africa, a low- to middle-income country, indicated that 29.8% of men and 54.9% of women there were overweight or obese in 2007 (15) and projections indicate that overweight and obesity are likely to increase in South Africa in the future (16). National Health and Nutrition Examination Survey (NHANES) data predict that within the US 86.3% of adults will be overweight or obese and 51.1% of adults will be obese by 2030 (17). Overweight and obesity, which result from an energy imbalance characterized by increased caloric intake and decreased caloric expenditure (18), indicate body weights that are greater than what is generally considered healthy for a given height and have been shown to be

associated with numerous health problems (19). Body mass index (BMI) is calculated using both height and weight measurements with the formula BMI = (mass in kilograms)/((height in meters)^2) and is generally accepted to correlate to the amount of body fat in most people. The Centers for Disease Control and Prevention (CDC) defines four categories of BMI: underweight (BMI<18.5), healthy weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), and obese (BMI>30) (19). Obesity is associated with a large number of health conditions and diseases, including insulin resistance, glucose intolerance, type II diabetes, hypertension, cardiovascular disease, arthritis, sleep apnea and some cancers (2, 19-23). It is thought that many of the detrimental health consequences associated with obesity are due to the hormonal and metabolic changes related to both increased adipose tissue mass and increased storage of triacylglycerol and other complex lipids within adipose tissue and other tissue types (24-26). For example, obesity is associated with higher circulating levels of the proinflammatory cytokines interleukin (IL) 6 and tumor necrosis factor-α (TNFα) (27). Levels of IL6 and TNFα in the blood directly correlate with the amount of adipose tissue mass on the body (27), and it is hypothesized that the high levels of these cytokines play a role in the pathogenesis of many of the overweight- and obesity-associated health conditions. Finally, in addition to all the health conditions and diseases associated with obesity, numerous studies have shown that obesity itself is solely and directly correlated with a decreased life expectancy, as elevated BMI is a risk factor for all cause mortality (20, 28-31).

Obesity and Immune Function in Animal Models

Although obesity is a recognized risk factor for increased morbidity and mortality, its specific effects immune function, both humoral and cellular, are as of yet incompletely understood. A previously published study from our lab investigated the effects of obesity on the immune response to influenza virus (11). Diet-induced obese and lean control mice were infected with a mouse-adapted strain of influenza A and the immune responses were monitored. The diet-induced obese mice had a dramatically higher mortality rate than the lean control mice. At 9 days after infection, only 50% on the diet-induced obese mice were still alive compared to over 95% of the lean control mice. In addition, the diet-induced obese mice had elevated lung pathology, altered antiviral and proinflammatory cytokine expression in the lungs, and substantial reductions in natural killer cell cytotoxicity. This groundbreaking study shows that obesity impairs the ability to respond to the influenza virus and suggests that obesity may result in greater morbidity and mortality from influenza infection (11). In a similar study from our lab (32), dendritic cell numbers from diet-induced obese mice were lower overall and had impaired antigen presentation, although antigen uptake and migration appeared to be intact. In addition, IL2, IL12, and IL6 were altered in the diet-induced obese mice, thereby affecting the number and frequency of $CD3^+$ and $CD8^+$ T cells in the lung. These data suggest that obesity impairs the cellular immune responses to influenza virus infection (32).

In addition, there are two studies published regarding memory T cells and obesity,

both from our lab (12, 33). The first indicates that following a secondary influenza viral challenge, obese mice display reduced levels of influenza-specific $CD8^+$ effector memory T cell in the lung, compared to lean mice, and the influenza-specific $CD8^+$ effector memory T cells in the obese mice express less CD122, the IL2β receptor, compared to cells from the lean mice (12). Obese mice also had lower levels of the cytokine IL7 in lungs following the primary influenza infection (12). Another study following a secondary influenza viral challenge indicated that obese mice had a 25% higher mortality rate and increased lung pathology and viral lung titers, compared to lean mice (33). Obese mice also had lower levels of interferon (IFN)-γ expression and IFNγ-producing influenza-specific $CD8^+$ T cells than lean mice. Even when memory $CD8^+$ T cells from obese mice were stimulated with influenza-pulsed dendritic cells from lean mice, IFNγ expression was lower (33). A recently published study, also utilizing an obese mouse model, showed that obese mice had higher mortality and higher initial viral titers in the lungs following infection with pH1N1 than lean control mice, possibly due to dysregulated cytokine production associated with obesity (34).

Obesity and Immune Function in Humans

There is evidence in humans suggesting that obesity negatively impacts the immune response. The most pertinent and telling are the data gathered during the 2009 pH1N1 influenza pandemic. One of the unusual characteristics of the pH1N1 pandemic was the populations that were affected most by the virus (35, 36). With typical seasonal influenza epidemics, approximately, 90% of deaths occur in adults aged 65 years or older, however,

with the pH1N1 pandemic, the highest influenza-associated mortality was seen in adults 25 to 49 years of age, followed by adults 50 to 64 years of age, and then individuals who were 5 to 24 years of age (35, 36). Interestingly, a recent study showed that obesity was associated with more severe pH1N1 infection in children hospitalized for influenza (37) and in non-hospitalized school age children (38). As was expected, severe infection with pH1N1 was seen in populations with underlying risk factors, including cardiovascular disease, chronic lung disease, hypertension, diabetes and pregnancy (39, 40). However, epidemiological evidence of the pH1N1 pandemic showed, for the first time, that obesity was an independent risk factor for morbidity and mortality from influenza infection (41). In fact, in adults over 20 years of age obesity was identified as the major risk factor associated with pH1N1 death, in the absence of other risk factors, and in adults with morbid obesity, there was a very strong association with death and hospitalization from pH1N1 (41). Another study found that in adults admitted to the intensive care unit during the first wave of the pH1N1 pandemic, the obese and morbidly obese patients with severe influenza infection had a longer duration of stay and a higher risk of developing pneumonia than healthy weight patients (42).

Conditions and Disease States Associated with Obesity

More generally, obesity is linked to various health conditions and disease states (43). Obesity is a risk factor for a number of different respiratory conditions, including asthma (44), obstructive sleep apnea (45), and chronic obstructive pulmonary disease (45), as well

as respiratory infections, including community-related respiratory tract infections (46), such as pneumonia (47). In addition, obese individuals are more likely to develop nosocomial infections; specifically obese individuals who have undergone surgery have a higher incidence of nosocomial infections including infection of wounds, pneumonia, bacteraemia, and *Clostridium difficile* colitis, compared to healthy weight individuals (48, 49). Obese individuals who have undergone surgery also have a higher risk of developing an infection at the surgical site, compared with healthy weight individuals (50). Other infections of the skin have also been found to be increased in obese individuals, compared to healthy weight individuals, which include candidiasis, cellulitis, erythrasma, folliculitis, intertrigo, and even necrotising fasciitis (51, 52). The latter may be related to the higher incidence of *Staphylococcus aureus* living in the nasal passages of obese individuals, which is also a risk factor for surgical-site infections (53). In addition to skin infections and surgical site infections, obese individuals also exhibit impaired wound healing following surgery or trauma (54, 55). Finally, obesity is known to accelerate thymic aging in both mice, who display replacement of lymphostromal thymic zones with adipose tissue, and in humans, who show decreased numbers of naive T cells that originate from the thymus (56).

Obesity and the Response to Vaccination

There are also a small number of studies that have found that obese individuals show an impaired response to vaccination compared to healthy weight individuals. One such study found that following vaccination with the tenanus toxoid, there were reduced

tetanus antibody titers in overweight children, compared to healthy weight children (10). Another such study found that following three doses of hepatitis B virus plasma vaccine, obese individuals had a much lower levels of detectable antibody to hepatitis B surface antigen in serum, compared to healthy weight individuals (9). These data were confirmed by a number of other studies (57, 58). However, in a subsequent study, it was shown that obese adolescents who were immunized against hepatitis B with a 1.5-inch needle achieved significantly higher antibody titers than obese adolescents immunized with a 1-inch needle (59). As such, a 1.5-inch needle was utilized for the vaccination studies presented in this dissertation.

Importantly, using an obese mouse model, it was shown that following immunization to pH1N1, obese mice had lower antibody titers, and following a post-vaccination infection, had higher pulmonary virus titers, higher levels of inflammation and proinflammatory cytokines in the lungs, and a higher mortality rate, compared to lean control mice (60), although there are no data specifically examining the response to pH1N1 vaccination in a human population.

Obesity and PBMC Populations

Finally, obesity and overweight have been hypothesized to alter the populations of PBMCs circulating in the blood, however, the evidence in the literature concerning this issue continues to be mixed and inconclusive. Studies have reported that obesity decreases

overall circulating T cell numbers (56, 61), increases decreases overall circulating T cell numbers (62), and that obesity has no effect on T cell numbers (63). Some studies provide evidence that although overall PBMC numbers or T cell numbers are not changed by obesity, the subpopulations may be altered. One such study that demonstrated there was a reduced frequency of $CD8^+$ T cells, but an increased frequency of $CD4^+$ T cells (64), while a different study indicated that obesity promotes $CD4^+$ T cell differentiation to the T_H17 subtype (65). A study in females from Saudi Arabia found that there was a positive correlation between BMI and $CD4^+$ T cell numbers, but not $CD8^+$ T cell numbers (66), while a similar study in women from the US found that morbid obesity was associated with increased percentages of both $CD4^+$ and $CD8^+$ T cell numbers (67). More recently, a study showed that $CD4^+$ T cells from morbidly obese individuals were skewed towards a T_H2-dominated phenotype (68). Another such study indicated that obesity was associated with increased numbers of leukocyte and altered lymphocyte subset numbers (69). Although it is unknown how obesity affects numbers of circulating PBMCs, it does appear to impair function of certain cell populations. Natural killer cells from obese adult have been shown to exhibit decreased overall numbers, impaired anti-tumor activity, and increased susceptibility to cigarette smoke, compared to natural killer cells from healthy weight adults (70, 71). Furthermore, when reactive oxygen specific production was measured in PBMCs, the levels were significantly higher in cells from obese adults compared to from healthy weight adults (72). It has been shown that obesity results in decreased proliferation and decreased response of T cell and B cell populations following mitogen stimulation (73, 74). In addition, PBMCs from obese adults stimulated with toll-like receptor (TLR) ligands

showed an impaired ability to produce the antiviral cytokines IFNα and IFNβ, compared to PBMCs from healthy weight adults (75), which is similar to data generated in diet-induced obese mouse studies (11). With morbid obesity, dendritic cell numbers were found to be lower numbers and function was found to be impaired (76). Another study showed that overweight and obesity were associated with increased expression in PBMCs of TLR2, TLR4, and myeloid differentiation factor 88 (MyD88), and an even higher increase in expression of those markers with type II diabetes (77). Similarly, it was found that there was elevated TLR2 expression in PBMCs obtained from pregnant women with gestational diabetes compared to pregnant women with normal glucose tolerance (78). Interestingly, there is some evidence suggesting that weight loss may correct impairments in immune function. The production of monocyte chemoattractant protein (MCP) and IFNγ following stimulation were measured in morbidly obese adults with an average BMI of 62.4 before and one year after gastric bypass surgery. The weight loss completely normalized the ability to produce MCP and IFNγ, to levels similar to those seen in healthy weight adults (79).

The Type II Diabetes Epidemic

Type II diabetes is a chronic disease that occurs when the body is not able to effectively use the insulin that it produces and is often a result excess body weight and inadequate physical activity (80). Hyperglycemia is a consequence of uncontrolled diabetes, which can result in serious damage to numerous tissues and organs in the body, has both acute and chronic effects (80). The WHO estimates that in 2004, approximately 3.4 million

people throughout the world died from consequences of hyperglycemia (80). Reports from the CDC indicate that the risk for death among people with diabetes is approximately twice that of people without diabetes of similar age within the US (81). Appropriate treatment can drastically improve the outcome of type II diabetes and access to treatment is certainly influenced by economics, as more than 80% of diabetes deaths occur in low- and middle-income countries (80). Obesity is an independent risk factor for type II diabetes, however some individuals develop type II diabetes who are not obese (82). Adults with a BMI equal to or greater than 30 have a significantly higher risk (60 to 80 times higher risk) of developing type II diabetes (83). Conversely, losing weight can improve the risk of type II diabetes. A study found that in adults with a BMI equal to or greater than 34, weight loss of about 6% combined with an increase in physical activity resulted in a 58% decrease in diabetes incidence of (84). Similar to obesity, type II diabetes, has become a chronic disease of epidemic proportions, as over 347 million people worldwide are currently estimated to have diabetes and the WHO projects that deaths from diabetes will increase by 75% between 2008 and 2030 (80). Within the US, the CDC estimated that 25.8 million people suffered from diabetes in 2010, which is approximately 8.3% of the US population (81). Type II diabetes is much more common than type I diabetes, as about 90% of people who suffer from diabetes worldwide have type II diabetes (80). The long-term consequences of type II diabetes include heart disease, stroke, hypertension, blindness and eye problems, kidney disease, and nervous system disease (81).

The Pathogenesis of Type II Diabetes

Type II diabetes is associated with and characterized by insulin resistance, glucose intolerance, hyperinsulinemia, and hyperglycemia (85). Obesity is hypothesized to induce insulin resistance, leading to type II diabetes, in two ways, via increased levels of lipid intermediates (86, 87) or via increased production of inflammatory cytokines (86, 88), which thought to counteract insulin action or interfere with insulin signaling. One of the strongest links between excess adiposity and insulin resistance is the amount of fat stored in non-adipose tissues (89). When excess dietary calories are consumed, some of this energy is converted to fatty acids or triacylglycerol and other complex lipids for storage. Fat storage should occur predominantly in adipose tissue, but when the storage capacity of the adipose tissue is challenged, storage can occur in other tissues of the body, including muscle, liver, and heart tissue, as well as many other tissues and organs of the body (90). Evidence shows that the adipose tissue of obese individuals releases higher amounts of non-esterified fatty acids into circulation than that of healthy weight individuals (91). It is thought that higher amounts of non-esterified fatty acids cause an increase in flux through lipid synthesis and lipid breakdown pathways in both adipose and non-adipose tissues, leading to increased levels of intracellular fatty acid metabolites, such as diacylglycerol, fatty acyl-coenzyme A, and ceramides (91). In humans, obesity-associated impairments in insulin sensitivity may result from a high dietary intake of saturated fatty acids, such as palmitic acid, or from a low dietary intake of *cis*-monounsaturated fatty acids, such as oleic acid, both of which are characteristic of the so-called unhealthy Western diet (92). Some evidence suggests that

saturated fatty acids, but not *cis*-monounsaturated fatty acids, may increase insulin resistance by increasing production of detrimental lipid metabolites or by stimulating inflammatory signaling (91, 92). This suggests that it may be the increase in intracellular content of lipid metabolites or the alterations in fatty acid residues in the lipid metabolites, or perhaps both, that lead to impairments in insulin sensitivity. As adipose tissue accumulates large quantities of triacylgylcerol, there is a concurrent increase in the production and release of proinflammatory adipocytokines, such as TNFα (93). TNFα may circulate through the body in the bloodstream and elicit harmful effects on non-adipose tissues. It is thought that TNFα directly antagonizes insulin's effects by initiating cascades that result in inappropriate phosphorylation of insulin receptor substrate, rendering it inactive (94). In addition, TNFα is proinflammatory cytokine and has important roles in innate immunity. It is one of the primary mediators of the acute inflammatory response to host cell infection with influenza virus. TNFα receptors are present on most cell types in the body; TNFα receptor binding initiates signaling cascades that include activation of caspase-8, which initiates apoptotic pathways, or association with TNF receptor-associated factors (TRAF), followed by activation of transcription factors such as nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1) (95). As part of normal local inflammatory responses to viral infection, TNFα signaling induces endothelial cells and macrophages to secrete chemokines and activates neutrophils and macrophages to destroy infection cells (95). High levels of TNFα can circulate through the body in the bloodstream and have unintended systemic effects on many tissues of the body. Interestingly, another one of the effects of TNFα is to initiate a signaling pathway that upregulates transcription of the

sialyltransferases enzymes, which catalyze the addition of sialic acid residue to the terminal sugar groups of glycoproteins and glycolipids (96). These are the sialic acid containing molecules that are found in the plasma membrane of nasal epithelial cells, where they act as receptors for the influenza virus (97). TNFα has important roles in innate immunity in the respiratory mucosa; the increased expression induces neutrophil and eosinophil recruitment to the site (98). It is interesting to hypothesize that increased numbers of receptors for influenza virus could lead to an increased amount of virus taken up into the cell, increased rate of viral replication, and poorer overall health outcomes. A simple way to begin assessing this possibility would be to test expression of TNFα in respiratory epithelia cells.

Type II Diabetes and the Response to Influenza Infection and Vaccination

In addition, there is evidence that links type II diabetes with impaired responses to both influenza infection and influenza vaccination. After the pH1N1 pandemic of 2009, it was determined that patients with diabetes were three times more likely to be hospitalized following a pH1N1 infection and four times more likely to be admitted to the intensive care unit for complications with the infection (99). A study that analyzed the outcomes of diabetic patients with diabetes who w3ere hospitalized with pH1N1 infection showed that although more of the diabetic patients died and were admitted to the intensive care unit, compared with non-diabetic patients, the worse outcomes may be due to existing comorbidities, and may not be attributed to solely diabetes (100). Another similar study

that examined in-hospital mortality risk, length of hospital stay, and costs found that although diabetes was not an independent risk factor associated with in-hospital fatality risk, the independent factor that increased the risk of death the most among diabetic patients was underlying obesity (101). However, in a retrospective cohort study, it was found that women with type II diabetes had increased rates of influenza-associated pneumonia (102). In addition, there are some data to suggest that type II diabetes may impair the response to vaccination as well. When patients type II diabetes were given a single-dose adjuvanted, inactivated, pandemic H1N1 vaccination, a lower seroconversion rate was associated with a longer duration of diabetes (103). In addition, the immune response to seasonal influenza virus vaccination in adults with type II diabetes was analyzed for six weeks following vaccination and although there were less activated cells expressing IL2 receptors at 72 hours after vaccination, the antibody titers were similar between the adults with and without type II diabetes throughout the six weeks (104). However, it is important to note that it is not known how these responses may have changed over the next year, including the 8 months of influenza season during which protective levels of antibodies need to be maintained. There is also evidence showing no differences in post-vaccination titers following vaccination with seasonal TIV in participants with diabetes and healthy weight participants, although in participants with type 1 diabetes, there was a higher percentage of non-responders (105). Interestingly, in participants with higher concentrations of glycosylated hemoglobin, the delayed type hypersensitivity reaction to influenza was lower (105), suggesting a possible mechanism for impairments in antibody function with higher blood glucose levels. There are also data showing that type II diabetes

can impair the immune response to other viral infections. In genetically-induced obese/type II diabetes mice infected with West Nile virus, there was impaired viral clearance, increased mortality, delayed induction of IFNα, and lower levels of West Nile virus-specific IgM and IgG antibodies, compared with lean, non-diabetic control mice (106). While this study provides information about the combined effects of obesity and type II diabetes, it would be interesting to separate the effects of obese and type II diabetes, perhaps by adding a genetic mouse model of obesity without alterations in glucose or insulin metabolism.

Type II Diabetes and PBMC Populations

In addition, as one of the hallmark symptoms of type II diabetes is elevated blood glucose, the populations and function of PBMCs circulating in the blood may be altered. When PBMCs from adults with and without type II diabetes were stimulated with a mitogen, there was a great percentage of total $CD8^+IFN\gamma^+$ T cells from the adults with type II diabetes, but the cells had lower overall levels of $IFN\gamma^+$, as measured by mean fluorescence intensity (107). Gene expression studies of PBMCs from adults with and without type II diabetes showed that a number of genes in the c-Jun NH(2)-terminal protein kinase (JNK) signaling pathway were upregulated and multiple genes involved in the mitochondrial pathways of oxidative phosphorylation and the electron transport chain were downregulated, possible due to hyperglycemia-induced oxidative stress (108). Similarly, PBMCs from type II diabetics showed much higher levels mitochondrial DNA oxidation and elevated levels of superoxide dismutase activity, than in PBMCs from non-diabetics, again

suggesting higher levels of oxidative stress (109). Following stimulation with a mitogen and with a bacterial antigen, PBMCs from adults with type II diabetes had much higher intracellular levels of IL12 and IFNγ, compared to from adults without type II diabetes, respectively (110, 111). Lymphocytes from patients with polycystic ovarian disease, acanthosis nigricans, and type II diabetes activated with a mitogen showed impaired pyruvate dehydrogenase responsiveness to both low and high levels of insulin stimulation, compared to non-diabetic patients (112). Finally, T cells from patients with type II diabetes showed decreased glucose uptake compared to T cells from patients without type II diabetes, suggesting impairments in glucose metabolism (113).

Influenza

Influenza is an acute viral infection, primarily characterized by respiratory illness, which is caused by an influenza virus (8). Symptoms include sudden onset of high fever, cough, headache, muscle and joint pain, severe malaise, sore throat, and runny nose (8). Influenza viruses are highly contagious and can be spread through airborne droplet transmission or direct contact with infected individuals or contaminated objects. There are a number of complications associated with influenza virus infections, including secondary viral or bacterial pneumonia and dehydration; people with chronic diseases, young children, and elderly adults are at particularly risk for such complications (114).

Seasonal Influenza Epidemics

There are widespread outbreaks of influenza virus infection throughout the world each year. The WHO estimates that worldwide, seasonal influenza epidemics result in three to five million cases of severe illness and 250,000 to 500,000 deaths each year, even in the absence of a major pandemic (8). Within the US, it is estimated that 5% to 20% of the population gets the flu each year, of which over 200,000 people are hospitalized due to flu-related complications and approximately 36,000 people die from flu-related causes (7, 115). In 2010, the CDC modified the method by which estimates of deaths associated with influenza were reported, indicating that the influenza-associated deaths from respiratory and circulatory causes ranged from 3,349 to 48,614 per year (116). In addition, deaths associated with influenza infection are thought to be the seventh leading cause of death in the US (117).

The typical seasonal influenza virus outbreaks within the US usually occur during the cooler, winter months, generally starting in November and ending March, however the duration, timing, and severity of the influenza season can fluctuate significantly each year. Countries in the southern hemisphere usually experience seasonal influenza virus outbreaks during the opposite months of the year, during their cooler season, so influenza viruses are generally circulating somewhere in the world throughout most of the year.

Pandemic Influenza Outbreaks

In addition to seasonal influenza virus epidemic outbreaks, worldwide influenza virus pandemic outbreaks can occur. There were three influenza virus pandemics that occurred in the 20th century, the Spanish influenza pandemic in 1918-1919, the Asian pandemic in 1957, and the Hong Kong in 1968-1969 pandemic, each of which caused millions of deaths worldwide (118-120). The first pandemic of the 21st century occurred in 2009 when a novel H1N1 influenza A virus circulated around the globe. This H1N1 strain is hypothesized to have formed from reassortment of swine influenza A viruses from Europe, Asia, and North America (121). In April of 2009, the US declared a public health emergency due to the high pathogenicity and rapid spread of this influenza virus (122). The CDC estimated that between April 2009 and April 2010, there were 61 million clinical cases of influenza and that 274,000 persons were hospitalized and 12,500 died due to influenza-related complications within the US (123). In addition, as mentioned above, epidemiological data collected during the pH1N1 pandemic identified obesity an independent risk factor for morbidity and mortality from influenza infection for the first time (41).

Influenza Virus Biology

Influenza viruses are negative sense single-stranded segmented RNA viruses. Each RNA segment is encapsidated by a protective nucleoprotein (NP) to form a nucleocapsid or a ribonucleoprotein NP (RNP) complex, which are transcriptionally active (124). There are

three types of influenza viruses: A, B, and C, based on differences in expression of the internal proteins NP and matrix protein (M1) (8). Influenza viruses B and C are not classified by subtype, however influenza virus A is further classified by strains (8). The influenza virus A genome is comprised of 8 RNA segments that code for 11 different proteins, two of which are the two cell surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Influenza virus A is divided into subtypes based on the expression and antigenic differences of these two proteins (8). There are 16 different HA glycoproteins (H1-H16) and 9 different NA glycoproteins (N1-N9) (125). HA plays an important role in influenza virus infection because it binds to sialic acid residues found in carbohydrate side chains of target cell surface glycoproteins and glycolipids via hydrophobic interactions and hydrogen bonds (96, 126). HA glycoproteins form homotrimers that have receptor binding sites and membrane fusion activity (96, 126). Influenza viruses that infect humans express HA glycoproteins with receptor binding sites that bind to sialic acid residues found in an α-2,6-linkage to galactose, while avian and equine viruses express HA glycoproteins that bind to α-2,3-linked sialic acid residues; swine viruses are able to bind sialic acid in both linkages (125). NA has important roles in both influenza virus uptake into target cells and virus efflux from infected host cells. NA glycoproteins form homotetramers with enzyme active sites in each subunit (125). NA cleaves the sialic acid residue from the target cell surface and promotes viral uptake via endocytosis into the cell (96). NA also functions to remove the sialic acid residues from the host cell surface to prevent newly assembled viruses from binding to the host cell and to allow virus budding and release (125). The other proteins coded for by the influenza virus genome include PA, PB1, PB1-F2, PB2, M1, membrane protein-2 (M2), non-structural

protein-1 (NS1), and non-structural protein-2 (NEP) (127). PA, PB1, and PB2 together comprise the RNA polymerase complex, which mediate viral genome replication (127). PB1-F2 is theorized to play a role in initiating cell death in infected host cells (127). M1 is an internal protein that provides structure to the virus particle and acts as a link between the lipid membrane and the RNP complexes, while M2 functions as an ion channel and is essential for effective viral entry into host cells, viral assembly, and viral budding (127). NS1 is involved in evasion of the influenza virus from host cell defenses, while NS2 is important for the translocation of the newly formed RNP complexes to the lipid membrane during replication (127).

Antigenic Drift and Shift of the Influenza Virus

The influenza viral genome is susceptible to frequent mutations both because of evolutionary pressures imposed by host cell immune defenses and because there are no proofreading proteins or mechanisms built into the makeup of the influenza virus, therefore any errors that occur during RNA replication are unable to be corrected and are passed along to progeny viruses (128, 129). When enough mutations occur, they result in changes in amino acids, thereby forming antigenically altered influenza strains. Amino acid changes can occur in all 11 influenza virus proteins, but have particular importance when they occur in the surface glycoprotein HA (128). These mutation-induced changes in the HA protein occur so frequently they result in new influenza strains being constantly formed. This antigenic drift is why there are often changes in the influenza strains that circulate in the

seasonal epidemics from year to year (128, 129). While antigenic drift is constantly occurring, another type of mutation occurs only rarely and at random intervals (128, 129). This second type of mutation of the influenza virus, antigenic shift, is much more dramatic and results in the introduction of a completely novel influenza A subtype into the human population, which may have unique HA and NA proteins (128, 129). Antigenic shift can occur in two ways, either from reassortment between two different influenza viruses in a cell infected with two or more strains of influenza virus, which can sometimes occur in an intermediate host such as swine, or from the direct transfer of a new avian influenza virus to humans (128, 129). If the human population is immunologically naive to this new influenza virus, a pandemic can occur. The three influenza virus pandemics that occurred in the 20th century, the Spanish influenza pandemic in 1918-1919, the Asian pandemic in 1957, and the Hong Kong in 1968-1969 pandemic, are theorized to have resulted from dramatic antigenic shifts of the influenza A virus (118-121, 130). The influenza virus that caused the recent 2009 pH1N1 pandemic theorized to have formed by antigenic shift from reassortment of swine influenza A viruses from Europe, Asia, and North America (121). Specifically, the influenza viral genome segments that code for the HA, NP, PA, PB1, PB2, NS1, and NEP proteins were derived from swine H1N2 and H3N2 influenza A viruses found in the late 1990s in North America, while the influenza viral genome segments that code for the NA, M1, and M2 proteins were derived from swine influenza A viruses isolated in the early 1990s in Europe (121).

Influenza Infection

In humans, influenza can infect any cells that express the α-2,6-linked sialic acid residues (131), including nasal mucosal, tonsil, trachea and lung cells (132), and can be contracted via airborne, droplet, or contact transmission (131). Following the interaction between the influenza viral HA protein and α-2,6-linked sialic acid residues, the bound virus is taken up into the cell via receptor-mediated endocytosis (96). The low pH of the virus-containing endosome causes a change in HA conformation, which promotes fusion with the endosomal membrane and the release of the viral genome-containing nucleocapsid into the cytosol (96, 133).

After the nucleocapsid is released into the cytoplasm, it is transported into the nucleus of the host cell, where replication and transcription occur (133). Within the nucleus, viral RNA polymerases associate with the negative sense single-stranded viral RNA, which is transcribed to make complementary positive sense RNA (133). In addition to the replication of the viral genome, the influenza virus also promotes the transcription and translation of HA and NA proteins, both of which localize to the host cell plasma membrane and consequently the viral envelope, in addition to M1, matrix protein, and M2, an ion channel protein (134). Since the influenza virus assembles and buds from the plasma membrane, all the viral components synthesized within the host cells must be directed to the apical domain of the plasma membrane (134). Both HA and NA possess the biochemical determinants in their structures that target them to the apical plasma membrane (134). HA

and NA proteins are synthesized in the same compartment of membrane-bound polyribosomes and are transported together from the endoplasmic reticulum through the Golgi network to the plasma membrane, while M1 and M2 are synthesized on free cytosolic polyribosomes and then move to the plasma membrane (134). As the infection progresses, more and more viral proteins accumulate on the plasma membrane indicating that the release of influenza virus progeny from the host cell is imminent (134).

In the later part of the infection cycle, the viral RNA genome, polymerase subunits, and nucleoprotein will form RNP complexes, which will then be exported from the nucleus to the cell membrane to form new virions, which will exit the host cell via budding, enveloped in a lipid coating obtained from the host cell plasma membrane (133). RNP complexes congregate near the areas of the plasma membrane with the highest concentrations of the influenza virus proteins HA, NA, M1, and M2 (133). The exportation process is initiated by viral activation of the intracellular mitogen-activated protein kinase (MAPK) signaling cascade (133). Evidence suggests that it is the accumulation of HA in the plasma membrane that actually triggers the activation of the MAPK signaling pathway via protein kinase C activation, resulting in RNP complex export from the nucleus (133). Virus bud formation is a complex process that involves membrane bending at the budding site, which is facilitated in part by the increased viscosity and asymmetry of the lipid bilayer in the areas of the plasma membrane with the highest concentrations of influenza virus proteins (134). After the opposing membranes of the virus bud fuse, the virus particle will

separate from the host plasma membrane and be released into the extracellular environment (134).

Influenza Virus Recognition and Host Cell Signaling

When viral entry occurs, the infected host cell is able to detect viral genomes at initial entry or after replication by pattern recognition receptors, which include TLRs and retinoic-acid-inducible gene I-like receptors (RLRs) (135). TLRs and RLRs activate the infected cell's immune response via complex (136) signaling pathways, which result in the induction of antiviral proteins, including IFNα and IFNβ (136). IFNα and IFNβ are essential for the early antiviral response of target epithelial cells by interfering with viral replication and spread (137), and have even been suggested to be important factors in the impaired response to influenza with obesity (138). In addition, IFNα and IFNβ regulate innate and adaptive immunity by mediating hundreds of IFN stimulated genes, many with antiviral effects (137). Although the TLR signaling pathways are under continued investigation, it is thought that TLR3, which is expressed on intracellular endosomes of target cells, responds to the presence of double stranded RNA influenza virus, present in the cell following replication, by binding with TRIF (Toll/IL-I receptor domain-containing adaptor). This leads to the activation of tank-binding kinase-I (TBK-I), which phosphorylates IFN response factor (IRF)-3 that dimerizes, translocates into the nucleus, and upregulates expression of IFNβ (137, 139). The TLR3 signaling pathway can also initiate at least two other cascades; one results in the translocation of NF-κB into the nucleus, where it upregulates expression of

IFNα and IFNβ (137), while the other results in activation of phosphoinositol-3-kinase (PI3K) and Akt, which phosphorylate and activate IRF-3, resulting in the upregulation of IFNβ expression (139). Of note, the latter PI3K/Akt signaling cascade is also used by insulin (140), which can become dysregulated with obesity-induced insulin resistance, which may be an explanation for the decreased IFNα and IFNβ expression seen in obese mice (11). TLR7, which is also found on intracellular endosomes, is stimulated by single-stranded RNA influenza virus, which activates the MyD88 signaling pathway that phosphorylates IRF-7 (137). IRF-7 can then dimerize, translocate into the nucleus, and upregulate expression of IFNα (137). The RLR retinoic-acid-inducible gene I (RIG-I) is present in the cytosol of target cells and detects the presence of single-stranded RNA influenza virus (137). After activation, RIG-I associates with IFNβ promoter stimulator-1 (IPS-1), initiating a signaling cascade that results in phosphorylation of IRF-3 and IRF-7, which upregulate IFNα and IFNβ expression (136). Figure 2.1 below depicts each of these pathways.

Figure 2.1 Signaling Pathways Initiated with Influenza Infection

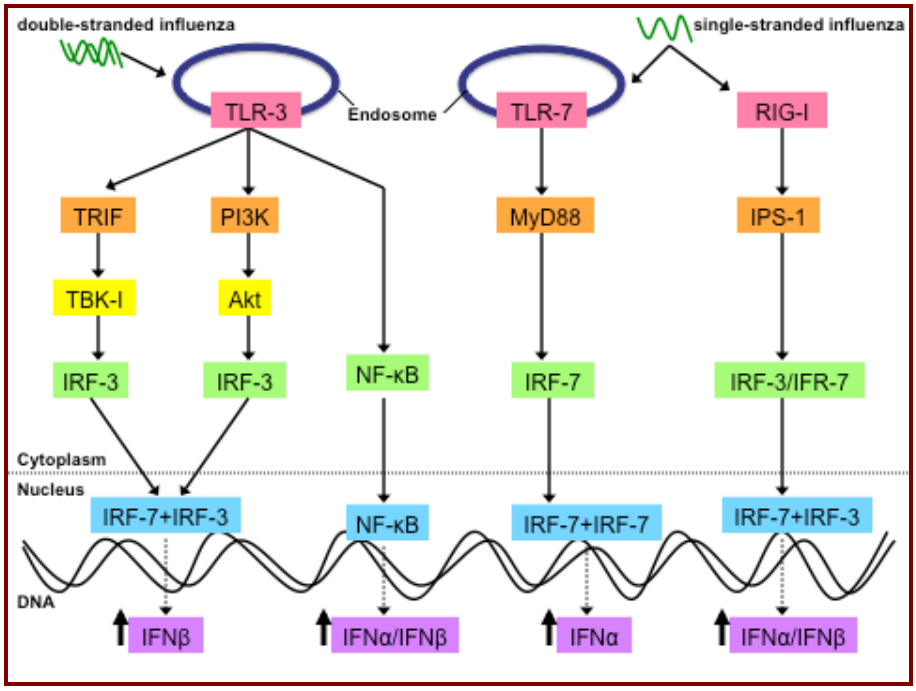


Figure 2.1: TLR3 is activated by double-stranded influenza viral RNA, while TLR7 and RIG-I are activated by single-stranded influenza viral RNA. The signaling pathways upregulate expression of the antiviral cytokines IFNα and IFNβ.

Influenza Vaccination

Vaccination against influenza virus is the most effective method available for decreasing susceptibility to influenza infection and increasing the ability to limit pathology when infected. The trivalent influenza vaccine (TIV) contains the three strains of influenza the WHO predicts to be the most common strains in circulation during the upcoming flu season (115, 141). Successful vaccination produces robust levels of antibodies to the

influenza strains and generates protective lymphocytes that can respond to cross-reacting influenza strains in future encounters and will elicit a rapid, amplified, and effective immune response (141, 142).

The most commonly used influenza vaccine in the US is the inactivated subunit TIV (141, 143). To create the TIV, viruses are grown in embryonated chicken eggs and are then inactivated and purified (144). Because influenza has a segmented genome, the virus strain selected for the upcoming vaccine can be grown in eggs in conjunction with a donor virus, influenza A/Puerto Rico/8/34, which has a very high growth rate in eggs (145). It is a subunit formulation designed to contain primarily the purified surface glycoproteins HA and NA, although suggest that small amounts of other viral proteins, including internal influenza proteins, are present even after inactivation and purification (146-150). The level of protection conferred by vaccination is dependent on the antigenic match between the virus used to create the vaccine and the virus circulating in the population during the influenza season and the quantity and quality of the humoral and cellular immune responses in each individual (115, 141). Vaccination is especially recommended for young children and older adults, as well as for individuals who have a medical condition that could be highly exacerbated by an influenza infection, such as people with chronic cardiovascular or pulmonary disease, pregnant women, and immunosuppressed patients (115, 141). Although there is no definite level of serum antibodies produced in response to vaccination that will ensure protective immunity, many articles indicate that a ≥1:40 serum HAI (hemagglutination inhibition) assay result suggests protective seroconversion (141-143,

151, 152), as that level of serum HAI titer is associated with a low frequency of influenza infections following vaccination (153, 154).

There are some questions about the effectiveness of the influenza vaccine preventing influenza infection in certain groups of people. In healthy younger adults, seasonal influenza vaccination is estimated to be 70% to 90% effective in preventing influenza infection in any given year (155). In adults aged 65 year or older the effectiveness of the seasonal influenza vaccination is thought to be closer to 60% in preventing influenza infection in any given year, due to age-related decreased in immunity, although it does reduce influenza-associated mortality by 80% in this population (155).

Humoral Immune Response to Influenza

Humoral immunity is the primary defense against extracellular microbes and their toxins and is mediated by antibodies produced by B cells. B cells recognize extracellular and cell surface antigens, become activated, differentiate into plasma B cells, and initiate production of antibodies specific to the antigen (142, 156). Antibodies recognize microbial antigens and bind to them, thereby neutralizing the infectivity of the microbes and marking them for elimination (142, 156). Antibodies are able to recognize and bind both soluble and cell-associated antigens (142, 156). B cells differ from T cells in that they are able to recognize numerous types of antigens, including proteins, nucleic acids, polysaccharides, lipids, small chemicals, as well as peptides; T cells however are only able to recognize

peptide antigens (157-159).

The B cell response to influenza virus is mediated via $CD4^+$ helper T cell-dependent antibody responses to influenza virus protein antigens (157, 158). Both B cells and $CD4^+$ T cells must be activated by antigen before the B cell-$CD4^+$ T cell interaction can occur, which mediates the subsequent $CD4^+$ T cell induced activation of the B cell (157, 158, 160). Activated B cells begin expressing CD40, which can bind CD40 ligand (CD40L) on T cells, thereby creating an interaction that is necessary for optimal B cell antibody production and isotype switching (161). Naive B cells are present in and circulate amongst the follicles of the nodes of the peripheral lymphoid organs (157). B cell receptor (BCR) complexes are present in the membranes of naive B cells, and are comprised of IgM or IgD molecules and the noncovalently associated Igα and Igβ molecules, which are linked to each other via disulfide bonds (157, 162). Antigen activation of the BCR complex can occur by three routes. Influenza virus protein antigens can be captured by dendritic cells and displayed on the cell surface in an intact form, where antigen-specific B cells can access them (163). Influenza virus protein antigens can also be transported into B cell follicles by the complement C3 protein and its receptors (164). In addition, it is possible for soluble influenza virus protein antigens to enter follicles and be recognized by B cells (163).

Signaling in the Humoral Immune Response

Antigen-induced signaling via the BCR complex is actuated by cytoplasmic domains of

the Igα and Igβ molecules, which contain tyrosine residues that can be phosphorylated by the nearby tyrosine kinases of the Src family, including Lyn, Fyn, and Blk (165). Following the phosphorylation of the Igα and Igβ molecules, Syk is phosphorylated and activated, which result in the activation of multiple downstream signaling pathways (165, 166). These pathways include the protein kinase C-γ (PKCγ) pathway, which results in increases in the second messengers diacylglycerol and Ca^{2+}, which activate PKC and other enzymes (167), in addition to the Ras-MAPK pathway which activates extracellular signal-regulated kinase (ERK) and JNK (168). These BCR complex signaling pathways lead to the activation of transcription factors such as NFAT, NK-κB, and AP-1, which regulate the proliferation and differentiation of B cells (169, 170). BCR complex signaling also results in the uptake of the antigen via receptor-mediated endocytosis; the antigen is then processed in an endolysosome and the resulting peptides are presented on the B cell surface in complex with class II major histocompatibility complex (MHC) molecules (171). Furthermore, BCR complex signaling upregulates expression of the chemokine receptor C-C-C chemokine receptor type 7 (CCR7) and downregulates expression of the chemokine receptor chemokine (C-X-C motif) receptor 5 (CXCR5) (172). The ligand for the CXCR5 chemokine receptor, C-X-C motif chemokine 13 (CXCL13), is secreted by follicular dendritic cells, present only in the follicle of lymph nodes, thus allowing the activated B cell to migrate from the follicle towards the T cell zone (173).

Role of Dendritic Cells and $CD4^+$ T Cells in the Humoral Immune Response

Dendritic cells internalize and process the influenza virus antigen proteins, move to lymph nodes, and then present them in association with class II MHC molecules to naive $CD4^+$ T cells, thereby activating them (174-176). $CD4^+$ T cell activation is required for a robust B cell response (159, 160). The dendritic cells are also induced to express B7-1 and B7-2, also known as CD80 and CD86, together forming B7, which provide second signals for $CD4^+$ T cell activation (177, 178). The activated $CD4^+$ T cells begin to proliferate, upregulate expression of CD40L (176), and increase secretion of cytokines (160). They also downregulate expression of the CCR7 chemokine receptor and upregulate expression of the CXCR5 chemokine receptor, which causes the activated $CD4^+$ T cells to leave the T cell zone and migrate towards the follicle (159, 160, 179). The interaction between the activated B cells and $CD4^+$ T cells occurs via binding between the antigen-class II MHC complex and CD40 trimers expressed on the B cell and the TCR and CD40L expressed on the $CD4^+$ T cell (159-161). When the CD40L binds the CD40 trimers, an intracellular signaling cascade is initiated that starts with the association of TRAFs with the cytoplasmic domain of CD40; these CD40-mediated signaling cascades are required for B cell proliferation, differentiation, and especially germinal center formation (180). In addition, the $CD4^+$ T cells are able to secrete numerous cytokines that are able to bind their receptors on the B cell in close proximity, including IL2 (181), IL4 (181), IL21 (182), B-cell activating factor (BAFF) (183), and a proliferation-inducing ligand (APRIL) (184) which mediate and regulate B cell proliferation and differentiation, and IL6, which acts as a B cell growth factor (160, 182).

B Cell Class Switching

Following the B cell-$CD4^+$ T cell interaction at the interface between the follicle and the T cell zone, activated B cells migrate deeper into the lymphoid follicle, where germinal center formation occurs, which is the site for heavy chain isotype (class) switching, B cell affinity maturation, and memory cell generation (185, 186). Class switching occurs within the germinal center (186). Without cytokine input from $CD4^+$ T cells, B cells will only produce antibodies of the IgM and IgD classes (159, 186). $CD4^+$ T cells are able to direct the humoral immune response that will best counteract the specific invading microbe through the types of cytokines they produce and secrete (159), as shown in Figure 2.2. One of the key enzymes involved in class switching is activation-induced deaminase (AID), which is upregulated via CD40 activation (187, 188). The influenza virus particularly activates T_H1 cell subset of $CD4^+$ T cells, which produce high levels of IFNγ (159, 189, 190). This cytokine is a potent inducer of B cell class switching to IgG antibodies, the primary antibody found in the serum and of great importance in the humoral response to the influenza virus (191).

There are a number of different subclasses of IgG antibodies, including IgG1, IgG2a, IgG2b, and IgG3 (192). Interesting, severe pH1N1 infection was found to be correlated with a deficiency of IgG2 subtype antibodies; the authors hypothesized that pregnancy-related reductions in IgG2 levels would explain this relationship in some, but not all pregnant patients (193). Another study suggested that there were lower levels of both IgG1 and IgG2

subtype antibodies in patients with severe pH1N1 infection (194).

Figure 2.2 Dendritic Cell Presentation of Influenza Peptides to B Cells and CD4$^+$ T Cell-induced Class Switching

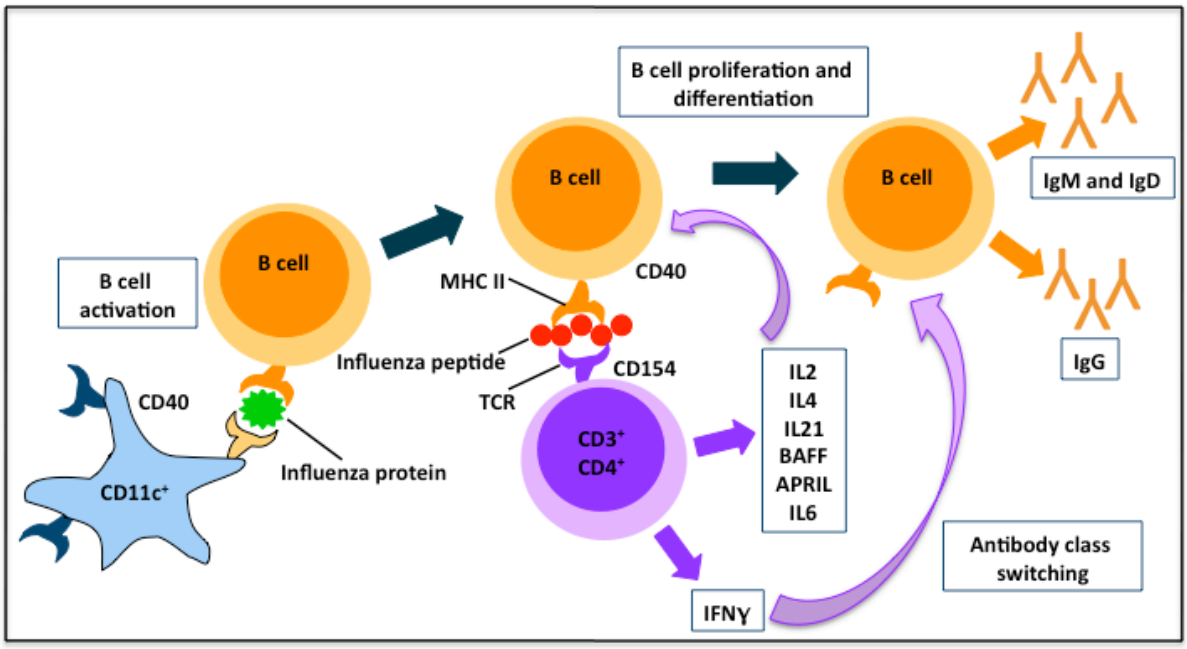


Figure 2.2: Once activated, CD11c$^+$ dendritic cells present influenza proteins to B cells, thereby activating them and inducing their differentiation and proliferation, as well as upregulating antibody production. CD4$^+$ T cells are able to secrete numerous cytokines that are able to bind their receptors on the B cell in close proximity, including IL2, IL4, IL21, BAFF, and APRIL which mediate B cell proliferation and differentiation, and IL6, which acts as a B cell growth factor. CD4$^+$ T cells also produce high levels of IFNγ, which is a potent inducer of B cell class switching to IgG antibodies, the primary antibody found in the serum and the most important antibody in the humoral response to the influenza virus.

In addition, location within the body plays a role in class switching. In mucosal-associated lymphoid tissues, production of transforming growth factor-β (TGF-β) induces antibody class switching to IgA antibodies (195). IgA antibodies are localized to epithelia via transport through mucosal secretions and function to protect these epithelia from invading

microbes (196). This is important for influenza virus infection, which often is initiated in the upper respiratory mucosa (196). B cell affinity maturation also occurs in germinal centers, where antigen-specific B cells rapidly proliferate and experience high levels of somatic mutations in the Ig genes, resulting in the production of B cells that produce slightly different antibodies with varying levels of affinities to the antigen (197). Follicular dendritic cells display the antigen and the B cells producing the antibodies with the highest affinity to the antigen are selected to survive (197). The cytokines IL2, IL4, and IL6 produced by $CD4^+$ T cell further promote and regulate antibody synthesis and secretion by the selected B cells (181, 182). Antibody secreting plasma B cells can migrate from the germinal center to the bone marrow where they can reside for long periods of time and still produce the antigen-specific antibodies (198). In addition, some antibody-secreting B cells can become memory cells, which are able to survive in the periphery for long periods of time, even in the absence of antigen (199). If the antigen is encountered in the future, the memory B cells will be able to act to mediate a faster and more effective humoral immune response for many years (199).

Antibody Function in the Humoral Response

IgG antibodies are able to neutralize infectivity of the influenza virus by preventing uptake into target cells by binding to HA and NA on the viral membrane and physically preventing them from interacting with the sialic residues on target cells (196, 200). IgG antibodies also promote destruction of the influenza virus by binding to HA and NA, thereby

initiating the opsonization process that marks the influenza virus particle for phagocytosis (196, 200). In addition, IgG antibody binding to HA and NA on the influenza virus can also trigger complement activation, which results in the formation of the membrane attack complex that destroys the virus (196, 200). IgG antibodies can also initiate antibody-dependent cell-mediated cytotoxcity, which marks an influenza-infected cell for destruction by natural killer cells (196, 200).

Humoral Immune Response to Influenza Vaccination

The humoral immune responses to influenza infection and inactivated TIV are somewhat similar. Influenza viral proteins are either introduced to the body as a natural infection via the mucosa in the nasal and respiratory epithelia or as part of a vaccination administered to the deltoid muscle. At either location, the influenza proteins are taken up by dendritic cells and carried to lymphoid organs where they can interact with B cells and $CD4^+$ T cells. An influenza infection initiates the humoral immune response as described above, resulting in populations of B cells that produce antibodies that target all the major influenza proteins (201). The production of antibodies to HA can prevent further infection as these antibodies can neutralize the virus and prevent it from entering other potential target cells, while antibodies to NA predominantly act to impair virus budding from infected host cells, thereby preventing further spread of the infection (202). The influenza virus invades the body via the upper and lower respiratory tracts. IgA antibodies specific to viral proteins provide protection in the mucosal secretions of the upper respiratory tract, while

IgG antibodies specific to viral proteins provide protection via the serum in the lower respiratory tract (203). Antibodies specific to influenza virus proteins can be detected in the peripheral blood within 10-14 days after infection initiation; IgG antibody levels peak 4-6 weeks after the start of the infection, while IgA and IgM antibodies reach their highest levels at about 2 weeks following the start of the infection (204). The inactivated TIV is injected intramuscularly in the deltoid muscle of the arm and the humoral immune response proceeds as described above, primarily directed towards the influenza virus membrane glycoproteins HA and NA (205). The humoral response to vaccination is rapid, as increases in influenza-specific antibodies can be seen in serum 2-6 days following vaccination (205, 206). The predominant antibody detected in the serum in response to influenza vaccination is IgG, with IgA and IgM antibodies detected at lower levels (205, 207, 208). Even though subunit vaccines are purified formulations that primarily contain the surface glycoproteins HA and NA, vaccination with subunit vaccines can stimulate antibody production specific to other internal viral proteins, such as M1 and NP (209), which is interesting to note when interpreting data from our studies.

Cellular Immune Response to Influenza

Cellular immunity is the primary defense against intracellular microbes, including viruses, and is mediated by T cells. It is interesting to note that the most severe cases of pH1N1 in humans were characterized by defective T cell responses (210). T cells promote killing of infected cells, thereby eliminating reservoirs of infection. Two types of T cells are

$CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. $CD4^+$ helper T cells produce cytokines, which function to promote inflammation, proliferation, and differentiation of T cells, as well as activation other immune cells such as macrophages and B cells; $CD8^+$ cytotoxic T cells directly destroy infected cells. Both types of T cells have a restricted specificity for antigens, recognizing only peptide antigens that are bound to MHC molecules on the surface of other cells. There are two main types of MHC molecules: class I MHC molecules (MHC-I) and class II MHC molecules (MHC-II). Class I MHC molecules are expressed on all nucleated cells, while class II MHC molecules are expressed only on limited types of cells, including dendritic cells, B cells, and macrophages (211). In addition, the two types of MHC molecules function to sample different pools of peptide antigens. Class I MHC molecules access peptide antigens derived from cytosolic proteins that were degraded in proteasomes (211). These peptide antigens become associated with class I MHC molecules in vesicles in the cytosol; the antigen-class I MHC complexes then move to the cell surface and present to $CD8^+$ T cells (211). Class II MHC molecules access peptide antigens from extracellular antigens that have been endocytosed into vesicles and degraded in endolysosomes (211, 212). These peptide antigens move to the endoplasmic reticulum where they become associated with class II MHC molecules; the peptide-class II MHC complexes then move to the cell surface and present to $CD4^+$ T cells (211, 212).

Dendritic Cells in Cellular Immunity

Dendritic cells are specialized or "professional" antigen-presenting cells and process microbial antigens from the external environment; they are found in many areas of the body including lymphoid organs, as well as the epithelium of the skin, gastrointestinal, and respiratory tracts (174, 175). Immature dendritic cells express CD209, a receptor that binds the mannose-type carbohydrates typically found on viruses, such as the influenza virus (213). After binding, dendritic cells can internalize and process antigen protein (174, 175). Activated dendritic cells localize to draining lymph nodes where they present antigen peptide-MHC complexes to naive T cells, initiating the adaptive immune response (174, 175). CD40, a transmembrane surface receptor, is constitutively expressed at low levels on non-activated dendritic cells; its expression is substantially upregulated when dendritic cells process antigen and is therefore used as marker of activation (214). CD40 signaling promotes expression of MHC-II and the costimulatory molecules CD80 and CD86 (177), thereby increasing the capacity of dendritic cells to effectively present antigen. CD80, present only on mature dendritic cells, and CD86, an early marker of dendritic cell activation, together form B7, and bind CD28 on T cells and act as costimulators for T cell activation (178). Mature dendritic cells secrete IL12, which promotes differentiation to the T_H1 cell subtype and IFNγ production in $CD4^+$ T cells (215, 216) and enhances cytotoxic activity and IFNγ production in $CD8^+$ T cells (217), and IL7, which is required to effectively trigger the T cell response to influenza virus (218). Dendritic cells have the unique ability to cross-present peptide antigens to both $CD4^+$ T cells and $CD8^+$ T cells (219). They are able to

ingest infected cells and present extracellular antigens derived from these cells in association with class I MHC molecules to $CD8^+$ T cells, in addition to the more conventional route of presentation, in which the extracellular antigens are associated with class II MHC molecules and presented to $CD4^+$ T cells (212), as shown in Figure 2.3. This is especially important with influenza viral infections to ensure that $CD8^+$ cytotoxic T cell responses can be initiated despite the fact that the influenza virus is an intracellular microbe.

Figure 2.3 Dendritic Cell Presentation of Influenza Peptides to $CD4^+$ and $CD8^+$ T Cells

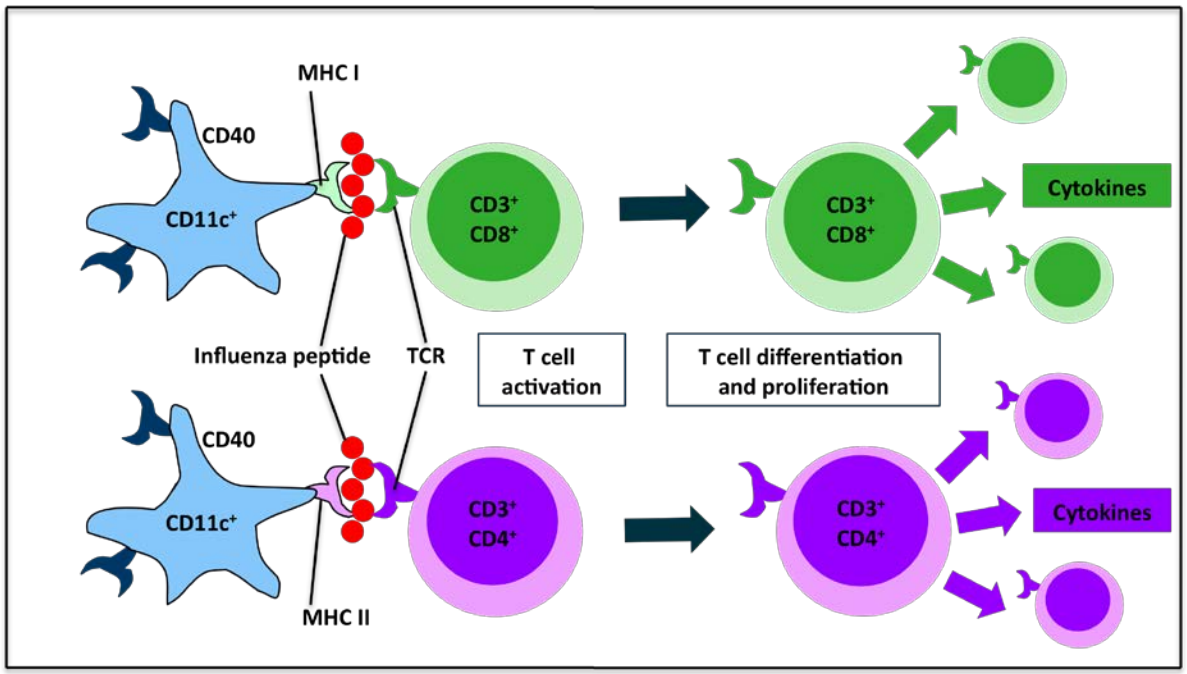


Figure 2.3: Once activated, $CD11c^+$ dendritic cells process influenza proteins and present antigenic influenza peptides to $CD8^+$ T cells in association with class I MHC molecules and to $CD4^+$ T cells in association with class II MHC molecules. Presentation of influenza peptides activates $CD8^+$ T cells and $CD4^+$ T cells and induces their differentiation and proliferation, as well as upregulates cytokine production.

$CD4^+$ and $CD8^+$ T Cells in Cellular Immunity

The CD4 and CD8 molecules are transmembrane glycoproteins and coreceptors; they function to bind with MHC molecules and to enhance TCR signaling. $CD4^+$ T cells and $CD8^+$ T cells recognize and interact with class II MHC-peptide complexes and class I MHC-peptide complexes, respectively (211). $CD4^+$ T cells and $CD8^+$ T cells both express CD3, a transmembrane protein with intracellular and extracellular domains (220). The T cell-specific Src family tyrosine kinase Lck is closely associated with the cytoplasmic regions of CD4 and CD8 and when activated will function to phosphorylate the tyrosine residues in CD3 on the T cells, thereby stimulating the T cell activation cascade (221). Many $CD4^+$ and $CD8^+$ T cells also express CD28, a costimulatory receptor that binds with B7 on dendritic cells and functions to initiate a signaling cascade that promotes activation of T cells (222). Activation of CD4 and CD8 results in proliferation and expansion of antigen-specific T cells and differentiation into effector and memory T cells. Dendritic cells further support expansion and differentiation of the T cell pool via the secretion of IL12 (223). Activated $CD4^+$ and $CD8^+$ T cells both produce and secrete IL2, which has autocrine functions to promote expansion and differentiation (224). T cells can migrate to the location of infection where they act to eliminate the source of infection.

T cells mediate and execute the cellular response to influenza virus. $CD4^+$ T cells provide help, in the form of cytokine synthesis and secretion, to promote the activation and

cytotoxic function of $CD8^+$ T cells and the activation and antibody production of B cells. It is primarily the T_H1 subset of $CD4^+$ T cells that mediates the immune response to influenza (189, 190). The main functions of $CD8^+$ T cells are to kill influenza-infected cells, thereby limiting the spread and severity of infection, and also to secrete cytokines that act to regulate the immune response. The higher risk for influenza-related complications in obese individuals could be caused by defects in the $CD4^+$ and $CD8^+$ T cell responses to the virus.

The activation of $CD4^+$ and $CD8^+$ T cells is a highly coordinated process. CD69 is a T cell activation marker and its expression increases substantially and early in the activation process (225). CD28, the receptor for the costimulatory molecules CD80 and CD86, also increases with T cell activation and signals to promote proliferation, expansion, sensitivity to antigen, and survival of T cells (226). CD40L expression is upregulated in mature T cells and it binds and activates CD40 on dendritic cells (227). In addition, the IL12 receptor (IL12R) becomes enriched in the cell surface of activated T cells (228).

Effector $CD4^+$ T Cells

Effector $CD4^+$ T cells secrete large amounts of diverse cytokines that have numerous effects in the immune response. $CD4^+$ T cells play a role in the activation and differentiation of $CD8^+$ T cells, by acting directly on $CD8^+$ T cells and by acting on antigen-presenting cells to enhance their ability to stimulation $CD8^+$ T cell differentiation. $CD4^+$ T cells produce TNFα, which binds to CD40 on antigen-presenting cells that makes them better able to stimulate

differentiation on $CD8^+$ T cells (229). $CD4^+$ T cells can also activate macrophages to destroy the microbes that they have taken up by phagocytosis (230). $CD4^+$ T cells can be further divided into a number of subsets, including T_H1, and T_H2 cells. Differentiation of $CD4^+$ T cells into $CD4^+$ T_H1 cells is primarily mediated by IL12, which signals via the janus kinse-2-signal transducer and activator of transcription-4 (JAK2-STAT4) pathway (231-233), and by IFNγ, which signals via STAT1 (234, 235). One of the most important functions of differentiated $CD4^+$ T cells is to produce numerous cytokines; T_H1 cells produce high amounts of IFNγ, particularly in response to influenza (189, 236), and lesser amounts of IL3, IL10, and granulocyte/macrophage-colony-stimulating factor (GM-CSF) (236-238), while T_H2 cells produce large amounts of IL4, IL5, and IL13 and lesser amounts of IL3 and GM-CSF (237, 238). IL5, secreted predominantly by the T_H2 subset of $CD4^+$ T cells, is more closely associated with allergic responses rather than viral pathogens (239). It is primarily the T_H1 cells that mediate the immune response to influenza infection; in addition, T_H1 cells promote the $CD8^+$ T cell-mediated responses to influenza vaccination and help to maintain the vaccination-specific memory $CD8^+$ T cell population (240).

Effector $CD8^+$ T Cells

Effector $CD8^+$ T cells have cytotoxic function and act to destroy cells that have been infected with intracellular microbes. The differentiation of $CD8^+$ T cells primarily involves the development of the cytoplasmic granules that contain the proteins to be used for killing target cells, namely perforin and the granzymes, mediated via changes in gene transcription

(241). Signals from $CD4^+$ T cells may be required for differentiation of $CD8^+$ T cells, such as the binding of CD154 on $CD4^+$ T cells to CD40 on antigen-presenting cells, making them more efficient at stimulating differentiation of $CD8^+$ T cells (242). The primary function of differentiated $CD8^+$ T cells is to act to destroy cells that have been infected with intracellular microbes. $CD8^+$ T cells form an immunological synapse with the infected antigen-expressing target cell (243). $CD8^+$ T cells have granules in their cytoplasm containing substances that function to destroy target cells. Upon cytoskeleton rearrangement the granules are translocated to the immunological synapse, where they fuse with the plasma membrane and releases their contents (243). The granules contain perforin and granzymes. Perforin forms a pore in the target cell, which allows the granzymes to move into the infected cell (243), a process called degranulating, of which the marker CD107a is an indicator (244). One of the most potent granzymes is GrB, which executes the cytotoxic function of $CD8^+$ T cells. GrB is a serine protease enzyme that cleaves peptides after aspartyl residues and activates both the caspase and Bcl-X_L inhibitory protein BH3 interacting-domain (Bid) signaling cascades, resulting in the fragmentation of nuclear and mitochondrial DNA, respectively (245). GrB activates caspase-3 and the Bcl-2/Bid, which triggers the mitochondrial pathway of apoptosis (245). Bcl-2 is a mitochondrial protein and an inhibitor of the mitochondrial pathway of apoptosis (245). Bid can bind to Bcl-2 and inactivate it, thereby decreasing its apoptotic inhibition (245). Bid can also bind and opens the mitochondrial membrane Bax channel, which allows the mitochondrial protein cytochrome C to move into the cytoplasma where it helps to activate caspase-9 (245). $CD8^+$ T cells also express the Fas ligand membrane protein, which can bind to the death receptor Fas on target cells. Fas activation

initiates a signaling pathway that activates caspase-8 (245). Caspases are powerful proteolytic enzymes that cleave proteins at aspartate residues, leading to destruction of the infected target cell (245). Another function of differentiated $CD8^+$ T cells is to produce cytokines, including IFNγ, lymphotoxin, and TNFα, which promote inflammation and activate phagocytic cells (246).

Memory T Cells

Memory T cells are specific to a unique antigen and can persist for years. When an antigen is encountered again, memory T cells allow for a more rapid and amplified immune response to that antigen (247). Memory T cells can be derived from both $CD4^+$ and $CD8^+$ T cells, and can further be divided into different subclasses of memory T cells, central memory T cells and effector memory T cells. Central memory T cells express CCR7 and L-selectin, while effector memory T cells do not express either (248). Central memory T cells reside in lymphoid tissue, while effector memory T cells reside in peripheral tissues (248). When central memory T cells are exposed to a repeat antigen, they can undergo proliferation quite rapidly to produce high numbers of effector cells (248). When effector memory T cells encounter a repeat antigen, they produce large amounts of cytokines such as IFNγ (248). The maintenance of memory T cells is dependent on low-level, long-term proliferation, mediated by the appropriate cytokine milleau (248, 249). $CD4^+$ memory T cells require IL7, while $CD8^+$ memory T cells require both IL7 and IL15 (249).

Humoral and Cellular Responses to Influenza

The scope of the cellular response to influenza is dictated by the antigenic peptides that are presented to $CD4^+$ cells and $CD8^+$ T cells in the lymph nodes by dendritic cells or other antigen-presenting cells. The cellular response to influenza infection and vaccination is different than the humoral response as it is focused on the destruction of virus-infected cells, rather than infection prevention. The cellular response to infection is primarily mediated via the helping functions of $CD4^+$ T cells through production of cytokines and via the cytotoxic functions of $CD8^+$ T cells which directly kill infected cells. Infection with influenza virus activates a significant $CD4^+$ T cell response, particularly the T_H1 $CD4^+$ T cell response, which functions to upregulate B cell influenza-specific antibody production (159, 186). During infection, T cells specific to the influenza virus have been found in the peripheral blood and lower respiratory tract secretions in humans (250). Numbers of influenza-specific $CD8^+$ T cells can be measured in peripheral blood 6-14 days following initiation of infection or vaccinated vaccination in humans (251).

T Cell Metabolism

When both $CD4^+$ and $CD8^+$ T cells become activated by an antigen and prepare to initiate an appropriate immune response, there are very unique and specific changes that occur in their energy metabolism (252, 253). At rest, in a non-activated, non-stimulated state, T cells get most of their ATP from a mixture of fuels oxidized in the mitochondria,

which includes the Kreb's cycle, the electron transport chain, and fatty acid β-oxidation (254). After activation, T cells rapidly switch from obtaining most of their ATP from oxidative phosphorylation to almost entirely deriving ATP from aerobic glycolysis (255), in addition to the pentose phosphate pathway and glutaminolytic pathways (256, 257). This tremendous flux through aerobic glycolysis results in the production of very high levels of lactate from pyruvate, surprisingly even under conditions of adequate oxygen (258-260), similar to the Warburg effect in cancer cells (261). Therefore the major metabolic byproduct from activated T cells is lactate (254, 262); as much as 85% of glucose consumed by activated T cells is converted to lactate (263), and much of that is then secreted from the T cells. If glucose uptake or flux through the glycolytic pathway is suboptimal, T cells cannot synthesize cytokines, such as IFNγ (264), cannot proliferate effectively (265), and proapoptotic proteins are upregulated, thereby increasing T cell death (265). Even under conditions of an excess of alternative energy sources, glucose limitations can prevent T cell proliferation and survival (264). Studies show that there is a direct positive correlation between proliferation and survival of T cells and increasing glucose concentrations (265). Furthermore, resting T cells express very low levels of the glucose transporter (GLUT) 1 (265), and the insulin receptor (266); however upon activation, expression of both GLUT1 and the insulin receptor is rapidly and significantly upregulated (265, 266). In addition, insulin may have anti-inflammatory effects on circulating immune cells and may drive T cell differentiation towards an anti-inflammatory T_H2 phenotype, which may suppress the pro-inflammatory T_H1 phenotype (267). Full activation of T cells requires two signals: stimulation of the TCR complex, which upregulates GLUT1 protein synthesis, and CD28

costimulation, which increases GLUT1 trafficking to the cell surface through PI3K/Akt signaling pathway (268). Stimulation of T cells with both anti-CD3 and anti-CD28 antibodies, but with neither alone, resulted in increase GLUT1 translocation to the cell surface and significant increases in glucose uptake rate (268). In fact, increases in glucose utilization into T cells are seen as soon as one hour after stimulation with anti-CD3 and anti-CD28 antibodies (262, 269, 270). Increases in GLUT1 gene transcription, GLUT1 protein translation, GLUT1 translocation to the cell surface, and GLUT1 activity are all essential to utilize the immense amounts of glucose required to sustain flux through aerobic glycolysis (268, 271). GLUT1 translocation to the cell surface requires appropriate activation of the PI3K/Akt signaling pathway (272). Phosphorylation of the amino acid residue threonine 308 (Thr308) leads to partial Akt activation, while full activation of Akt requires additional phosphorylation of amino acid residue serine 473 (Ser473) (273). Activation of the TCR complex and CD28 and stimulation of the insulin receptor will initiate a signaling pathway via PI3K/Akt that results in the phosphorylation of Ser473 (272) and the phosphorylation of Thr308 (140). In fact, when PI3K is inhibited in CD28-stimulated T cells, the upregulation of glycolysis is impaired, likely due to decreased translocation of GLUT1 to the cell surface (268).

Figure 2.4 PI3K/Akt Signaling Pathway in Activated T Cells

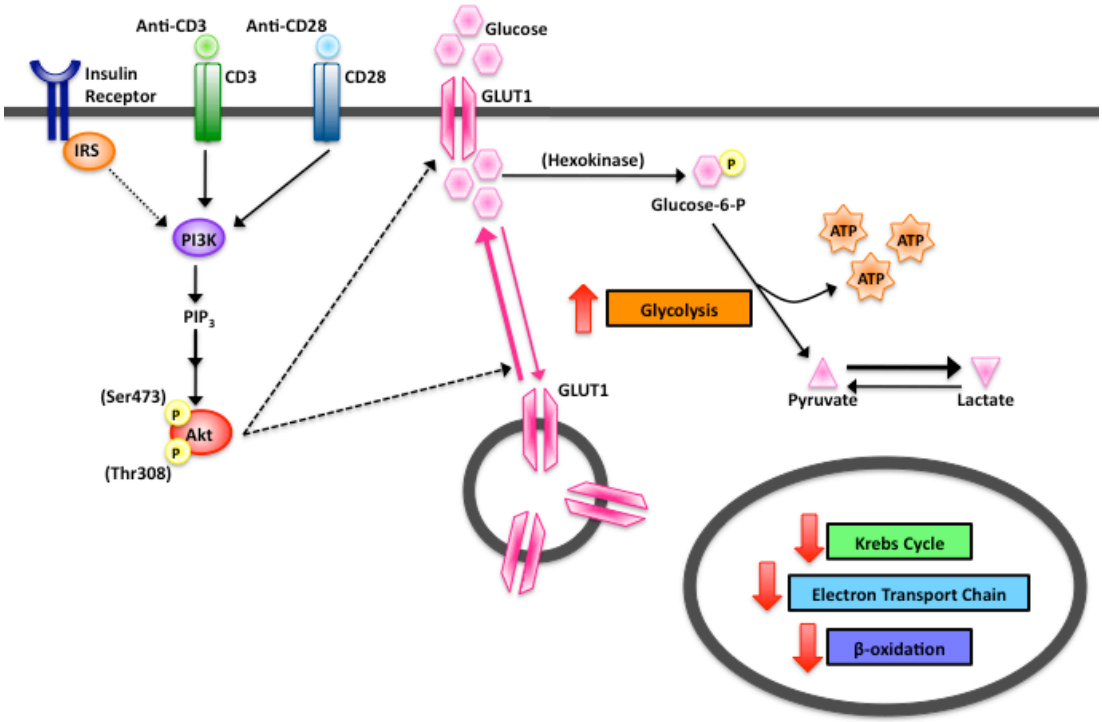


Figure 2.4: In order to accommodate the increased glucose needs of the activated T cell, expression of the glucose transporter GLUT1 and the insulin receptor are rapidly upregulated. Signaling through the PI3K/Akt pathway results in increased GLUT1 transcription, translation, translocation, and activity. Full activation of Akt requires phosphorylation at both the threonine 308 (Thr308) and serine 473 (Ser473) amino acid residues.

Significance

The specific mechanisms involved in the differential response of obese individuals with or without type II diabetes to influenza vaccination, influenza infection, or other stimuli are as of yet unknown, but likely involve alterations in the humoral, the cellular response, or

both. Potential humoral response mechanisms include defective maintenance of antibody-producing B cells or the production of less effective antibodies. Potential cellular response mechanisms include modifications to the proliferation, differentiation, or function of dendritic cells, effector $CD4^+$ T cells, or effector $CD8^+$ T cells or to the generation or maintenance of memory $CD4^+$ T cells, or $CD8^+$ T cells. Impaired humoral or cellular responses to influenza vaccination or influenza infection, resulting in increased susceptibility to an influenza infection or decreased ability to fight off an acquired influenza infection. There are widespread outbreaks of influenza throughout the world each year and with the dramatic increases in obesity, it is imperative that the biochemical mechanisms modulating the interplay between obesity and influenza be elucidated, to guide the development of more effective influenza infection treatment methods and appropriate influenza vaccination strategies for obese individuals.

CHAPTER III

OBESITY IS ASSOCIATED WITH IMPAIRED IMMUNE RESPONSE TO INFLUENZA VACCINATION IN HUMANS[1]

Overview

Obesity is an independent risk factor for morbidity and mortality from pandemic influenza H1N1. Influenza is a significant public health threat, killing an estimated 250,000 to 500,000 worldwide each year. More than one in ten of the world's adult population is obese and more than two-thirds of the US adult population is overweight or obese. No studies have compared humoral or cellular immune responses to influenza vaccination in healthy weight, overweight and obese populations despite clear public health importance. The study employed a convenience sample to determine the antibody response to the 2009-2010 inactivated TIV in healthy weight, overweight and obese participants at one and 11 months post vaccination. In addition, activation of $CD8^+$ T cells and expression of IFNγ and GrB were measured in influenza-stimulated PBMC cultures. BMI correlated positively with higher initial fold increase in IgG antibodies detected by ELISA to TIV, confirmed by HAI

[1] Previously published: Patricia A. Sheridan and Heather A. Paich (co-first authors), Jean Handy, Erik A. Karlsson, Michael G. Hudgens, Aileen B. Sammon, Lara A. Holland, Samuel Weir, Terry L. Noah, and Melinda A. Beck. *International Journal of Obesity* (London). 2012 Aug;36(8):1072-1077.

antibody in a subset study. However, eleven months post vaccination, higher BMI was associated with a greater decline in influenza antibody titers. PBMC's challenged *ex vivo* with vaccine strain virus demonstrated that obese individuals had decreased $CD8^+$ T cell activation and decreased expression of functional proteins compared with healthy weight individuals. These results suggest obesity may impair the ability to mount a protective immune response to influenza virus.

Introduction

Influenza causes some three to five million cases of severe illness and 250,000 to 500,000 deaths every year around the world, even in the absence of a major pandemic (8). Obesity is a growing health concern of epidemic proportions in many countries (1) ; more than one in ten of the world's adult population is obese (1) and more than two-thirds of the US adult population is overweight or obese (14). In addition to co-morbidities such as cardiovascular disease and diabetes, obesity itself is an immunosuppressive condition (3-5). During the recent pandemic of influenza A/H1N1/2009 (pH1N1), obesity was recognized for the first time as an independent risk factor for increased influenza morbidity and mortality (6, 41, 274-276).

Annual vaccination is the primary strategy available for decreasing the impact of influenza infection. No studies have examined how obesity may affect the response to influenza vaccination in humans. Obesity is associated with decreased antibody response to

hepatitis B vaccine (9, 277) and to tetanus toxoid (10). Our work with diet-induced obese mice found greater mortality following influenza infection and impaired innate immune responses (11), as well as an impaired $CD8^+$ T cell memory response that increased morbidity and mortality from a secondary influenza challenge (12). However, the effects of obesity on immune responses to influenza vaccine have not been characterized in humans. We therefore initiated a prospective observational study of the effect of BMI on humoral and cell mediated immune responses to influenza vaccination in humans. We here report data from the first two years of this study.

Methods

Study Design and Subjects

This is an ongoing, prospective observational study carried out at the University of North Carolina Family Medicine Center, an academic outpatient primary care facility in Chapel Hill, NC. Eligible subjects were adult (≥18years) patients at the Center scheduled to receive the 2009-2010 seasonal TIV. Enrollment and data analysis were conducted independently for each year because of the annual change in vaccine composition. Exclusion criteria were immunosuppression, self-reported use of immunomodulator or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillian-Barre syndrome, or use of theophylline preparations or warfarin (278, 279). All procedures were approved by the Biomedical

Institutional Review Board at the University of North Carolina. In year one of the study (September-November 2009), we enrolled 499 participants. At enrollment, informed consent, height, weight, and a baseline serum sample were obtained. One dose of 2009-2010 seasonal TIV [0.5mL Fluzone (Sanofi Pasteur, Swiftwater, PA, USA) containing A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008] was administered in the deltoid muscle. Participants (461, 92% completion rate) returned 28-35 days later for a post-vaccination blood draw. Pre- and post-vaccination serum samples were stored at -80°C until analyzed, while PBMCs were separated from a second heparinized blood sample on a Histopaque (Sigma, St. Louis, MO, USA) gradient and frozen in liquid nitrogen until analyzed. At the start of year two of the study, 74 participants who had participated in year one independently re-enrolled to participate in year two, providing us the additional opportunity to assess immune parameters 11 months after receipt of the 2009-2010 TIV.

ELISA and Haemagglutination Inhibition Assays

IgG antibodies were quantified by enzyme-linked immunosorbent assay (ELISA) using the 2009-2010 seasonal TIV as antigen. Vaccine was diluted and adsorbed to microtitration plates in a carbonate coating buffer. After washing, triplicate serum dilutions in PBS were allowed to react with antigen, and bound antibodies were detected by a peroxidase-conjugated goat anti-human IgG (Abcam, Cambridge, MA, USA), followed by a chromogenic substrate. Color intensity was measured by absorbance at 450nm. Internal control sera

were included in each run. Pre- and post-vaccination sera from each participant were tested in the same run. The intra-assay coefficient of variation using this assay is 4%. In order to determine serotype-specific antibody responses to the three components of the seasonal TIV, a subset of pre- and one month post-vaccine serum samples from 38 healthy weight and 38 obese participants, matched for race, age, and sex, was tested by Focus Diagnostics (Cypress, CA, USA) for HAI antibodies against the 2009-2010 seasonal TIV strains.

Activation and expression of IFNγ and Granzyme B by influenza-stimulated PBMCs

PBMC samples obtained from 61 participants 11 months post-vaccination (23 healthy weight (BMI 22.2 +/- 1.7), 17 overweight (BMI 27.6 +/- 1.5), and 21 obese (BMI 35.7 +/- 4.5)) were thawed and cultured in AIM-V serum-free media supplemented with 1% penicillin/streptomycin and 1% glutamine. We have previously determined that our freezing method did not alter lymphocyte cell subset numbers in samples from healthy weight or obese individuals (data not shown). Cells were plated at 1 X 10^6 cells per well for 120 hours with or without stimulation with 6.4 hemagglutination units of influenza A/Brisbane/59/2007 (H1N1). For the last 6 hours, GolgiPlug, a protein transport inhibitor containing Brefeldin A, (BD Biosciences, San Jose, CA, USA) was added to all PBMC samples. PBMCs were stained with fluorochrome-conjugated antibodies and analyzed for CD3, CD8, CD69, IFNγ, and GrB expression using a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA). Appropriate isotype controls were used for each stain and data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). CD69 is an early-activation

marker expressed on the surface of activated lymphocytes. $CD8^+$ cytotoxic T cells function to destroy infected cells and mediate this targeted killing through a variety of mechanisms, including production of the functional proteins IFNγ and GrB.

Statistics

Associations between baseline variables and antibody response (at one month and 11 months post-vaccination) were assessed using Spearman's rank correlation coefficient for continuous variables (age and BMI) and the Kruskal-Wallis test for categorical variables (diabetes, race, sex, smoking status). Baseline variables that were marginally associated with ELISA antibody fold increase at one month post-vaccination were included in a multivariate linear regression model, with $\log_{10}$ antibody fold increase as the response variable. For the matched pairs HAI substudy, response frequencies (defined as ≥ 4-fold increase, or seroconversion) between healthy weight and obese participants were compared using McNemar's test. Comparisons of HAI titer fold increase between healthy weight and obese participants were assessed by applying the Wilcoxon signed rank test to the log of the fold increase ratio between healthy weight and obese participants within a pair. Fold increase between paired unstimulated and stimulated PBMC expression of CD69, GrB, and IFNγ was assessed using the Wilcoxon signed rank test. All reported p-values are two-sided.

Results

Characteristics of the Study Population

The demographics of the study population are presented in Table 3.1. Our participants were 29.7% healthy weight, 33.4% overweight, and 35.5% obese. The subset of 74 participants who were studied 11 months after receipt of the 2009-2010 vaccine were demographically similar to the overall study population (Table 3.1).

ELISA and HAI Titers 1 Month Post Vaccination

As others have reported (280), age was negatively correlated with antibody response as measured by ELISA ($P<0.001$). BMI was positively correlated with antibody fold increase ($P<0.001$). Antibody response was higher in females ($P=0.001$), consistent with previous studies of gender effect on vaccine response (281). There was a marginally significant association between race and antibody response ($P=0.01$), suggesting that African Americans had slightly higher responses than whites. There was no association of antibody response with smoking status or diabetes. In a multivariate model of $\log_{10}$ antibody fold increase including BMI, age, race, and sex, both BMI and age remained significant predictors of fold increase ($P=0.002$ and $P<0.001$, respectively), while race was marginally significant ($P=0.03$), and sex was borderline significant ($P=0.08$). Based on the fitted model, a ten unit increase in BMI was associated with 13% greater fold increase in

antibody titer, while a ten year increase in age was associated with an 8% lower fold increase in antibody titer. Age and BMI did not interact in the model. Pre- and post-vaccination antibody titers for the three vaccine strains were not significantly different between the healthy weight and obese participants. As shown in Figure 3.1, when examining fold increase between pre- and post-vaccination antibody titers, there was no difference for the A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) strains (P=0.014 and P=0.09, respectively), however there was a higher fold increase in obese compared to healthy weight participants for B/Brisbane/60/2008 (P=0.04). Spearman-rank correlations between the ELISA and HAI fold increases demonstrated that the ELISA was positively correlated with HAI results for all three of the vaccine strains: a correlation of 0.57 (P<0.001) for A/Brisbane/59, 0.50 (P<0.001) for A/Brisbane/10, and 0.52 (P<0.001) for B/Brisbane/60.

ELISA and HAI Titers 11 Months Following Vaccination

As shown in Figure 3.2 A, increasing BMI was associated with a larger drop in antibody titer to 2009-2010 seasonal TIV as measured by ELISA 11 months after vaccination. To confirm this finding using HAI, we tested 17 matched pairs of healthy weight (BMI 22.6 +/- 1.9) and obese participants (BMI 35.4 +/-5.4) 1 and 11 months post vaccination for HAI antibodies against each vaccine strain of virus. Although the majority of both healthy weight and obese vaccinated participants had a decrease in antibody titer during this interval, a larger percentage of obese participants had a 4-fold or greater drop in HAI titer at 11

months compared to healthy weight participants (Figure 3.2 B). Thus, these results indicate that obese individuals have a steeper decline in vaccine antibody over time compared with healthy weight individuals.

Decreased activation of influenza-specific $CD8^+$ T cells in PBMCs obtained from obese individuals

In order to test the cellular response to influenza vaccination, PBMCs at 11 months post vaccination were challenged *ex vivo* with live vaccine strain influenza A/Brisbane/59/2007 H1N1. PBMCs from obese participants exhibited a significantly lower percent increase in $CD8^+$ T cells expressing the early activation marker CD69, than PBMCs from healthy weight participants (P=0.015) (Figure 3.3 A), although the total numbers of $CD8^+$ T cells were similar (data not shown).

Decreased expression of functional proteins in influenza-specific activated $CD8^+$ T cells in PBMCs obtained from obese individuals

In addition to upregulating activation markers upon stimulation, $CD8^+$ T cells generate IFNγ and express GrB in order limit influenza replication and rapidly clear the virus. PBMCs from obese participants exhibited a significantly lower percent increase in activated $CD8^+$ T cells expressing GrB than PBMCs from healthy weight participants (P=0.026) (Figure 3.3 B). PBMCs from obese and overweight participants exhibited a lower percent increase in activated $CD8^+$ T cells expressing IFNγ, than PBMCs from healthy weight participants (P=0.006 and P=0.047, respectively) (Figure 3.3 C). These data indicate that obesity, and

overweight in the case of IFNγ, results in a decreased production of the proteins IFNγ and GrB.

Discussion

During the 2009 H1N1 influenza pandemic, obesity was recognized as an independent risk factor for increased influenza morbidity and mortality (41, 274, 275). Influenza vaccination is the single most effective method for reducing morbidity and mortality from influenza. Despite recognition that obesity is immunosuppressive (3), this is the first study to examine antibody and $CD8^+$ T cell responses to influenza vaccination in healthy weight, overweight, and obese individuals.

Because obesity reduces antibody responses to hepatitis B vaccine in adults and to tetanus vaccine in children (3, 9, 10, 277), elevated antibody response to influenza vaccination in our obese study participants was unexpected. Our data show that obese individuals mount a vigorous initial antibody response to TIV. However, a vaccine is protective only if the antibody titer is maintained throughout the period when influenza virus is circulating in the population. To examine the level of antibody maintenance after vaccination, we measured antibody levels 11 months after vaccination. Increases in BMI were positivity correlated to decreases in antibody titer. More than 50% of the obese participants had a ≥4-fold decrease in HAI titers to A/Brisbane/10 and B/Brisbane/60, and 47% had a ≥4-fold decrease in HAI titer to A/Brisbane/59 at 11 months compared to one

month post-vaccination. By comparison, less than 25% of healthy weight participants had a ≥4-fold decrease in HAI titer to A/Brisbane/59 and B/Brisbane/60. The objectives of our ongoing study include more precise definition of the kinetics of this differential decline in antibody titer, as well as follow-up of participants to determine whether BMI influences actual rates of laboratory-confirmed influenza in vaccinated individuals. In addition to stimulating production of influenza antigen-specific antibodies, influenza vaccination also functions to generate a $CD8^+$ T cell response. The importance of a robust $CD8^+$ T cell memory response has been appreciated, and there is great interest in developing influenza vaccines that can promote a heightened T cell memory response. Our own work in a murine diet-induced obesity model demonstrated an impaired $CD8^+$ T cell memory response leading to increased morbidity and mortality from an influenza challenge (12). In addition, it has also been suggested that there is an obesity-associated decrease in naive T cells and T cell diversity (56), which could contribute to the impaired $CD8^+$ T cell response seen in out study.

Influenza-specific $CD8^+$ T cells do not protect against infection, but instead act to limit progression of disease, allow for more rapid viral clearance, and to lessen the severity of disease (282). While the targets for antibodies are the proteins on the surface of the influenza virus, the targets for $CD8^+$ T cells are located on the internal, highly conserved proteins of the virus, which allow for extensive cross-reactivity against multiple strains of influenza virus. The influenza virus surface proteins have a tendency to change frequently; as such, an antibody-based vaccine may be protective for one year, but not the next (283).

Because memory $CD8^+$ T cells are specific to internal influenza proteins which vary little from year to year, it is likely more effective and efficient to develop influenza vaccines which expand memory $CD8^+$ T cell populations, in addition to invoking a robust antibody response (284). Indeed, $CD8^+$ T cell cytotoxic activity correlates better with influenza protection than antibody titer in an elderly population (285).

We found that percentages of influenza-activated $CD8^+$ T cells were decreased in the obese participants, and two markers of functional $CD8^+$ activity, IFNγ and GrB, were also significantly decreased in the obese participants. $CD8^+$ T cells kill virus-infected cells by release of perforin and GrB (286) and inhibit viral replication via release of IFNγ (287). The fact that influenza-stimulated $CD8^+$ T cells from obese individuals were deficient in the expression of both of these proteins strongly suggests that protection from an influenza infection may be not be optimal in the obese population.

We report here for the first time that influenza vaccine antibody levels decline significantly and $CD8^+$ T cell responses are defective in obese compared to healthy weight individuals. These findings suggest a mechanism for the increased risk of severe disease from pH1N1 infection in the obese population. If antibody titers and influenza vaccination-induced memory $CD8^+$ T cell populations are not maintained over time in obese individuals, they may be at risk for suboptimal vaccine response. Additional studies are needed to determine the risk of influenza infection in a vaccinated obese population.

Tables and Figures

Table 3.1 Demographic Characteristics of 2009-2010 and 2010-2011 Returning Study Participants

		Underweight[a]	Healthy Weight	Overweight	Obese	Total
Year 1[b]						
Enrolled[b]		6 (1.3)	137 (29.7)	154 (33.4)	164 (35.5)	461
Age*		45.6 +/-24.0	59.7+/-17.8	52.5 +/-15.6	51.6+/-12.8	
Gender						
	Male	0 (0)	41 (8.9)	73 (15.8)	46 (9.9)	160 (34.7)
	Female	6 (1.3)	96 (20.8)	81 (17.5)	118 (17.5)	301 (65.2)
Race						
	White	5 (1.1)	105 (22.8)	104 (22.6)	95 (20.6)	309 (67.0)
	AA	0 (0)	19 (4.1)	43 (9.3)	63 (13.7)	125 (27.1)
	Other	1 (0.2)	13 (2.8)	7 (1.5)	6 (1.3)	27 (5.9)
Diabetes						
	Yes	0 (0)	8 (1.7)	29 (6.3)	54 (11.7)	91 (19.6)
	No	6 (1.3)	129 (28.0)	125 (27.1)	110 (23.9)	370 (80.4)
Year 1[c]						
Enrolled[c]		0	24 (32.4)	23 (31.0)	27 (36.5)	74
Age*		-	47.6+/-17.5	54.6 +/-14.1	54.8+/-11.9	
Gender						
	Male	-	6 (8.1)	10 (13.5)	10 (13.5)	26 (35.1)
	Female	-	18 (24.3)	13 (17.6)	17 (23.0)	48 (64.9)
Race						
	White	-	17 (22.9)	17 (22.9)	15 (20.2)	49 (66.2)
	AA	-	6 (8.1)	5 (6.8)	11 (14.9)	22 (29.7)
	Other	-	1 (1.4)	1 (1.4)	1 (1.4)	3 (4.1)

[a]BMI: Underweight (<18.5), Healthy Weight (18.5-24.9), Overweight (25-29.9), Obese ($\geq$30)
[b] Subjects who completed the 2009-2010 study.
[c] Subset of Year 1 Subjects who returned for the 2010-2011 study.
* Mean +/- SD

Figure 3.1 Obese Participants Do Not Have an Impaired Initial Response to Influenza Vaccination

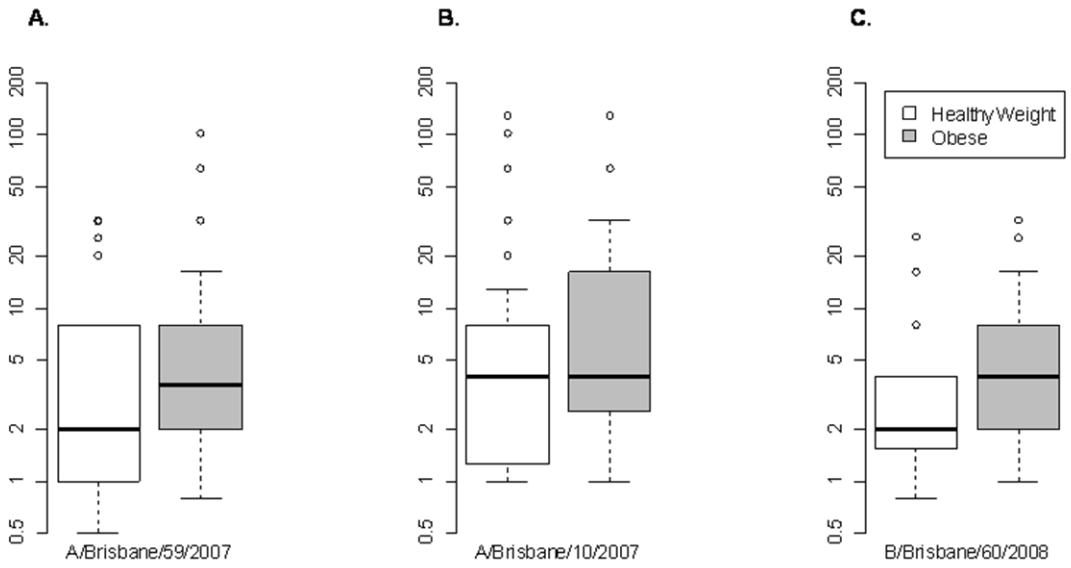


Figure 3.1: Boxplots of the fold-increase of the geometric mean titers of HAI response for each vaccine strain. Wilcoxon signed rank test of fold-increase of Healthy weight vs. Obese: (A) A/Brisbane/59/2007, P=0.14; (B) A/Brisbane 10/2007, P=0.09; (C) B/Brisbane/60/2008, P=0.04. Healthy weight n=40, obese n=40.

Figure 3.2 Obesity Results in a Greater Decline of Influenza Antibodies

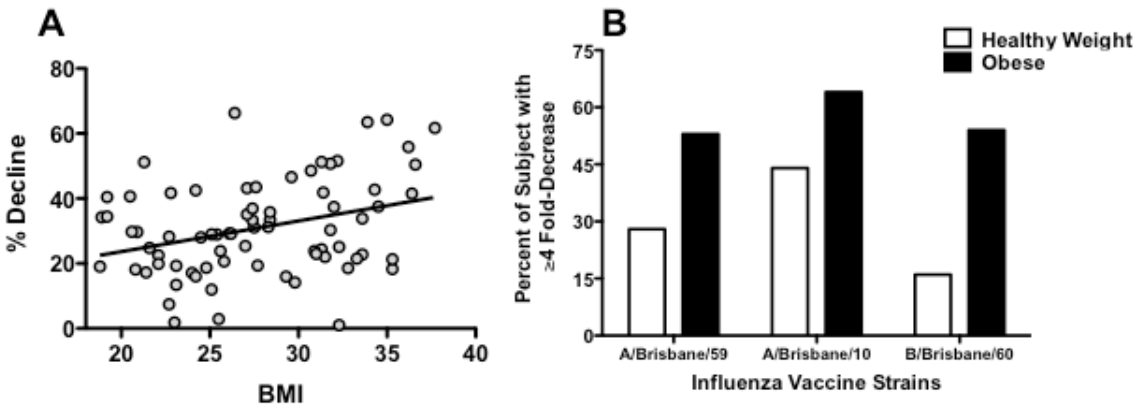


Figure 3.2: (A) Correlation between BMI and percent antibody drop of ELISA titers. As the BMI increases, the drop in antibody at 11 months post vaccination is increased. Spearman-rank correlation: r=0.29 (P=0.01). n=74; (B) More obese individuals have a greater than 4 fold drop in HAI titer at 11 months post-vaccination compared with healthy weight individuals (McNemar's test P=0.16 for A/Brisbane/59, P=0.32 for A/Brisbane/10 and P=0.03 for B/Brisbane/60). Healthy weight n=17, obese n=17.

Figure 3.3 Obesity Results in Defective $CD8^+$ T Cell Activation and Production of the Functional Proteins Granzyme B and IFNγ by Influenza-stimulated PBMCs

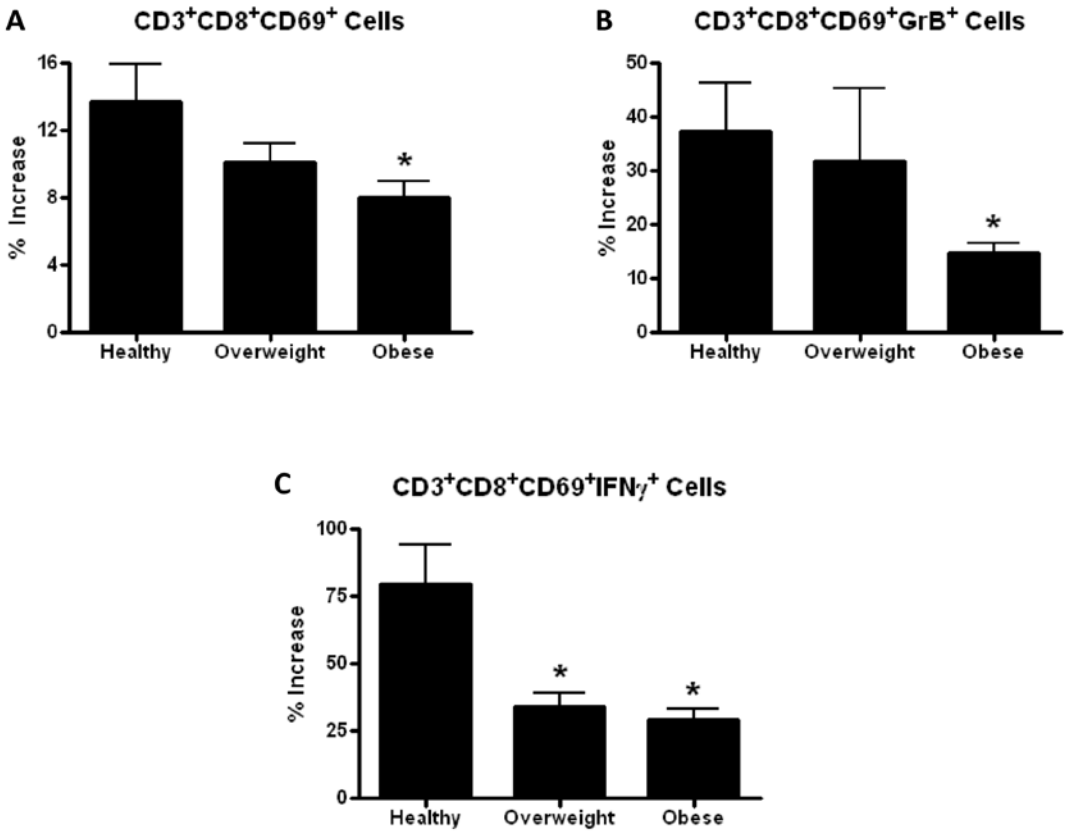


*Figure 3.3: (A) PBMCs from obese participants display a lower percent increase in activated CD69-expressing $CD8^+$ T cells (P=0.015) and (B) a lower % increase in activated T cells that express Granzyme B (P=0.026) compared to healthy weight. (C) PBMCs from overweight and obese participants display a lower percent increase in activated $CD8^+$ T cells that express IFNγ (P=0.047 and P=0.006, respectively). The percent increase in cell number for each population of cells was calculated between PBMCs incubated with plain media and PBMCs incubated with influenza A virus. As such, each individual sample was compared to its own control. Bar graphs show mean percent increase and standard error for the three groups. Healthy weight n=23, overweight n=17, obese n= 21. * indicates p-value is <0.05 compared to the healthy weight group. GrB=Granzyme B.*

CHAPTER IV

OVERWEIGHT AND OBESE ADULT HUMANS HAVE A DEFECTIVE CELLULAR IMMUNE RESPONSE TO PANDEMIC H1N1 INFLUENZA A VIRUS[2]

Overview

Obese adults have a greater risk of morbidity and mortality from infection with pandemic H1N1 influenza A virus (pH1N1). The objective of the present study was to elucidate the specific mechanisms by which obesity and overweight impact the cellular immune response to pH1N1. We stimulated PBMCs from healthy weight, overweight, and obese individuals *ex vivo* with live pH1N1 and then measured markers of activation and function using flow cytometry and cytokine secretion using cytometric bead array assays. Our data indicate that $CD4^+$ and $CD8^+$ T cells from overweight and obese individuals expressed lower levels of CD69, CD28, CD40 ligand, and IL12 receptor, as well as produced lower levels of IFNγ and GrB, compared to healthy weight individuals, suggesting deficiencies in activation and function. Dendritic cells from the three groups expressed similar levels of major histocompatibility complex-II, CD40, CD80, and CD86, as well as

[2] Previously published: Heather A. Paich, Patricia A. Sheridan, Jean Handy, Erik A. Karlsson, Stacey Schultz-Cherry, Michael G. Hudgens, Terry L. Noah, Samuel Weir, and Melinda A. Beck. *Obesity* (Silver Spring). 2013 Mar 20. doi: 10.1002/oby.20383. [Epub ahead of print]

produced similar levels of IL12. The defects in $CD4^+$ and $CD8^+$ T cells may contribute to the increased morbidity and mortality from pH1N1 in obese individuals. These data also provide evidence that both overweight and obesity cause impairments in immune function.

Introduction

There are over 1.4 billion overweight adults and approximately 500 million adults that are obese worldwide (1); reports indicate that obese adults have a greater risk of morbidity and mortality from infection with pandemic H1N1 influenza A virus (pH1N1) (41, 275). In 2009, for the first time, the CDC recognized obesity as an independent risk factor for influenza complications (274). However, little is known about the mechanisms mediating the obesity-associated increase in risk of complications and death from influenza infection. Recently, we have shown that there is an obesity-associated decrease in $CD8^+$ T cell responses and a decline in antibody levels 12 months after immunization with seasonal TIV in humans (288).

The cellular immune response to influenza virus infection requires appropriately functioning dendritic cells, $CD4^+$ T cells, and $CD8^+$ T cells (Figure 4.5). Dendritic cells present antigen to and promote activation of influenza-specific $CD4^+$ T cells and $CD8^+$ T cells. Once activated, $CD4^+$ T cells provide help, in the form of cytokine synthesis and secretion, to promote $CD8^+$ T cell activation and cytotoxic function and B cell activation and antibody production. It is primarily the T_H1 subset of $CD4^+$ T cells that mediates the immune response

to influenza (189) and seems to have a particularly important role in responding to pH1N1 (190). In addition, $CD4^+$ T cells have been shown to have cytotoxic activity against pH1N1-infected target cells (289). $CD8^+$ T cells limit the spread and severity of influenza infection by inducing apoptosis in influenza-infected cells, and may have an especially significant function in cross-reactive immune responses to pH1N1 (290).

To further understand how obesity and overweight impact the cellular response to influenza virus in humans, we stimulated PBMCs from healthy weight, overweight, and obese individuals *ex vivo* with live pH1N1. We demonstrate that influenza-stimulated $CD4^+$ and $CD8^+$ T cells from both overweight and obese adults have significant deficiencies in markers of activation and function, while the associated dendritic cell markers of activation and function remain intact. These defects in $CD4^+$ and $CD8^+$ T cells could contribute to the increased morbidity and mortality from influenza infection in obese adults. Our data are particularly compelling because they provide evidence that both overweight and obesity cause impairments in immune function.

Methods and Procedures

Study population and samples

Participants were recruited as 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, NC (288). Eligible participants were adult (≥18 years of age) patients who received the 2010-2011 seasonal TIV. Exclusion criteria were immunosuppression, self-reported use of immunomodulator or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillian-Barre syndrome, or use of theophylline preparations or warfarin (288). All procedures were approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill.

A total of 454 participants were enrolled in the study between September 2010 and December 2010. At enrollment, informed consent, demographic characteristics, height, weight, and a blood sample were obtained. One 0.5mL dose of the 2010-2011 seasonal TIV (Sanofi Pasteur) containing A/Perth/16/2009(H3N2) and B/Brisbane/60/2008, as well as A/California/7/2009(pH1N1), was administered in the deltoid muscle using a 1.5-inch needle. Participants returned 28-35 days later for a post-vaccination blood draw. Serum and PBMCs were obtained from blood samples as previously described (288). Height and weight measurements were used to calculate BMI. Participants were classified by BMI as healthy weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), or obese (BMI ≥30.0) (1). Demographic characteristics of the 454 participants are presented in Table 4.1 A.

A sample group of 83 total participants, comprised of 28 healthy weight, 28 overweight, and 27 obese participants, was created by taking simple random samples without replacement from strata formed by age, race, gender, smoking status, and diabetes

status. Age was dichotomized into groups of individuals <53 or >54 years of age because 54 years of age was the median age in the overall study population. Race was dichotomized into African American or Caucasian. Smoking status was defined as non-smoker, previous smoker, or current smoker. Diabetes status was defined as either having diagnosed diabetes or not. Demographic characteristics of these 83 participants are presented in Table 4.1 B. PBMCs from these 83 participants obtained 30 days post-vaccination were used for the experiments presented in this manuscript.

A subgroup of 45 participants, comprised of 15 healthy weight, 14 overweight, and 16 obese participants, was created by taking simple random samples from the healthy weight, overweight, and obese groups without replacement from the sample group of 83 participants. PBMC supernates from these 45 participants were used to measure cytokines. Demographic characteristics of these 45 participants are presented in Table 4.1 C. A study overview is shown in Table 4.1 D.

PBMC stimulation

For 72 hours, PBMCs were cultured with or without stimulation with 20μL of 5μg protein/μL stock of live pH1N1 (equivalent to a multiplicity of infection of approximately 1) at 37°C in 5% CO_2. PBMC supernates were collected after 66 hours in culture and replaced with media containing GolgiPlug (BD Biosciences).

PBMC staining and FACS

PBMCs were stained with human fluorochrome-conjugated human antibodies as shown in Table 2. Sample data were acquired using an LSR II flow cytometer and FACSDiva software (BD Biosciences), and were analyzed using Kaluza analysis software (Beckman Coulter). The gating strategies are described in detail and a representative example of the gating strategy used to analyze $CD4^+$ T cells is shown in Figure 4.6.

Cytometric bead array assays

Cytometric bead array assays (BD Biosciences) were performed to measure levels of the following cytokines secreted in PBMC supernates: IL5, IL6, IL7, IL12, IFNγ, and TNFα. Sample data were acquired using an LSR II flow cytometer and FACSDiva software. Data were analyzed using FCAP Array software (BD Biosciences). Individual cytokine concentrations of each supernate were calculated by reference with a standard curve.

Statistical analyses

Statistical analyses were performed using JMP statistical software (SAS). Differences in cell populations, cytokine levels, and antibody titer levels between the healthy weight and overweight or obese groups were analyzed with a two-tailed Student's *t*-test. For each

individual, fold increase was calculated by dividing stimulated by unstimulated cytokine levels. Pairwise comparisons in fold increase between BMI groups were assessed using the Wilcoxon rank sum test. P values < 0.05 were considered statistically significant. No adjustment was made for multiple comparisons.

Results

Dendritic cell activation and function remain intact in PBMCs from overweight and obese individuals

To determine how obesity affects dendritic cells, flow cytometry was used to measure markers of activation and markers of function. As expected, there were increases in cell numbers between unstimulated PBMC samples and PBMC samples stimulated with pH1N1 in the three BMI groups for all the dendritic cell populations measured, showing clear evidence of increased proliferation. However, we found that there were no differences in total $CD3^{-}CD11c^{+}$ dendritic cell numbers (Figure 4.1 A), nor in activated dendritic cells expressing CD80 and CD86 (Figure 4.1 B), MHC-II (Figure 4.1 C), and IL12 (Figure 4.1 D) among any of the groups in either unstimulated or stimulated PBMCs.

These data show that overweight and obesity do not alter baseline levels or influenza-induced proliferation of dendritic cells and do not impair dendritic cell activation and expression of the costimulatory proteins CD80 and CD86, and of MHC-II. Furthermore, the data establish that dendritic cells from overweight and obese individuals express levels

of intracellular IL12 similar to levels expressed by dendritic cells from healthy weight individuals.

Activation and function of $CD4^+$ T cells are impaired in PBMCs from overweight and obese individuals

We next examined the T cell response to pH1N1 stimulation. As in dendritic cells, there were increases in cell numbers in all the $CD4^+$ T cell populations measured between unstimulated and stimulated PBMCs for all three BMI groups, again showing clear evidence of increased proliferation. Similarly, there were no differences in any of the $CD4^+$ T cell populations analyzed among the healthy weight, overweight, and obese groups in unstimulated PBMC samples. Total numbers of $CD4^+$ T cells were similar in stimulated PBMCs from healthy weight, overweight, and obese individuals (Figure 4.2 A), suggesting that overweight and obesity do not alter influenza-induced proliferation of $CD4^+$ T cells and that any differences in the $CD4^+$ T cell subpopulations are not simply a result of overweight and obese individuals having fewer $CD4^+$ T cells overall. When we examined numbers of $CD4^+$ T cells expressing the activation marker CD69 (Figure 4.2 B), as well as activated $CD4^+$ T cells expressing CD28 (Figure 4.2 C), CD40L (Figure 4.2 D), IL12R (Figure 4.2 E), IFNγ (Figure 4.2 F), both IFNγ and GrB (Figure 4.2 G), and CD28, CD40L, IFNγ, and GrB (Figure 4.2 H), we found that they were all significantly lower in stimulated PBMCs from overweight and obese individuals, compared to healthy weight individuals. These data suggest that when exposed to pH1N1, both overweight and obese individuals have a significant loss in ability to activate $CD4^+$ T cells responses, compared to healthy weight individuals.

Activation and function of $CD8^+$ T cells are impaired in PBMCs from overweight and obese individuals

Similarly, there were increases in cell numbers in all $CD8^+$ T cell populations measured between unstimulated and stimulated PBMCs for all three BMI groups, showing clear evidence of proliferation. There were no differences in any of the $CD8^+$ T cell populations analyzed among healthy weight, overweight, and obese groups in unstimulated PBMC samples. Total numbers of $CD8^+$ T cell numbers were similar in unstimulated PBMCs from healthy weight, overweight, and obese individuals; in stimulated samples, numbers were similar between healthy weight and obese individuals, while numbers were higher in overweight individuals (Figure 4.3 A). This suggests that overweight and obesity do not negatively impact pH1N1-induced proliferation of $CD8^+$ T cells and suggest that any differences in the $CD8^+$ T cell subpopulations are not a result of overweight and obese individuals having fewer total $CD8^+$ T cells. When we examined numbers of $CD8^+$ T cells expressing the activation marker CD69 (Figure 4.3 B) and IFNγ (Figure 4.3 C), as well as activated $CD8^+$ T cells expressing CD28 (Figure 4.3 D), CD40L (Figure 4.3 E), IFNγ (Figure 4.3 F), both IFNγ and GrB (Figure 4.3 G), and both CD28 and IL12R (Figure 4.3 H), we found that they were all significantly lower in stimulated PBMCs from overweight and obese individuals, compared to healthy weight individuals. As with the $CD4^+$ T cell populations, these data suggest that overweight and obese individuals do not activate their $CD8^+$ T cells in response to pH1N1 to the same level as healthy weight individuals.

No differences in levels of IL12 and IL7 secreted by PBMCs from healthy weight, overweight, and obese individuals

To determine if overweight or obesity altered PBMC cytokine production, we then measured protein levels of cytokines secreted into the PBMC culture media. Similar to the flow cytometry data, while there were increases in secreted IL12 between unstimulated and stimulated PBMCs, we found that there were no differences in IL12 (Figure 4.4 A) levels among the BMI groups when comparing unstimulated PBMCs with each other and stimulated PBMCs with each other. In addition, we found that there were no differences in IL7 (Figure 4.4 B) levels among the BMI groups, both in the unstimulated and stimulated samples. These findings suggest that dendritic cells from overweight and obese individuals secrete similar amounts of IL12 and IL7 as dendritic cells from healthy individuals. These data, along with the flow cytometry data, suggest that activation and function of dendritic cells are intact in PBMCs from overweight and obese individuals.

Higher levels of IL5 in supernates from obese individuals

We also found that IL5 levels were higher in supernates from obese individuals (Figure 4.4 C) and that IFNγ levels trended lower (Figure 4.4 D) in supernates from obese individuals, compared to healthy weight individuals. Because it is not known which cells produce which cytokines released into the supernate, any potential differences in IFNγ levels may have been mitigated, as it is known that dendritic cells have the ability to secrete IFNγ (291) in addition to T cells. The higher levels of IL5 in the supernates from obese

individuals suggest that the $CD4^+$ T cells from obese individuals are differentiating more to the T_H2 subset of $CD4^+$ T cells, which produce high amounts of IL5, and less to the T_H1 subset of $CD4^+$ T cells. Indeed, a recent study showed that $CD4^+$ T cells from morbidly obese individuals were skewed towards a T_H2-dominated phenotype (68).

Impaired upregulation of TNFα secretion in PBMC supernates from obese individuals

A part of the coordinated immune response to influenza virus includes an increased production of the proinflammatory cytokines TNFα and IL6. Although there were no differences in levels of TNFα (Figure 4.7 A) and IL6 (Figure 4.7 B) between the healthy weight and overweight groups and between the healthy weight and obese groups in unstimulated and stimulated PBMCs, we did find that the fold increase between unstimulated and stimulated PBMC supernates from obese individuals was lower for TNFα (Figure 4.4 E) and trended lower for IL6 (Figure 4.4 F), compared to healthy weight individuals. These data suggest that obese individuals may not be able to upregulate production of TNFα in response to pH1N1 as effectively as healthy weight individuals, perhaps due to resistance in the pathways that upregulate TNFα secretion associated with increased adiposity.

Discussion

Seasonal influenza virus strains typically affect the very young and the very old more

than young or middle-aged adults. However, pH1N1 disproportionately affected children, young adults, and pregnant women, while elderly adults maintained relatively low infection rates. In general, most of the deaths and cases with severe complications from pH1N1 occurred in adults less than 65 years of age, while deaths from seasonal influenza virus are characteristically highest in elderly adults over 65 years of age (292).

We found that there were no significant differences in pre-vaccination or post-vaccination serum titers to pH1N1 among the healthy weight, overweight, and obese groups (Figure 4.8). In the absence of cross-protective antibodies, the cellular immune response to influenza virus has a significant role in limiting the spread and severity of influenza symptoms and promoting clearance of the virus (293). Furthermore, studies have shown that the cellular immune response to influenza is a better predictor than the antibody-mediated immune response of protection from influenza (154). A number of studies have shown that previous natural infection or vaccination against seasonal influenza A viruses increase cellular immune responses against pH1N1 in the absence of humoral immune responses humans (289, 294, 295) and evidence from animal studies corroborates these data (290, 296).

We found that there were no impairments in markers of dendritic cell activation and function and no defects in dendritic cell cytokine secretion in PBMCs from overweight and obese participants. In contrast, we found that there were significant impairments in $CD4^+$ and $CD8^+$ T cell activation and function and alterations in T cell cytokine secretion in PBMCs

from overweight and obese participants. Expression of CD69, a T cell activation marker, was lower in $CD4^+$ and $CD8^+$ T cells, while expression CD40, a dendritic cell activation marker, was not impaired in dendritic cells from overweight and obese participants. CD40 signaling promotes expression of MHC-II and of the costimulatory molecules CD80 and CD86, which bind CD28 on T cells, thereby increasing the capacity of dendritic cells to effectively present antigen. While dendritic cells from overweight and obese individuals express levels of CD80 and CD86, which bind CD28 on T cells, similar to healthy weight individuals, $CD4^+$ and $CD8^+$ T cells from overweight and obese individuals are likely receiving reduced costimulatory signaling, due the decreased expression of CD28, which promotes proliferation, expansion, sensitivity to antigen, and survival of T cells. In addition, the $CD4^+$ and $CD8^+$ T cells from overweight and obese individuals may not be effecting optimal CD40-CD40L interactions due to the decreased expression of CD40L. However, it has been shown that activated dendritic cells can also produce CD40L, which can then act in a paracrine fashion to stimulate CD40 on other dendritic cells (297), thereby potentially bypassing the defective CD40 expression of T cells from overweight and obese individuals seen in our study. Despite comparable levels of IL12 production by dendritic cells from healthy weight, overweight, and obese individuals, the essential IL12R signaling pathway may not be optimally activated in $CD4^+$ and $CD8^+$ T cells from overweight and obese individuals, due to the decreased expression of IL12R, likely resulting in impairments in the downstream effects of IL12R signaling, including differentiation to the T_H1 cell subtype and IFNγ production in $CD4^+$ T cells (215) and cytotoxic activity and IFNγ production in $CD8^+$ T cells (217). There were also comparable levels of IL7 production by dendritic cells from healthy weight, overweight, and

obese individuals, which is required to effectively trigger the T cell response to influenza (218). Finally, the data indicate that overweight and obesity impair production of IFNγ, which is secreted in large amounts by both T_H1 $CD4^+$ T cells and $CD8^+$ T cells (189), and the production of GrB, suggesting that the respective anti-viral and apoptotic effects would be severely defective in the response to pH1N1. Interestingly, a previous study showed increased numbers of dendritic cells, but impaired antigen presentation, in the lungs of influenza-infected, diet-induced obese mice (32). However, in a mouse model of a secondary influenza infection, dendritic cells from diet-induced obese mice showed no impairments in antigen presentation (12).

In addition, secreted IL5 protein levels were higher in supernates and, although not statistically significant, IFNγ levels trended lower in supernates from obese individuals, in comparison to healthy weight individuals. In all immune responses, there needs to be a balance between the activities of T_H1 and T_H2 $CD4^+$ T cells; however, it is primarily the T_H1 $CD4^+$ T cells that mediate the response to influenza. IL5 is secreted predominantly by the T_H2 subset of $CD4^+$ T cells and is more closely associated with allergic responses rather than viral pathogens (239). These T cell data are similar to findings from studies utilizing diet-induced obese mouse models. In influenza-infected obese mice, there were lower levels of $CD8^+IFN\gamma^+$ T cells isolated from the spleen, compared to from lean control mice (32). During a primary influenza infection, increases in IFNγ mRNA expression in lung were both lower and delayed in obese mice, compared to lean control mice. During a secondary influenza viral challenge, diet-induced obese mice displayed reduced levels of influenza-specific $CD8^+$

effector memory T cells in lung, compared to lean control mice (12). Another study showed that during a secondary influenza viral challenge, diet-induced obese mice showed lower levels of IFNγ expression and IFNγ-producing influenza-specific $CD8^+$ T cells in lung tissue, compared to lean control mice. Even when memory $CD8^+$ T cells from obese mice were stimulated with influenza-pulsed dendritic cells from lean control mice, IFNγ expression was lower (12).

In addition to the anti-viral activity, controlled increases in inflammation are an important component of the immune response to influenza virus. We found that the fold increase in secreted cytokines between unstimulated and stimulated PBMC supernates from obese individuals was lower for TNFα, in comparison to healthy weight individuals. These data are similar to findings in animal models, showing that during a primary influenza infection, increases in TNFα and IL6 mRNA expression were both lower and delayed in obese mice, compared to lean control mice (11). In addition, when diet-induced obese mice were primed with a primary infection of the mouse-adapted influenza virus strain X-31 (H3N2), followed by a dose of influenza PR8 (H1N1) 4 weeks later, obese mice displayed a lower fold increase in mRNA expression of TNFα compared to lean control mice (12).

There are several significant strengths of the present study. The use of human samples and the *ex vivo* nature of the experiments enables the results to be immediately and directly applicable to human populations. There are also some limitations of the study. *Ex vivo* models are inherently limited in comparison to *in vivo* models; however, they are

often the best available option when the goal is to have direct relevance to human populations. We could not control for previous exposure to different strains of influenza virus, either through natural infection or vaccination. It would be very difficult to find a population in the US that was naive to all influenza virus strains that had cross-reactivity to pH1N1. However, this could also be considered a potential strength of our study, as our results are based on an intent-to-treat type of approach, which lends itself well to considering implications to the general US population. Finally, although our study has significant clinical implications for individuals exposed to, immunized against, or infected with pH1N1 influenza A virus, we were not able to directly assess whether the impairments in $CD4^+$ and $CD8^+$ T cell function seen in the PBMCs from overweight and obese individuals correlate with poorer clinical outcomes, although those studies will be important to conduct in the future.

The data from our combined experiments clearly indicate that $CD4^+$ and $CD8^+$ T cells from overweight and obese individuals have substantial defects in activation and function when stimulated *ex vivo* with pH1N1, despite the associated dendritic cell functions remaining intact. These defects likely contribute to the increased morbidity and mortality from pH1N1 in obese individuals. Our results are particularly compelling because they show that both overweight and obesity negatively impact immune function. With the dramatic increases in overweight and obesity worldwide and the heightened potential for influenza pandemics, these findings have important implications for understanding how adiposity affects the cellular immune response.

Tables and Figures

Table 4.1 Demographic Characteristics and Study Overview

Table 4.1 A Demographic Characteristics of the Study Population

		Healthy Weight	Overweight	Obese	Total
Enrolled		111 (24.4%)	145 (31.9%)	198 (43.6%)	454
BMI		22.3 ± 1.6	27.2 ± 1.5	37.8 ± 7.9	
BMI Range		18.5 – 24.9	25.0 – 29.9	30.0 – 76.5	
Age		50.0 ± 14.5	49.0 ± 13.7	51.0 ± 14.1	54.1 ± 15.3
Age Range		19 – 88	18 – 83	22 – 86	
Gender	*Female*	70 (25.4%)	80 (29.0%)	126 (45.7%)	276 (60.8%)
	Male	41 (23.0)	65 (36.5%)	72 (40.4%)	178 (39.2%)
Race	*White*	85 (27.7%)	108 (35.3%)	113 (37.0%)	306 (67.4%)
	AA	19 (14.0%)	35 (25.7%)	82 (60.3%)	139 (30.6%)
	Other	7 (58.3%)	2 (16.7%)	3 (25.0%)	12 (2.0%)
Smoking	*No*	66 (25.1%)	85 (32.3%)	112 (42.6%)	263 (57.9%)
	Previous	33 (40.9%)	45 (25.0%)	54 (34.1%)	132 (29.1%)
	Yes	12 (20.3%)	15 (25.4%)	32 (54.2%)	59 (13.0%)
Diabetes	*No*	103 (29.1%)	124 (35.0%)	127 (35.8%)	354 (77.0%)
	Yes	8 (8.0%)	21 (21.0%)	71 (71.0%)	100 (23.0%)

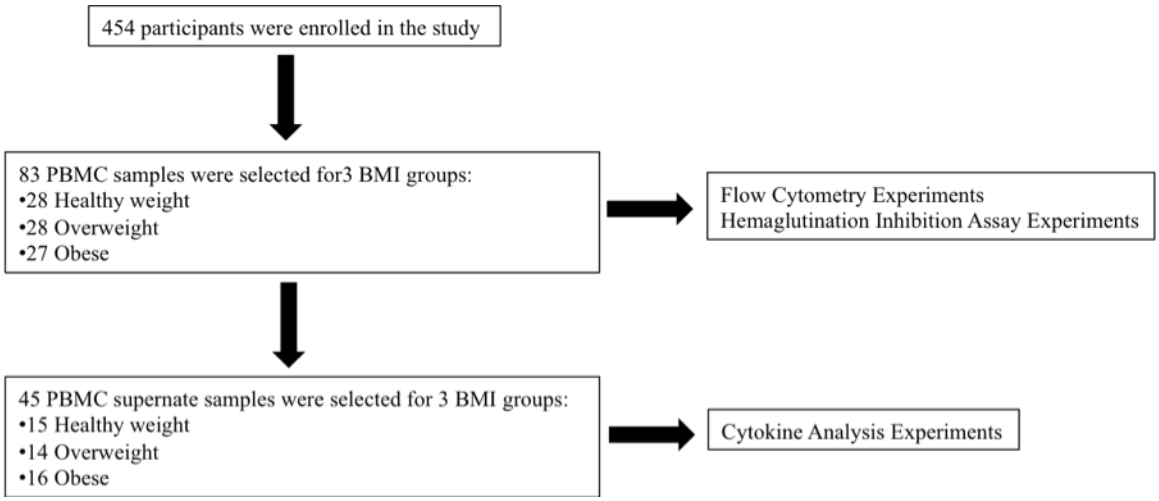
Table 4.1 B Demographic Characteristics of PBMC Samples for Flow Cytometry and Hemagglutination Inhibition Assay Experiments

		Healthy Weight	Overweight	Obese	Total
Participants		28	28	27	83
BMI		22.7 ± 1.7	26.8 ± 1.4	37.3 ± 7.8	
BMI Range		19.0 – 24.8	25.0 – 29.6	30.4 – 54.9	
Age		50.7 ± 14.2	49.2 ± 13.4	53.4 ± 12.9	50.4 ± 14.0
Age Range		19 – 69	23 – 70	24 – 70	
Gender	*Female*	16 (32.7%)	17 (34.7%)	16 (32.6%)	49 (59.0%)
	Male	12 (35.3%)	11 (32.3%)	11 (32.3%)	34 (41.0%)
Race	*White*	23 (33.3%)	23 (33.3%)	23 (33.3%)	69 (83.1%)
	AA	5 (35.7%)	5 (35.7%)	4 (28.6%)	14 (16.9%)
Smoking	*No*	15 (31.9%)	17 (36.2%)	15 (31.9%)	47 (56.6%)
	Previous	9 (40.9%)	7 (31.8%)	6 (27.3%)	22 (26.5%)
	Yes	4 (28.6%)	4 (28.6%)	6 (42.9%)	14 (16.9%)
Diabetes	*No*	25 (35.7%)	26 (37.1%)	19 (27.1%)	70 (84.3%)
	Yes	3 (23.1%)	2 (15.4%)	8 (61.5%)	13 (15.7%)

Table 4.1 C Demographic Characteristics of PBMC Supernates for Cytokine Analysis Experiments

		Healthy Weight	Overweight	Obese	Total
Participants		15	14	16	45
BMI		22.9 ± 1.7	27.1 ± 1.3	37.8 ± 7.2	
BMI Range		19.0 – 24.8	25.0 – 29.0	30.4 – 53.3	
Age		49.1 ± 16.3	48.6 ± 15.1	55.5 ± 11.3	51.2 ± 14.3
Age Range		19 – 69	23 – 66	19 – 69	
Gender	*Female*	7 (28.0%)	7 (28.0%)	11 (44.0%)	25 (55.6%)
	Male	8 (40.0%)	7 (35.0%)	5 (25.0%)	20 (44.4%)
Race	*White*	10 (27.0%)	12 (32.4%)	15 (40.5%)	37 (82.2%)
	AA	5 (62.5%)	2 (25.0%)	1 (12.5%)	8 (17.8%)
Smoking	*No*	8 (32.0%)	7 (28.0%)	10 (40.0%)	25 (55.6%)
	Previous	4 (33.3%)	6 (50.0%)	2 (16.7%)	12 (26.7%)
	Yes	3 (37.5%)	1 (12.5%)	4 (50.0%)	8 (17.8%)
Diabetes	*No*	14 (38.9%)	13 (36.1%)	9 (25.0%)	36 (80.0%)
	Yes	1 (11.1%)	1 (11.1%)	7 (77.8%)	9 (20.0%)

Table 4.1 D Study Overview



Tables 4.1: Demographic characteristics of the study population (A), PBMC samples for flow cytometry and hemaglutination inhibition assay experiments (B), and PBMC supernates for cytokine analysis axperiments (C). Healthy weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), obese (BMI ≥30) participants. Data presented are number of participants (% of total population), except for BMI and age, which are presented as mean ± s.d. (D) Study Overview.

Table 4.2 Fluorochrome-conjugated Antibodies Used for T Cell and Dendritic Cell FACS Panels

Table 4.2 A T Cell FACS Panel

Antibody	Fluorochrome	Manufacturer
Anti-CD3	V500	BD Biosciences
Anti-CD4	Qdot 605	Invitrogen
Anti-CD8	Qdot 655	Invitrogen
Anti-CD28	PE-Cy7	BioLegend
Anti-CD40L	ACP-Cy7	BioLegend
Anti-CD69	PE-Cy5.5	Invitrogen
Anti-IL12R	APC	BD Biosciences
Anti-IFNγ	FITC	BioLegend
Anti-GrB	PE-Texas Red	Invitrogen

Table 4.2 B Dendritic Cell FACS Panel

Antibody	Fluorochrome	Manufacturer
Anti-CD3	AmCyan	BD Biosciences
Anti-CD11c	Pacific Blue	BioLegend
Anti-CD40	PE-Cy5	BD Biosciences
Anti-CD80	Alexa Fluor 700	BD Biosciences
Anti-CD86	PerCP-Cy5.5	BioLegend
Anti-MHC-II	Pacific Orange	Invitrogen
Anti-IL12	PE	BioLegend

Tables 4.2: Fluorochrome-conjugated antibodies used for T cell (A) and dendritic cell (B) FACS panels, showing anti-human antibodies, fluorochrome conjugates, clones, and manufacturers.

Figure 4.1 Activation and Function of Dendritic Cells Remain Intact in PBMCs from Overweight and Obese Individuals

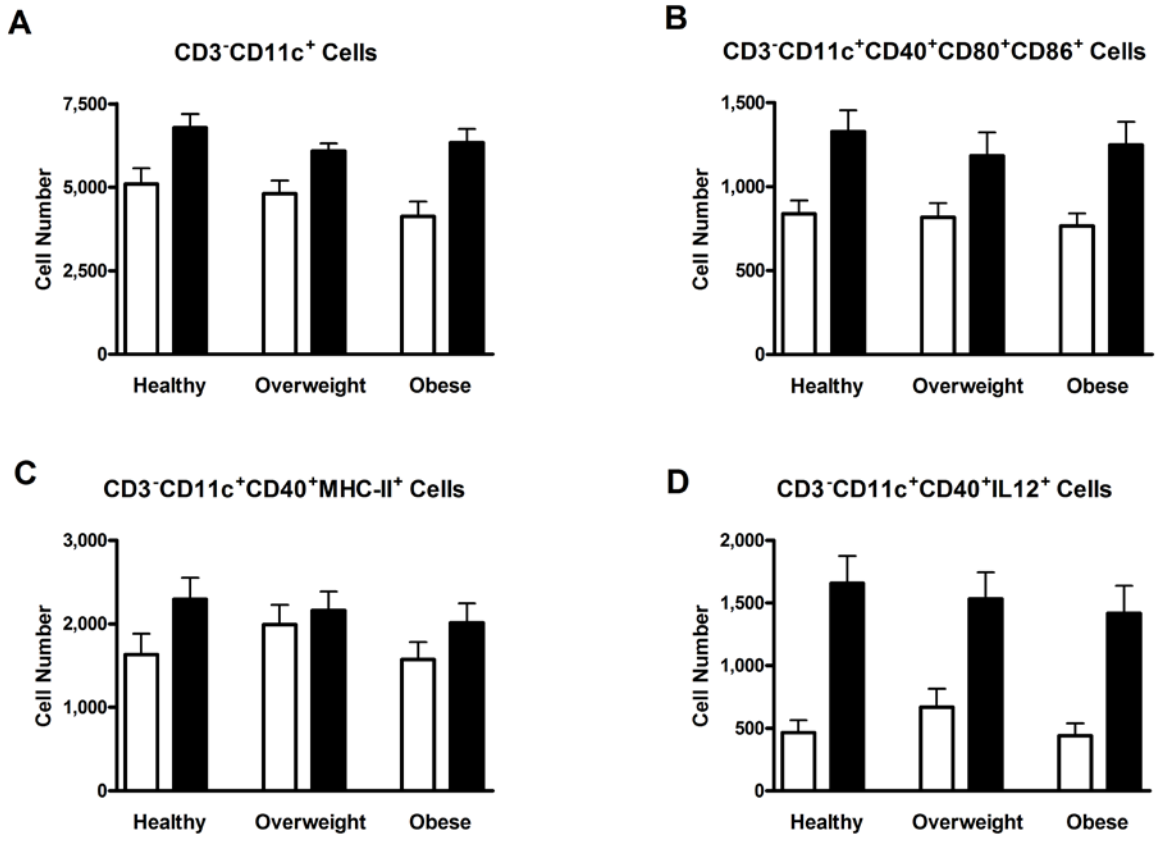


Figure 4.1: PBMCs were incubated with (filled bar) or without (open bar) live pH1N1 virus and dendritic cell populations were analyzed. There were no differences in (a) total $CD3^-$ $CD11c^+$ dendritic cells, nor in (b) activated dendritic cells expressing CD80 and CD86, (c) MHC-II, and (d) IL12 among any of the BMI groups in both unstimulated and stimulated PBMCs. Data were collected on approximately 30,000 events run in the dendritic cell/monocyte gate. Results are displayed as the mean ± s.e.m. (n=26-28 per group).

Figure 4.2 Activation and Function of $CD4^+$ T Cells are Impaired in PBMCs from Overweight and Obese Individuals

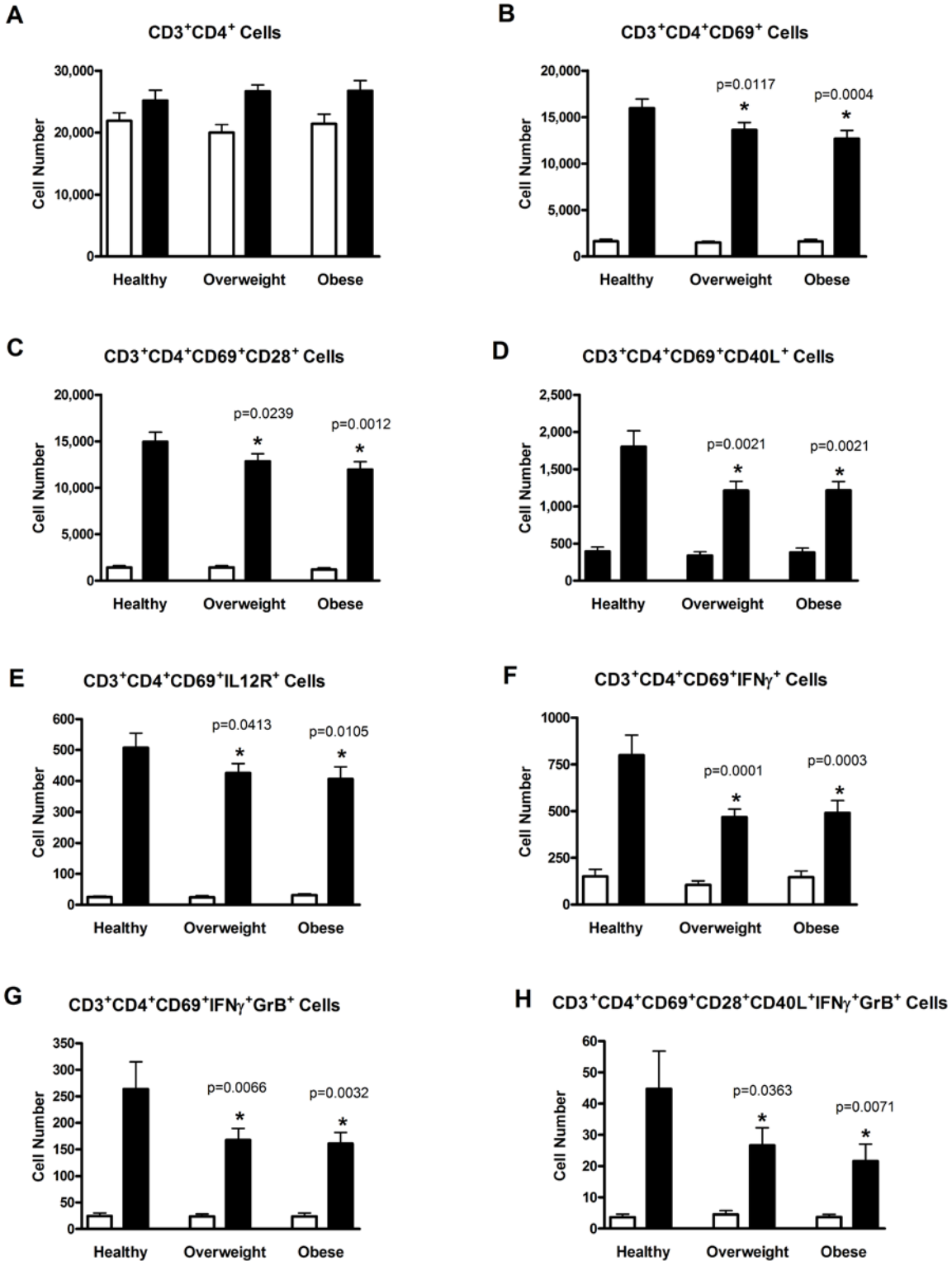


*Figure 4.2: PBMCs were incubated with (filled bar) or without (open bar) live pH1N1 virus and $CD4^+$ T cell populations were analyzed. Total $CD4^+$ T cells (A) were similar among groups, while $CD4^+$ T cells expressing CD69 (B), as well as activated $CD4^+$ T cells expressing CD28 (C), CD40L (D), IL12R (E), IFNγ (F), both IFNγ and GrB (G), and CD28, CD40L, IFNγ, and GrB (H), were significantly lower in stimulated PBMCs from overweight and obese individuals, compared to healthy weight individuals. There were no differences in unstimulated PBMCs among groups. Data were collected on approximately 50,000 $CD3^+$ events run in the lymphocyte gate. Results are displayed as the mean ± s.e.m. (n=26-28 per group). *P<0.05 compared to stimulated PBMCs from healthy weight individuals.*

Figure 4.3 Activation and Function of $CD8^+$ T Cells are Impaired in PBMCs from Overweight and Obese Individuals

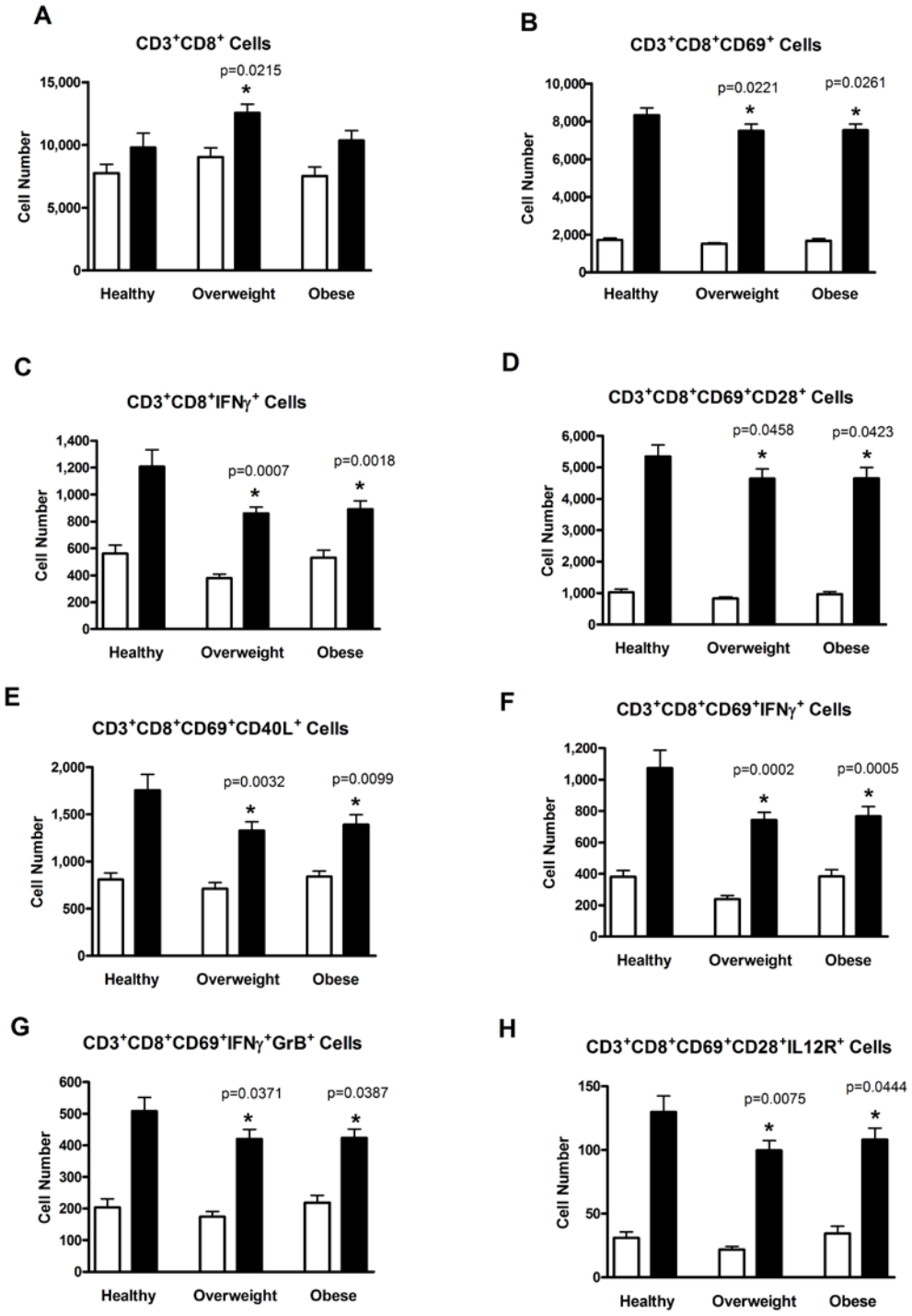


*Figure 4.3: PBMCs were incubated with (filled bar) or without (open bar) live pH1N1 virus and $CD8^+$ T cell populations were analyzed. Total $CD8^+$ T cells (A) were similar between healthy weight and obese individuals, while numbers were higher in overweight individuals. $CD8^+$ T cells expressing CD69 (B) and IFNγ (C), as well as activated $CD8^+$ T cells expressing CD28 (D), CD40L (E), IFNγ (F), both IFNγ and GrB (G), and both CD28 and IL12R (H), were significantly lower in stimulated PBMCs from overweight and obese individuals, compared to healthy weight individuals. There were no differences in unstimulated PBMCs among groups. Data were collected on approximately 50,000 $CD3^+$ events run in the lymphocyte gate. Results are displayed as the mean ± s.e.m. (n=26-28 per group). *P<0.05 compared to stimulated PBMCs from healthy weight individuals.*

Figure 4.4 PBMC Cytokine Secretion from Overweight and Obese Individuals Suggests a Shift Towards a T_H2-dominated Response

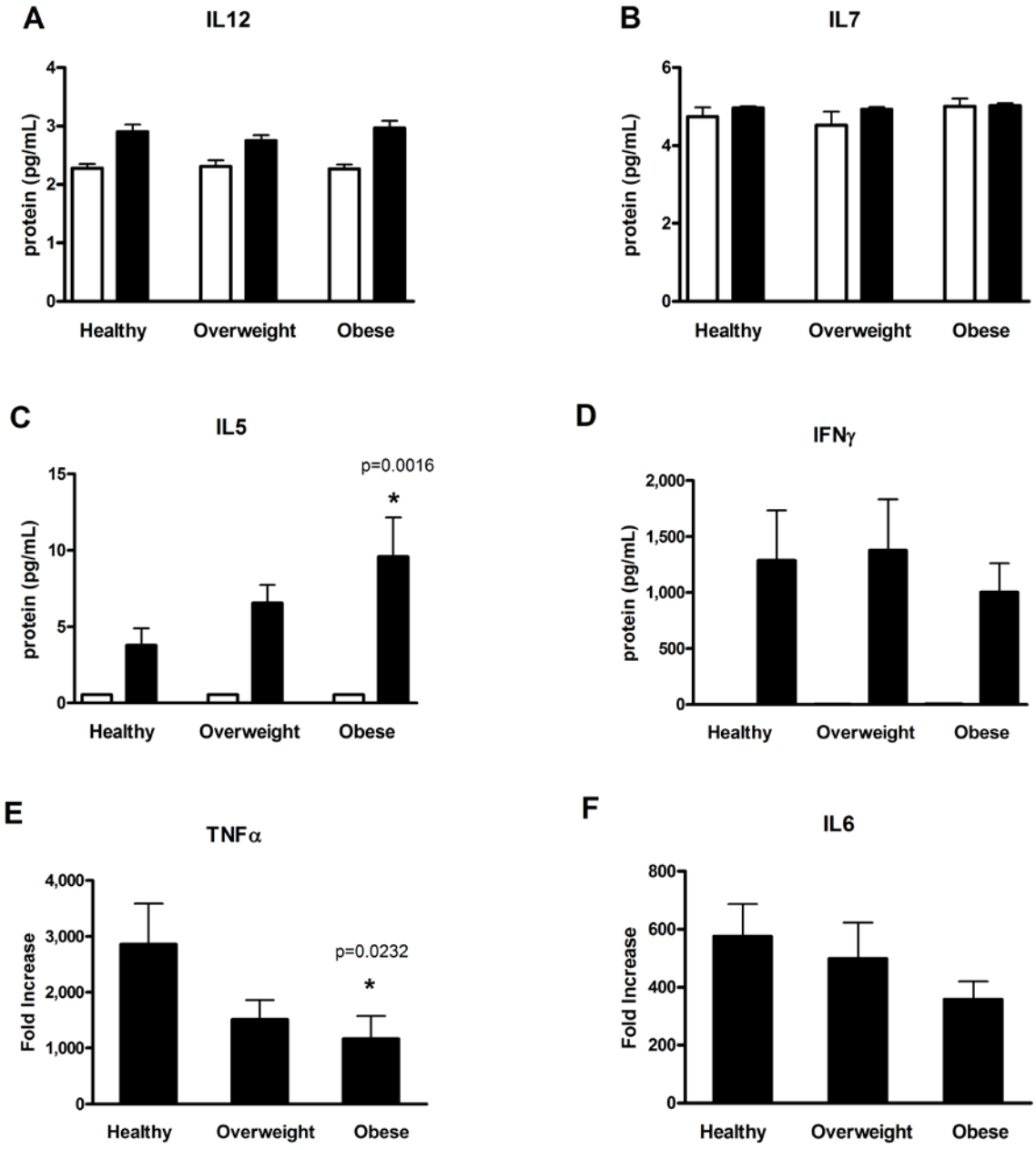


Figure 4.4: Secreted cytokines were measured in supernates from PMBCs incubated with (filled bar) or without (open bar) live pH1N1 virus. There were no differences in IL12 (A) and IL7 (B) levels between the healthy weight and overweight groups and the healthy weight and obese groups, both from unstimulated and stimulated PBMCs. IL5 levels (C) were higher and IFNγ levels (D) trended lower in stimulated PBMC supernates from obese individuals,

*compared to healthy weight individuals, while there were no differences in unstimulated PBMC supernates. Fold increase (filled bar) between unstimulated and stimulated PBMC supernates was lower for TNFα (E) and trended lower for IL6 (F), in stimulated PBMC supernates from obese individuals, compared to healthy weight individuals. Results are displayed as the mean ± s.e.m. (n=14-16 per group). Data below the limit of detection were assigned a value of half the lower limit of detection. The lower limits of detection of the assays were as follows: IL12, 0.6 pg/mL; IL7, 0.5 pg/mL; IL5, 1.1 pg/ml; and IFNγ, 1.8 pg/ml. *P<0.05 compared to PBMCs from healthy weight individuals within treatment group.*

Figure 4.5 Dendritic Cell Antigen Presentation to CD4+ and CD8+ T Cells

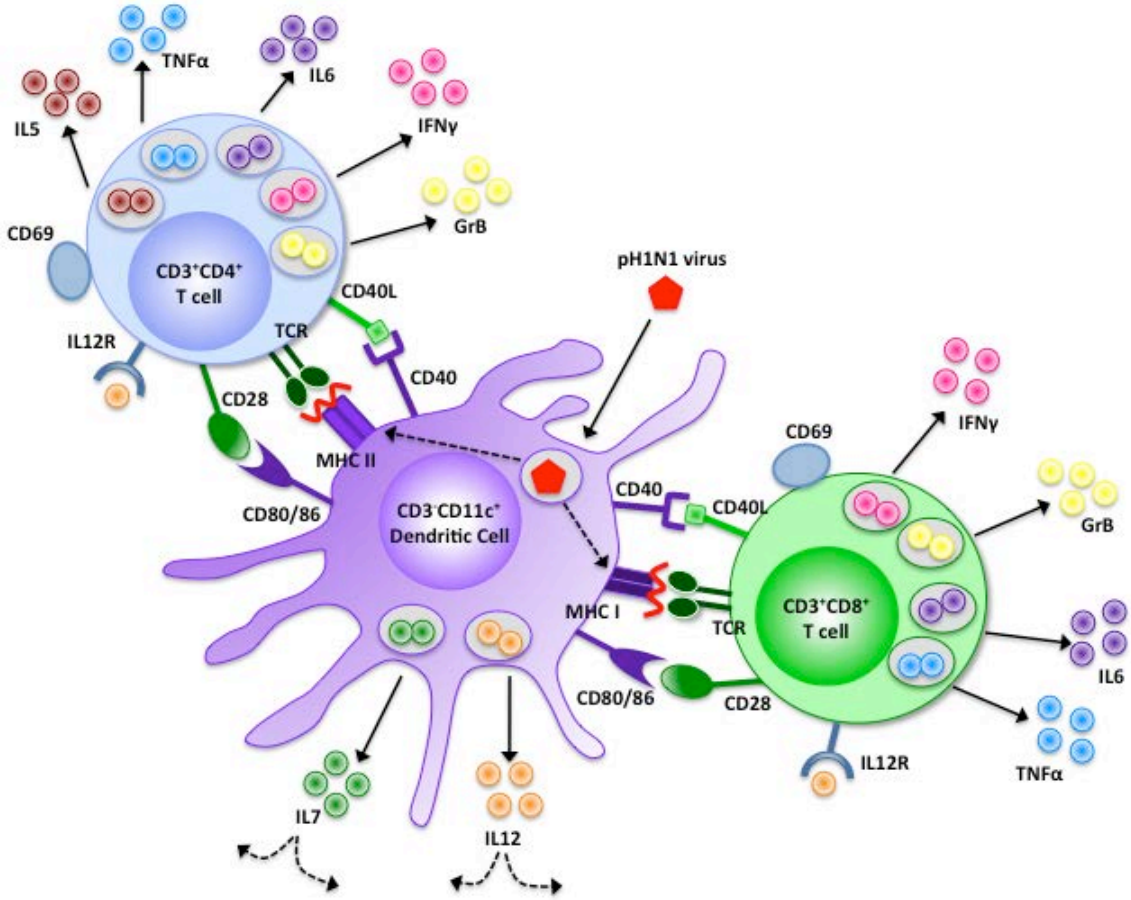


Figure 4.5: Dendritic cells process and present antigenic peptides to the TCR and its coreceptors on T cells, in association with MHC-II to CD4+ T cells and in association with MHC-I to CD8+ T cells. CD40 on dendritic cells binds CD40L on T cells. CD80 and CD86, expressed dendritic cells, bind CD28 on T cells. Activated dendritic cells secrete IL12. Activated CD4+ T cells and CD8+ T cells express CD69 and the IL12R and secrete IFNγ and GrB. MHC = major histocompatibility complex; TCR = T cell receptor; CD40L = CD40 ligand; IL12 = interleukin-12; IL12R = IL12 receptor; GrB = granzyme B; IFNγ = interferon-γ.

Figure 4.6 Representative Example of the Gating Strategy Used to Analyze $CD4^+$ T Cells

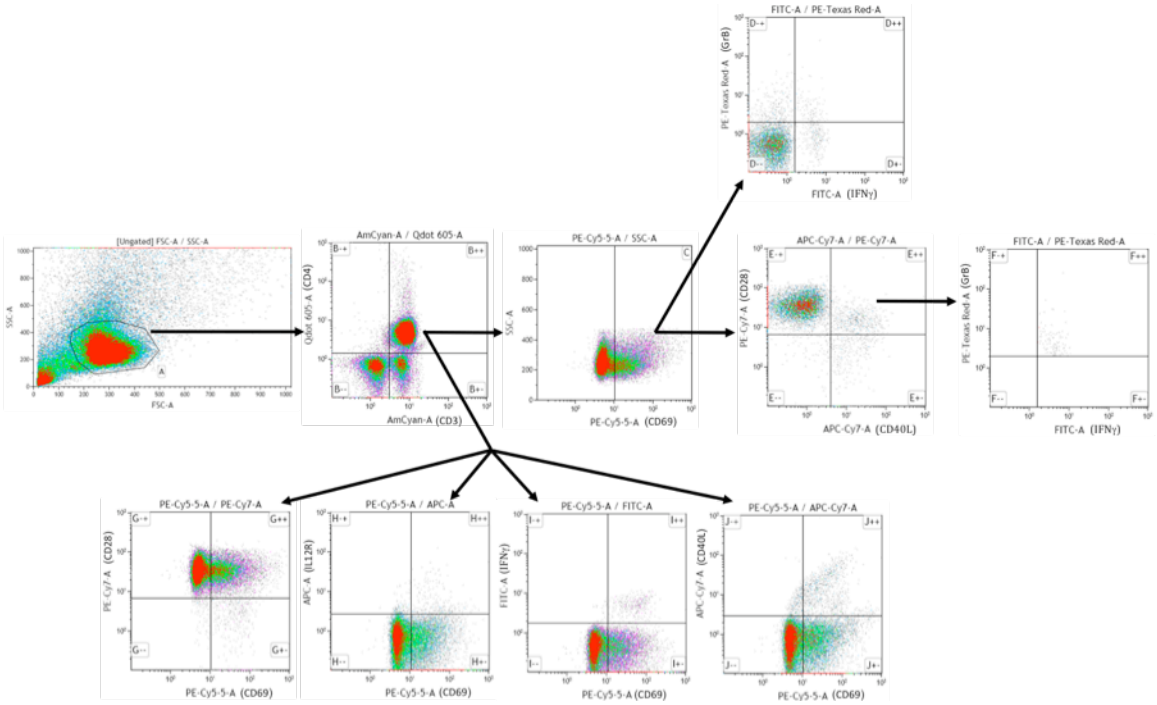


Figure 4.6: FACS gating strategies: $CD4^+$ and $CD8^+$ T cells were selected for analysis by creating a gate in the forward scatter versus side scatter plot that included only the lymphocyte population, followed by identification as $CD3^+CD4^+$ and $CD3^+CD8^+$, respectively. Cells were then analyzed for cell surface protein expression of CD28, CD40L, CD69, IL12R, and for intracellular protein expression of IFNγ and GrB. This is a representative example of the gating strategy used to analyze $CD4^+$ T cells; the gating strategy used to analyze $CD8^+$ T cells was similar. Dendritic cells were selected for analysis by creating a gate that included dendritic cells and monocytes, but excluded debris, platelets, and the entire lymphocyte population, thereby eliminating T cells, B cells, and NK cells from further analysis, followed by identification as $CD3^-CD11c^+$. Cells were then analyzed for cell surface protein expression of CD40, CD80, CD86, and MHC-II, and for intracellular protein expression of IL12. All antibodies were titered to determine the most appropriate concentration to use in the specific panel configurations in our experiments and with the BD LSR II flow cytometer at the UNC Flow Cytometry Core. Single-stained cells and single-stained compensation beads for every fluorochrome were used as compensation controls. Autofluorescence was calculated and included in the compensation adjustments. The use of monoclonal antibody conjugates at appropriate concentrations tend to have relatively low background staining (298). *In addition, in multi-color flow experiments used in the present study, the main source of background variation, after compensation has been calculated and applied, is caused by*

spillover (298). *As such, we utilized fluorescence-minus-one (FMO) controls for each stain to guide the gate placement to indentify positive and negative cell populations, as previously described* (299). *Data were collected on approximately 50,000 $CD3^+$ events run in the lymphocyte gate and approximately 30,000 events run in the dendritic cell/monocyte gate.*

Figure 4.7 PBMC Cytokine Secretion from Healthy Weight, Overweight, and Obese Individuals

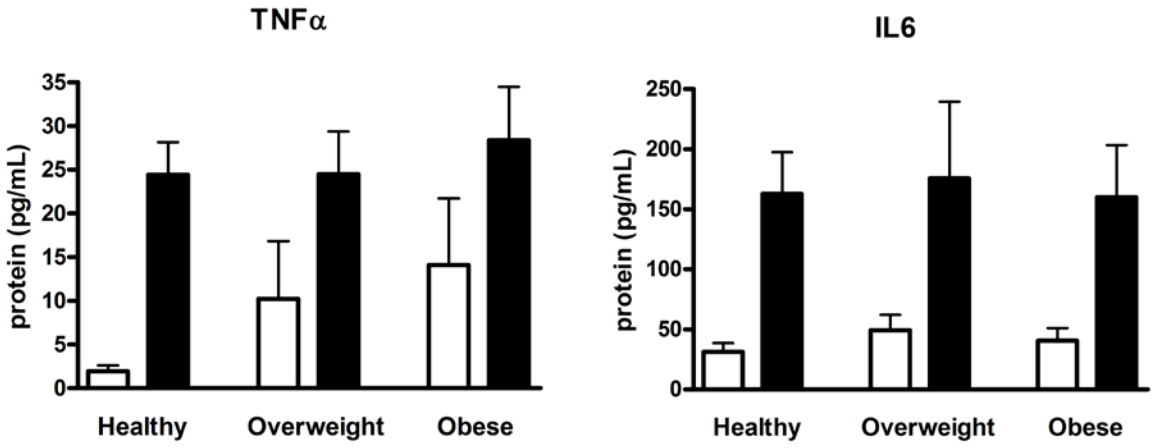


Figure 4.7: Secreted cytokines were measured in supernates from PMBCs incubated with (filled bar) or without (open bar) live pH1N1 virus. There were no differences in TNFα (A) and IL6 (B) levels between the healthy weight and overweight groups and the healthy weight and obese groups, both from unstimulated and stimulated PBMCs. Results are displayed as the mean ± s.e.m. (n=14-16 per group). Data below the limit of detection were assigned a value of half the lower limit of detection. The lower limits of detection of the assays were as follows: TNFα, 1.2 pg/ml and IL6, 1.6 pg/mL.

Figure 4.8 HAI Titers Measured in Serum from Healthy Weight, Overweight, and Obese Individuals

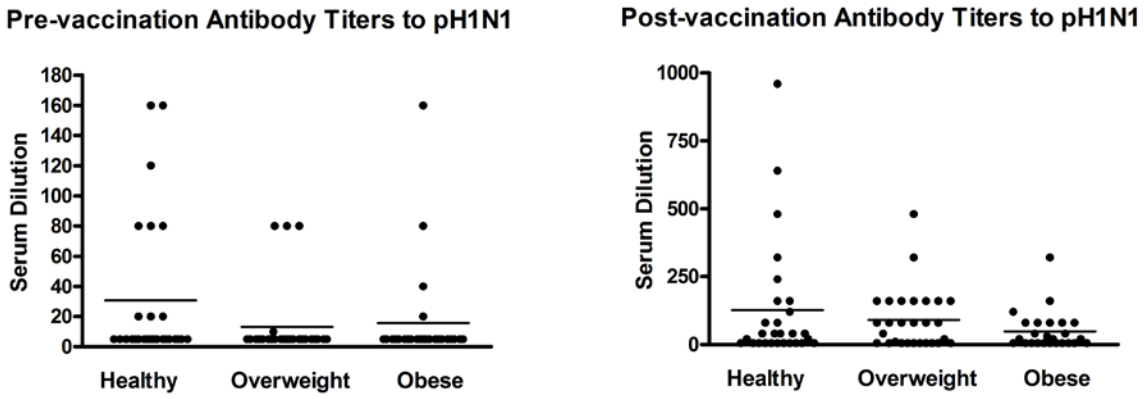


Figure 4.8: Hemagglutination inhibition (HAI) assays were conducted to determine the level of antibodies to pH1N1 in the sera, as previously described (294)*. Briefly, sera were treated with receptor destroying enzyme (Denka Seiken, Tokyo, Japan) overnight, followed by inactivation at 56°C for 1 hour, and dilution to 1:10 with PBS. Sera were then incubated in duplicate with influenza A virus A/California/4/2009 (H1N1) for 15 minutes at room temperature. After a 1 hour incubation at 4°C with 0.5% turkey red blood cells, HAI titers were determined to be the reciprocal dilution of the last well. Positive and negative controls, as well as back titrations of virus were included on each individual plate. Antibody responses specific to pH1N1 were assessed by HAI assay in pre-vaccination and post-vaccination serum samples from the 83 participants from whom the PBMC samples were obtained. Of the 83 participants, 80% had pre-vaccination serum titers to pH1N1 below the limit of detection of the test, while 6% of the participants had pre-vaccination serum titers to pH1N1 between 1:10 and 1:40, and 14% had pre-vaccination serum titers to pH1N1 equal to or above 1:40 (6 in the healthy weight group, 3 in the overweight group, and 3 in the obese group). Of the 83 participants, 39% had post-vaccination serum titers to pH1N1 below the limit of detection of the test, while 8% of the participants had pre-vaccination serum titers to pH1N1 between 1:10 and 1:40, and 53% had pre-vaccination serum titers to pH1N1 equal to or above 1:40 (15 in the healthy weight group, 15 in the overweight group, and 9 in the obese group). There were no significant differences in pre-vaccination or post-vaccination serum titers to pH1N1 among the healthy weight, overweight, and obese groups. Therefore, we were able to conclude that, prior to and following immunization, the immune responsiveness to pH1N1 was similar in the three groups, suggesting that exposure to pH1N1, infection with pH1N1, and vaccination effective against pH1N1 (i.e., the monovalent pH1N1 vaccination available in 2009) were equivalent in the three groups. Because of the evidence suggesting that cellular immune responses play the major role in protecting against pH1N1 and because the abilities to neutralize pH1N1 with antibodies were similar across the three BMI groups, we assessed the cellular immune responses in PBMC samples collected 30 days post-vaccination.*

CHAPTER V

THE EFFECTS OF TYPE II DIABETES ON GLUCOSE METABOLISM IN T CELLS

Introduction

Previous studies in this dissertation provide evidence of impairments in markers of T cell activation and function in response to influenza, but the underlying mechanism that mediated this differential effects is as of yet unknown. The experiments in Aim 3 of this dissertation were intended generate preliminary data about T cell glucose metabolism, in order to assess a potential mechanism for the results seen in the Aim 2 of this dissertation: that T cells from obese participants with and without type II diabetes would show alterations in T cell glucose metabolism, an essential pathway in activated T cells responding to an antigen such as the influenza virus. At rest, in a non-activated, non-stimulated state, T cells get most of their ATP from a mixture of fuels oxidized in the mitochondria, which includes the Kreb's cycle, the electron transport chain, and fatty acid β-oxidation (254). After activation, T cells rapidly switch from obtaining most of their ATP from oxidative phosphorylation to almost entirely deriving ATP from aerobic glycolysis (255), in addition to the pentose phosphate pathway and glutaminolytic pathways (256, 257). This tremendous flux through aerobic glycolysis results in the production of very high levels of lactate from

pyruvate, surprisingly even under conditions of adequate oxygen (258-260), similar to the Warburg effect in cancer cells (261). Therefore the major metabolic byproduct from activated T cells is lactate (254, 262); as much as 85% of glucose consumed by activated T cells is converted to lactate (263), and much of that is then secreted from the T cells. Because T cells circulating in the periphery are constantly exposed and bathed in whatever compounds and proteins are circulating the in bloodstream, and because with type II diabetes, there are often high levels of glucose and insulin in the bloodstream (85), we hypothesized that there would be differences in glucose metabolism, including glycolytic capacity, glycolysis rate, and glycolytic reserve, as well as a response to insulin, in T cells from obese individuals with and without type II diabetes.

Materials and Methods

Study population and samples

Participants were recruited as 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, NC (288). Eligible participants were adult (≥18 years of age) patients who received the 2010-2011 seasonal TIV. Exclusion criteria have been previously described (288). All procedures were approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill.

Participants were enrolled in the study between September 2010 and December 2010. At enrollment, informed consent, demographic characteristics, height, weight, and a blood sample were obtained. PBMCs were obtained from blood samples as previously described (288). Height and weight measurements were used to calculate BMI. Participants were classified by BMI as healthy weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), or obese (BMI $\geq$30.0) (1). Demographic characteristics of the 454 participants are presented in Table 5.1 A.

Two sample groups for the present study were created: obese participants with and without type II diabetes from our overall study population. BMI range was limited to 33.0 - 46.0, to ensure that participants were solidly in the obese category, rather than being closer to the overweight category, but also to exclude participants with extremely high BMIs, in the range on 47-76, based on the samples that were available for the study. From a pool of 44 obese participants without type II diabetes (with a BMI range of 33.3-45.9 and an age range of 40-69) and 35 obese participants with type II diabetes (with a BMI range of 33.3-45.4 and an age range of 22-70), participants were selected for each group. The groups were matched for BMI, age, gender, race, smoking status, and hypertensive medications. The groups differed in diabetes status, HbA1c levels, and diabetes medications. Participants from the obese diabetics group who used insulin to control their diabetes were excluded. Demographic characteristics of all 18 participants are presented in Table 5.1 and Table 5.2. Graphic depictions of age, BMI, and HbA1c levels are shown in Figure 5.1.

PBMC stimulation

T cells were isolated from thawed PBMCs by negative selection, which depletes B cells, NK cells, monocytes, platelets, dendritic cells, granulocytes, and erythrocytes. T cells were plated at 0.8 x 10^6 T cells/well for stimulated samples and 1.6 x 10^6 T cells/well for unstimulated samples in serum-free AIM-V media supplemented with 1% penicillin/streptomycin and 1% glutamine. For 24 hours, T cells were cultured with or without stimulation with anti-CD3/anti-CD28 beads at a ratio of 1 bead:1 cell at 37°C in 5% CO_2.

Metabolic Measurements

To examine cellular bioenergentics following the 24-hour incubation, T cells were transferred and adhered to a pre-conditioned Seahorse tissue culture plate in DMEM containing no glucose, glutamine, serum, or antibiotics. The plate with the T cells and the pre-conditioned, calibrated cartridge were placed in the Seahorse Analyzer. After equilibration and baseline measurements, the following compounds were injected into the well, followed by a standard mix:wait:measure cycle of 3 min:2 min:3 min: (1) 10mM glucose, which is a saturating concentration and allows for a measure of glycolytic flux; (2) 1µM insulin OR no insulin; (3) 0.5uM oligomycin, which is an ATP synthase inhibitor and shifts all energy production to glycolysis, which allows for a measure of glycolytic capacity; and (4) 100mM 2-deoxy-D-glucose, which is a glucose analog that inhibits glycolysis, as it is

phosphorylated by hexokinase to 2-deoxy-D-glucose and cannot be further metabolized, leading to the accumulation of 2-deoxy-D-glucose and the depletion of cellular ATP, thereby allowing a measure of glycolytic reserve. The two measurements taken by the Seahorse Analyzer were extracellular acidification rate (ECAR), which is the extrusion of protons via flux through the glycolytic pathway into the surrounding medium, and the O_2 consumption rate (OCR), which is a measure of mitochondrial respiration, including flux through the Kreb's cycle, fatty acid β-oxidation, and electron transport chain. The hypothesized results for ECAR and OCR in stimulated T cells from non-diabetic humans are shown in Figure 5.2. The injection of 10mM glucose is expected result in an increased in ECAR, due to the increased availability of substrate for the glycolytic pathway, and a corresponding decreased in OCR, due to the decreased reliance on mitochondrial oxidative phosphorylation for energy production. Following the injection of 1μM insulin, a similar was hypothesized to be observed: an increase in ECAR and a corresponding decrease in OCR, due to insulin upregulating GLUT1 translation, translocation, and activity, thereby bringing more glues into the cell and decreasing flux through mitochondrial oxidative phosphorylation. The injection of 0.5uM oligomycin was hypothesized to induce a significant increase ECAR, because with the inhibition of ATP synthase all of the energy production would be shifted to glycolysis, with a corresponding decreased in OCR. Finally, the injection of 100mM 2-deoxy-D-glucose, would be hypothesized to result in a dramatic decrease in ECAR and a corresponding increase in OCR, due to the inhibition in the glycolysis pathway and the concurrent shift to increased flux through mitochondrial oxidative phosphorylation.

Statistical analyses

Statistical analyses were performed using JMP statistical software (SAS). Differences in ECAR and OCR for the average of three measurements per treatment time period between the obese with type II diabetes and obese without type II diabetes group were analyzed with a two-tailed Student's *t*-test. P values < 0.05 were considered statistically significant.

Results

ECAR in Unstimulated T Cells

The ECAR measurements of unstimulated T cells without added insulin were significantly higher after the injection of 100mM 2-deoxy-D-glucose, from obese participants with type II diabetes, compared to T cells from obese participants without type II diabetes, as shown in Figure 5.3 A. This suggests that unstimulated T cells from obese participants with type II diabetes may have a higher glycolytic reserve than from obese participants without type II diabetes.

The ECAR measurements of unstimulated T cells treated with insulin were significantly higher after the injection of 10mM glucose, after the injection 1µM insulin, and

after the injection of 0.5uM oligomycin, from obese participants with type II diabetes, compared to T cells from obese participants without type II diabetes, as shown in Figure 5.3 B. This suggests that unstimulated T cells treated with insulin from obese participants with type II diabetes may have a higher glycolytic flux after glucose treatment and after insulin treatment, and a higher glycolytic capacity after oligomycin treatment.

ECAR in Stimulated T Cells

The ECAR measurements of stimulated T cells without added insulin and treated with insulin were not different after the injections of any of the compounds, as shown in Figure 5.4. This suggests that with stimulation from anti-CD3/anti-CD28 antibodies there are no differences in baseline glucose metabolism, or in glycolytic flux, glycolytic capacity, or glycolytic reserve.

OCR in Unstimulated T Cells

The OCR measurements of unstimulated T cells without added insulin and treated with insulin were not different after injections of any of the compounds, as shown in Figure 5.5, although the measurements trended higher in the T cells from obese type II diabetic participants throughout the experiment. This suggests that there are no differences in mitochondrial oxidative phosphorylation between unstimulated T cells from obese participants with and without type II diabetes.

OCR in Stimulated T Cells

The OCR measurements of stimulated T cells without added insulin and treated with insulin were not different after injections of any of the compounds, as shown in Figure 5.6, although the measurements trended higher in the type II diabetic samples at all timepoints. This again suggests that there are no differences in mitochondrial oxidative phosphorylation between unstimulated T cells from obese participants with and without type II diabetes.

Discussion

These experiments are the first to assess glucose metabolism in stimulated and unstimulated T cells in the context of obesity and type II diabetes. The higher ECAR measurements during the assessment of glycolytic reserve in unstimulated T cells not treated with insulin from obese participants with type II diabetes suggests that flux through aerobic glycolysis is increased, even without stimulation, perhaps due to higher production of substrates from the pentose phosphate pathway and the glutaminolytic pathways. In addition, the higher ECAR measurements during the assessments of glycolytic flux and glycolytic capacity in unstimulated T cells treated with insulin from obese participants with type II diabetes also suggest that even without external stimulation from anti-CD3/anti-CD28 antibodies, flux through the glycolytic pathway is increased. The fact that the

glycolytic flux remains higher in type II diabetic T cells even following insulin treatment was the opposite of what was hypothesized to happen. Perhaps with type II diabetes, there is already maximal flux through glycolysis and it is unable to be fine-tuned with the addition of excess external substrate, the 10mM glucose, or hormonal stimulation, the 1μM insulin.

There were no differences in ECAR measurements when examining baseline glucose metabolism, glycolytic flux, glycolytic capacity, and glycolytic reserve between stimulated T cells treated with insulin from obese participants with and without type II diabetes. This may be due to the type of stimulation that was used to activate the T cells. Anti-CD3/anti-CD28 antibodies induce a potent mitogenic stimulation, where all T cells in the culture are activated. This is different than an antigen-specific stimulation, where only a fraction of the total T cells present, those that have been primed against the specific antigen would be activated. It is possible that by using such a potent stimulation, the T cells from both obese participants with and without type II diabetes were already metabolizing glucose at the maximal rate possible and therefore any potential differences were not able to be seen.

Although there were no statistically significant differences in OCR measurements when examining baseline glucose metabolism, glycolytic flux, glycolytic capacity, and glycolytic reserve between unstimulated T cells from obese participants with and without type II diabetes and between stimulated T cells from obese participants with and without type II diabetes, each of the measurements consistently trended higher in the T cells from obese participants with type II diabetes. This trend suggests that both unstimulated and

stimulated T cells from obese participants with type II diabetes are more metabolically active overall and that even though energy production through the glycolytic pathway is increased, flux through mitochondrial oxidative phosphorylation energy-generating pathways is also elevated.

Although further studies in this area are needed, it is interesting to hypothesize that the impairments in markers of T cell activation and function seen in PBMCs from obese humans may be associated, at least in part, to defects in T cell glucose metabolism. As mentioned previously, the epidemiological data from the pH1N1 pandemic show that both obesity and type II diabetes impair the immune response. It will be important to determine whether there are additive, synergistic effects when obesity and type II diabetes occur in the same individual, in addition, to learning what the effects of obesity and type II diabetes have on the immune response as singular, unique variables. The significant and trending data from this aim indicate that T cells from obese participants with type II diabetes have both higher flux through glycolysis and higher flux through mitochondrial oxidative phosphorylation, in both the unstimulated and stimulated states, compared to T cells from obese participants without type II diabetes. This suggests that in the unstimulated state, T cells from obese participants with type II diabetes are not able to downregulate flux through glycolysis and continue to rely on glucose for energy, even through T cells from healthy weight, non-diabetic individuals are hypothesized to use almost solely mitochondrial oxidative phosphorylation for energy. This is analogous to liver and muscle cells in the insulin-resistant, hyper-glycemic state, which continue to utilize (liver and

muscle cells), and produce and secrete (liver cells) glucose during a fasting state. In addition, in a stimulated state, T cells need to minimize flux through mitochondrial oxidative phosphorylation and significantly increase flux through glycolysis. Again the significant and trending data from this aim indicate that stimulated T cells from obese participants with type II diabetes are not able to upregulate and downregulate flux through glycolysis and mitochondrial oxidative phosphorylation, respectively, to the same extent as T cells from obese participants without type II diabetes. The regulation of glucose metabolism and mitochondrial oxidative phosphorylation in the unstimulated and stimulated states are critical to T cell survival and function. It is interesting to speculate that an inability to regulate metabolism through these pathways may alter T cell function to the extent that it resulted in increased morbidity and mortality from pH1N1 seen in obese individuals, although further testing is needed to determine if these effects have clinical relevance and impact.

Future Directions

The generated data will be analyzed by a number of additional statistical methods, including a comparison of the ECAR and OCR measurements between unstimulated and stimulated T cells for each individual participant and a comparison of fold changes in ECAR and OCR measurements from baseline to the other timepoints for each individual participant. Furthermore, additional similar experiments may be conducted using T cells from healthy weight individuals without type II diabetes. Because of the strong association

between obesity and type II diabetes and due to the challenges in differentiating pre-type II diabetic individuals from type II diabetic individuals, real differences between the obese with type II diabetes group and obese without type II diabetes group may have been minimized.

Tables and Figures

Table 5.1 Demographic Characteristics of the Individuals in the Study Population

Obese Participants With Type II Diabetes

Sex	Age	Race	Diabetes Diagnosis	BMI	Smoking	HbA1c
Female	65	Caucasian	Type II Diabetes	33.3	No	6.8
Female	58	African American	Type II Diabetes	38.0	Previous	8.0
Male	70	African American	Type II Diabetes	34.6	Previous	6.2
Female	50	African American	Type II Diabetes	35.4	Yes	9.1
Male	60	African American	Type II Diabetes	38.4	No	6.4
Female	55	Caucasian	Type II Diabetes	43.0	No	6.0
Male	56	African American	Type II Diabetes	38.0	No	9.0
Male	47	Caucasian	Type II Diabetes	42.2	No	10.2
Female	48	Caucasian	Type II Diabetes	39.9	No	7.2
Male	40	African American	Type II Diabetes	43.3	Yes	11.4
-	54.9	-	-	38.61	-	8.0

Obese Participants Without Type II Diabetes

Sex	Age	Race	Diabetes Diagnosis	BMI	Smoking	HbA1c
Female	69	Caucasian	No Diabetes	36.2	No	5.6
Male	51	Caucasian	No Diabetes	34.6	Yes	n/a
Male	60	African American	No Diabetes	37.6	Previous	5.6
Female	51	African American	No Diabetes	41.1	No	5.4
Male	50	Caucasian	No Diabetes	34.6	Yes	5.1
Female	45	African American	No Diabetes	38.4	No	n/a
Female	68	African American	No Diabetes	36.7	Previous	n/a
Male	43	Caucasian	No Diabetes	41.2	Previous	5.0
-	54.6	-	-	37.6	-	5.3

Table 5.1: Participants included in the study were categorized as either male or female, either Caucasian or African American, either having been diagnosed with type II diabetes or not having a diagnosis or other indications of type II diabetes, and as either a non-smoker (No), previous smoker (Previous), or current smoker (Yes). Age is measured in years, BMI is measured in kg/m^2, and HbA1c levels are measured in %. The bottom row in each section shows the average values for age, BMI, and HbA1c levels. There were no differences between the groups in gender, age, race, BMI, or smoking status. Diabetes status and HbA1c levels were different between the two groups.

Table 5.2 Demographic Characteristics of the Study Population

		Obese with Type II Diabetes	Obese without Type II Diabetes
n		10	8
Age		54.9 ± 2.8	54.6 ± 3.5
BMI		36.8 ± 1.1	37.6 ± 0.9
HbA1c		8.0 ± 0.6	5.3 ± 0.1 (based on 5)
Gender	*Female*	5 (50%)	4 (50%)
	Male	5 (50%)	4 (50%)
Race	*Caucasian*	4 (40%)	4 (50%)
	African American	6 (60%)	4 (50%)

Table 5.2: Characteristics of the two study groups. Data are shown as number (percent). There were no differences between the groups in gender, age, race, BMI, or smoking status, as determined by Student's t test with a significance level set at 0.05. Diabetes status and HbA1c levels were significantly different between the two groups.

Figure 5.1 Age, BMI, and HbA1c Levels

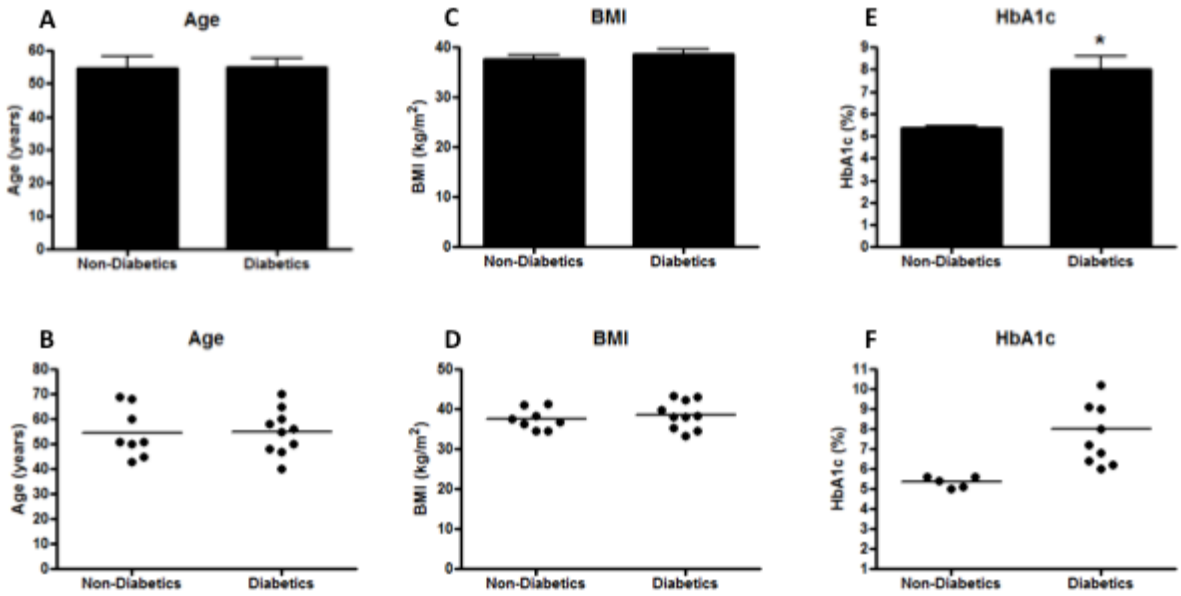


*Figure 5.1: Age, BMI, and HbA1c levels of the two groups. There are no differences in age (A, B) and in BMI (C, D). The HbA1c levels are higher in the obese with type II diabetes group, compared to the obese without type II diabetes group. *P<0.05 compared to the obese without type II diabetes group.*

Figure 5.2 Hypothesized Results for ECAR and OCR in Non-Diabetic Stimulated T Cells

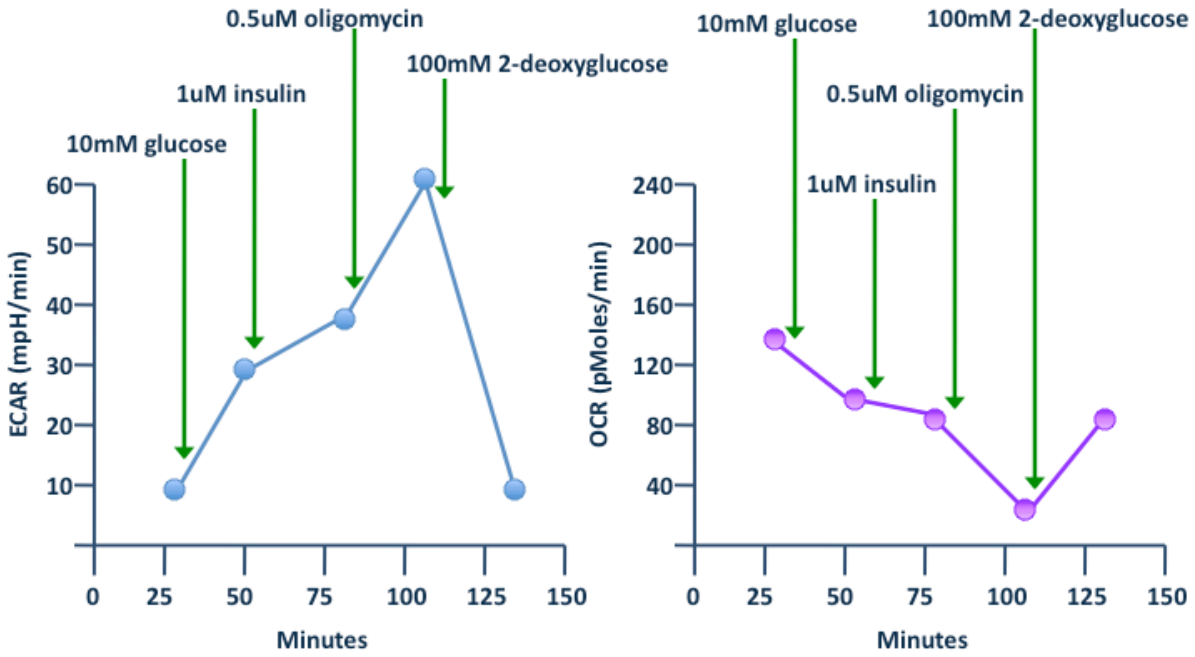


Figure 5.2: Hypothesized results for ECAR and OCR measurements for stimulated T cells from non-diabetics after injections of (1) 10mM glucose, which is a saturating concentration and allows for a measure of glycolytic flux; (2) 1μM insulin OR no insulin; (3) 0.5uM oligomycin, which is an ATP synthase inhibitor and shifts all energy production to glycolysis, which allows for a measure of glycolytic capacity; and (4) 100mM 2-deoxy-D-glucose, which is a glucose analog that inhibits glycolysis, as it is phosphorylated by hexokinase to 2-deoxy-D-glucose and cannot be further metabolized, leading to the accumulation of 2-deoxy-D-glucose and the depletion of cellular ATP, thereby allowing a measure of glycolytic reserve. ECAR is the extrusion of protons via flux through the glycolytic pathway into the surrounding medium and OCR is a measure of mitochondrial respiration, including flux through the Kreb's cycle, fatty acid β-oxidation, and electron transport chain.

Figure 5.3 ECAR in Unstimulated T Cells With and Without Insulin

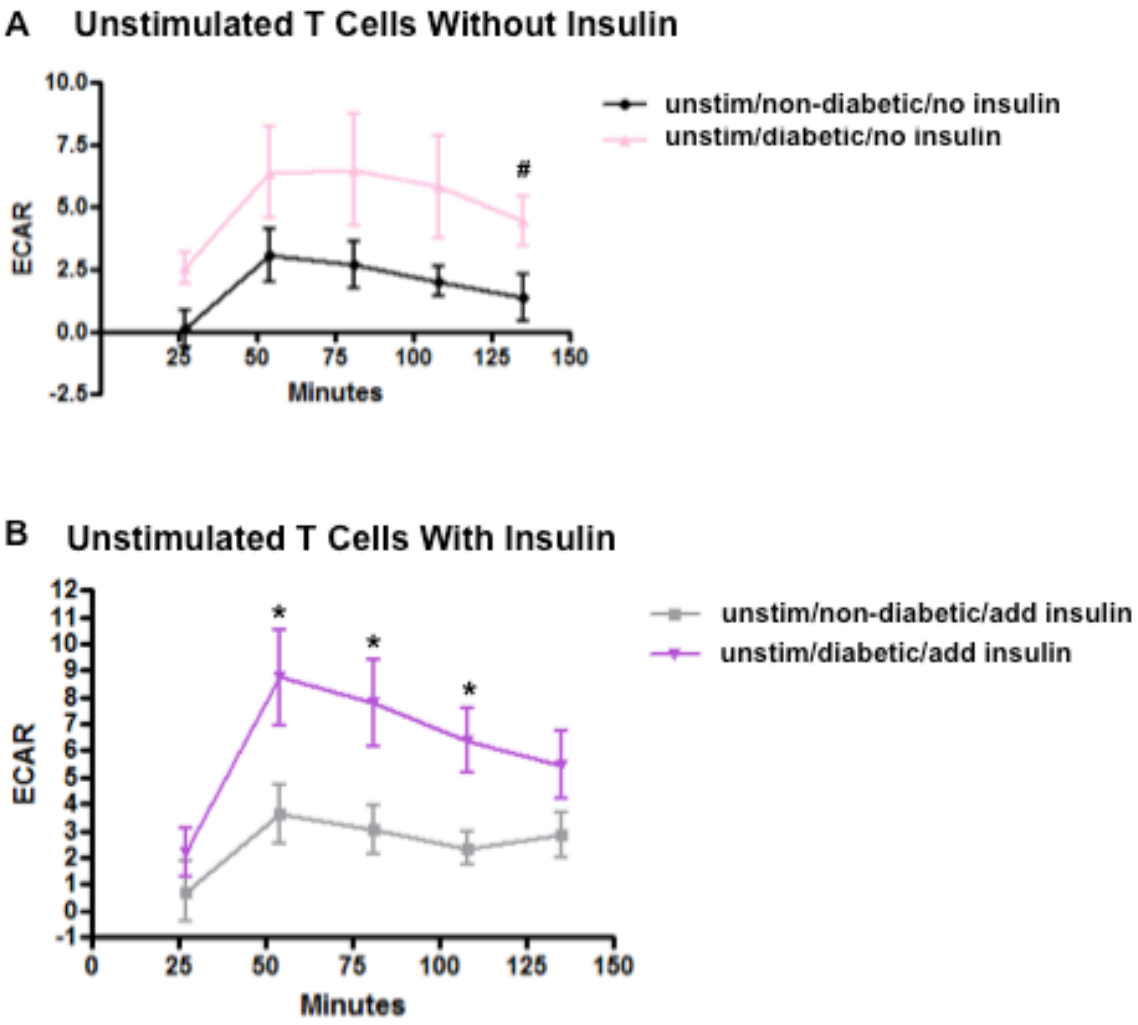


*Figure 5.3: The extracellular acidification rates (ECAR) of unstimulated T cells from obese participants with and without diabetes were analyzed in a Seahorse Analyzer. The first timepoint is a baseline measurement, the second timepoint is after an injection of 10mM glucose, the third timepoint is after an injection or not of 1µM insulin, the fourth timepoint is after an injection of 0.5uM oligomycin, and the fifth timepoint is after an injection of 100mM 2-deoxy-D-glucose. ECAR is measured in mpH/min. # = P<0.05 compared to the obese without type II diabetes without insulin group. *P<0.05 compared to the obese without type II diabetes with insulin group.*

Figure 5.4 ECAR in Stimulated T Cells With and Without Insulin

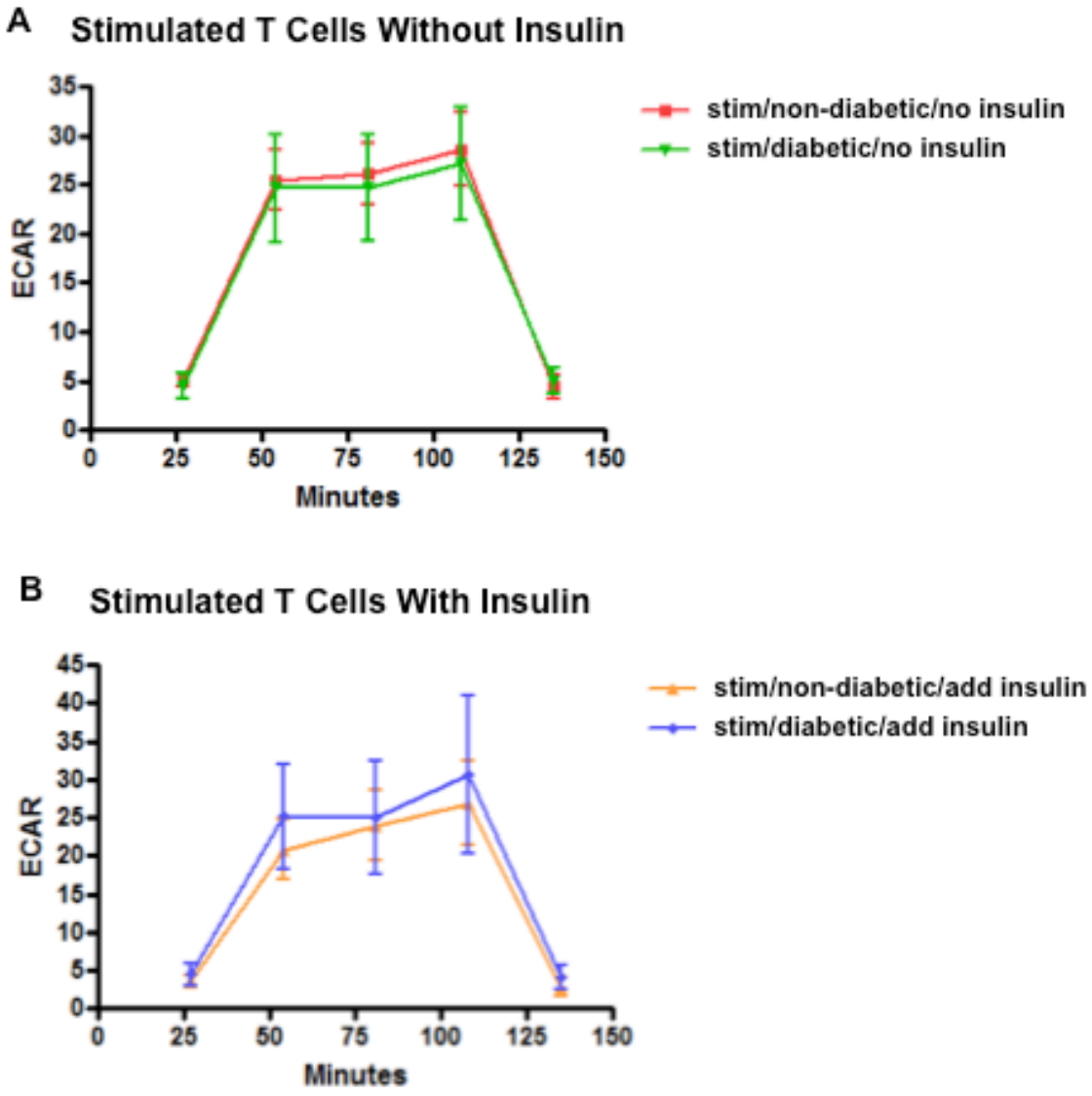


Figure 5.4: The extracellular acidification rates (ECAR) of T cells stimulated with anti-CD3/CD28 beads at a ratio of 1 bead:1 cell for 24 hours from obese participants with and without diabetes were analyzed in a Seahorse Analyzer. The first timepoint is a baseline measurement, the second timepoint is after an injection of 10mM glucose, the third timepoint is after an injection or not of 1µM insulin, the fourth timepoint is after an injection of 0.5uM oligomycin, and the fifth timepoint is after an injection of 100mM 2-

deoxy-D-glucose. ECAR is measured in mpH/min. There are no significant differences between groups.

Figure 5.5 OCR in Unstimulated T Cells With and Without Insulin

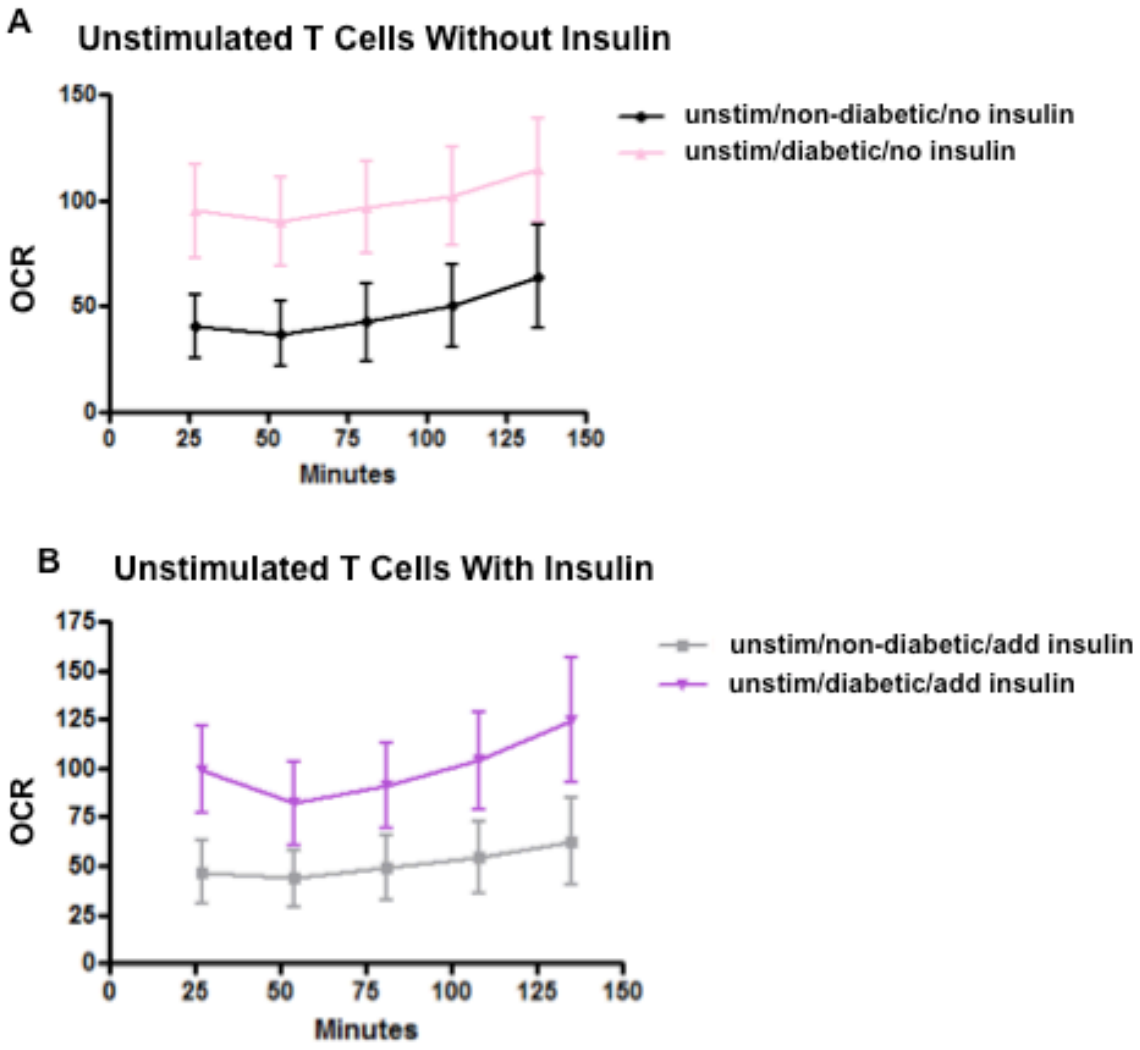


Figure 5.5: The oxygen consumption rates (OCR) of unstimulated T cells from obese participants with and without diabetes were analyzed in a Seahorse Analyzer. The first timepoint is a baseline measurement, the second timepoint is after an injection of 10mM glucose, the third timepoint is after an injection or not of 1µM insulin, the fourth timepoint is after an injection of 0.5uM oligomycin, and the fifth timepoint is after an injection of 100mM 2-deoxy-D-glucose. OCR is measured in pMoles/min. There are no significant differences between groups.

Figure 5.6 OCR in Stimulated T Cells With and Without Insulin

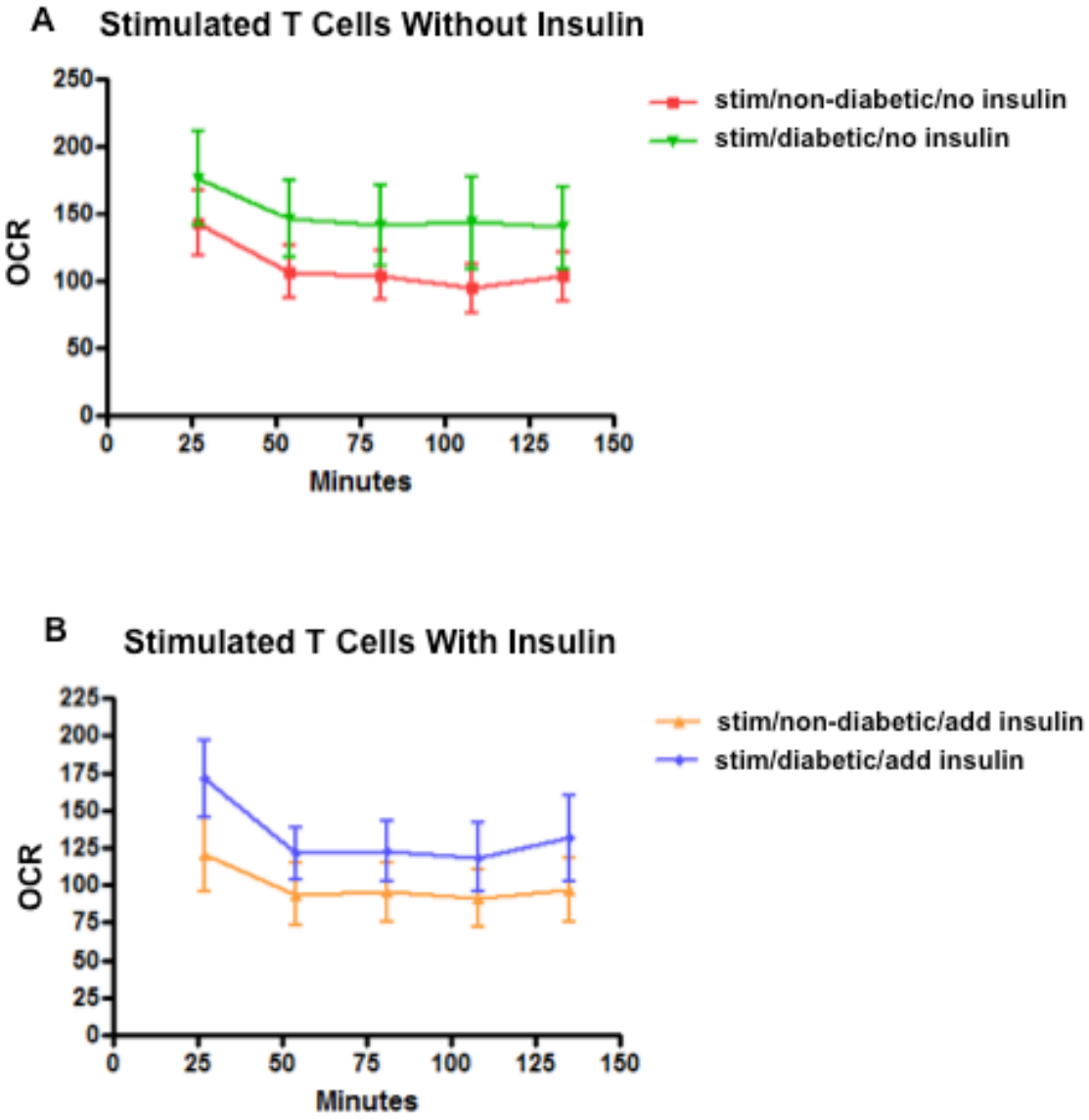


Figure 5.6: The oxygen consumption rates (OCR) of T cells stimulated with anti-CD3/CD28 beads at a ratio of 1 bead:1 cell for 24 hours from obese participants with and without diabetes were analyzed in a Seahorse Analyzer. The first timepoint is a baseline measurement, the second timepoint is after an injection of 10mM glucose, the third timepoint is after an injection or not of 1µM insulin, the fourth timepoint is after an injection of 0.5uM oligomycin, and the fifth timepoint is after an injection of 100mM 2-

deoxy-D-glucose. OCR is measured in pMoles/min. There are no significant differences between groups.

CHAPTER VI

SYNTHESIS

Overview of Research Findings

The data presented in this dissertation illustrate that the humoral and cellular immune responses to influenza virus are impaired with overweight and obesity, and that type II diabetes in conjunction with obesity may alter glucose metabolism in T cells. Specifically, we found that higher BMI was associated with a greater decline in antibody titers to influenza strains at eleven months post vaccination, suggesting that overweight and obese individuals may not be as protected throughout the duration of the flu season compared to healthy weight individuals. We also found that markers of dendritic cell activation and function were intact, while markers of T cell activation and function were significantly impaired with overweight and obesity, suggesting that overweight and obese individuals may not be able to fight off influenza infection as effectively as healthy weight individuals. Finally, we found that glucose metabolism may be altered in T cells from obese individuals with type II diabetes.

Potential Mechanism

Although the mechanisms mediating the impaired humoral immune response to influenza vaccination and cellular immune response to influenza virus seen in overweight and obese individuals presented in this dissertation are likely multi-factorial, it is interesting to speculate that dysregulations in leptin production and leptin action play a major causative role.

Circulating leptin levels can be affects by a number of factors, including adipose tissue mass, nutritional status (fed or fasted state), and also by infection. Adipocytes are the main producers of leptin in the body and circulating leptin levels are adipose tissue mass, so the higher the adipose tissue mass, the higher the serum leptin levels will be (300). Leptin mRNA expression is elevated in obese adults, compared to in healthy weight adults, although it is theorized that obese adults may be leptin resistant, characterized by decreased activation of intermediates in the leptin signaling cascade (301). Leptin is also secreted in response to a meal; serum leptin increases after a meal and then slowly decreases as the nutrients are moved into the tissues of the body (302). Leptin acts as a satiety factor, by binding with leptin receptors in the hypothalamus, thereby decreasing food intake (302). Finally, data from a previously published study from our lab show that serum leptin levels are elevated in lean control mice with influenza infection, although that same increase was no seen in diet-induced obese mice (11).

Leptin acts by binding to the leptin receptor expressed on the cell surface, thereby activating multiple intracellular signaling pathways, including a JAK-STAT cascade (303). Leptin predominantly activates JAK2 occurs by phosphorylating tyrosine residues in the cytoplasmic region of the receptor (303), although in PBMCs from obese adults, evidence indicates that leptin signaling is impaired and is characterized by lower levels of JAK2 phosphorylation (304). Following phosphorylation, STAT become associated with the leptin receptor (303). STAT can then be phosphorylated itself, which results in their dissociation from the leptin receptor (303). STATs are then able to form active dimmers, which can translocate into the nucleus where they act as transcription factors to regulate mRNA expression (303). Leptin has been reported to induce SOCS3 expression (305). However, the JAK/STAT signaling cascade is negatively regulated by SOCS3, as SOCS3 is able to directly inhibit tyrosine phosphorylation of the leptin receptor (306), resulting in an inhibition of STAT3 activity (307). Interestingly, SOCS proteins are chronically elevated with obesity (308) and leptin resistance is associated with high levels of SOCS3, resulting in decreased activity of the intermediates in the signaling cascade, most importantly decreased phosphorylation of STAT3, which results in dysregulation in gene expression in STAT3-responsive genes.

Leptin is known to have modulatory effects on the immune system (309-312). Scientists recently found that in an obese mouse model the blocking the actions of leptin with injections of anti-leptin antibody resulted in an increased survival rate following infection with mouse-adapted pH1N1, as well as decreased levels of IL6 and IL1β in the lungs, possibly suggesting that the chronic high levels of leptin circulating in the obese mice

elevated proinflammatory factors involved in the immune response and promoted the severe infection-associated injury in the lungs (34).

Leptin acts on B cells to induce cytokine synthesis and secretion, including that of TNFα and IL6 (313). Evidence suggests that leptin deficiency or leptin resistance can have significant effects on B cells. Mice that lack the leptin receptor (ob/ob mice) display much lower levels of total B cells compared to normal control mice; however when ob/ob mice were treated with leptin, there was a significant increase in B cell numbers (314). Similarly, mice in a starvation state have lower circulating levels of leptin, however intracerebroventricular leptin injections prevented the impairments in B cell development and proliferation in bone marrow (315). Although we did not directly measure B cell numbers or markers of B cell activation and function in our study, our data may be explained in part by downstream effects resulting from resistance in the leptin signaling pathway affecting the maintenance of memory B cells of obese and overweight individuals. Activated B cells migrate into the lymphoid follicle, where memory cell generation occurs (185, 186). Memory B cells then reside in the bone marrow where they can continue to produce antibodies specific to the influenza strains in the vaccine for years, a process which is sustained by low-level consistent proliferation of the memory B cells. Although there were no impairments in initial antibody responses to the influenza vaccine in overweight and obese individuals, perhaps over time, the vaccine-specific memory B cell population could not be maintained due to resistance in the leptin signaling pathways that are intended to sustain B cell proliferation in the bone marrow. As such, this would result in

lower numbers of memory B cells over time, thereby reducing the amounts of antibody levels found in the serum, such as we saw at 12 months post-vaccination in the overweight and obese individuals. Furthermore, leptin promotes IgG2a production from B cells (312), a subtype of antibody of particular importance in preventing influenza virus infection and induced by influenza vaccination. Perhaps chronic resistance in the leptin signaling cascade in overweight and obese individuals resulted in a lower production of antibodies over time, despite the fact that at one month post-vaccination there were no impairments in antibody production Taken together, it is interesting to hypothesize that deficiencies in B cell proliferation and antibody production, may have resulted from resistance in the leptin signaling pathway, the effects of which manifested most predominantly at 12 months post-vaccination. Furthermore, leptin has been proposed as a vaccine adjuvant to improve the body's immune response to different types of vaccines, including the influenza vaccine (316). Perhaps the administration of leptin to leptin resistance individuals concurrently with the influenza vaccine would result in increased B cell proliferation and increased antibody production over time.

In addition, human T cells are able to produce and secrete leptin, and T cells express the leptin receptor on their cell surface. In both $CD4^+$ and $CD8^+$ T cells, leptin promotes proliferation, activation, and cytokine production and secretion, in particular of IL2, which would further promote proliferation (309, 310, 312). Our data show that there are defects in markers of $CD4^+$ and $CD8^+$ T cell activation and function, including CD69, CD28, CD40L, IL12R, GrB, and IFNγ. Resistance in the leptin signaling pathway in overweight and obese

individuals may have affected the gene transcription and protein expression of these markers, and may be linked to the clinical outcomes of increased morbidity and mortality from pH1N1 seen in obese individuals. Leptin was shown to promote $CD4^+$ T cell differentiation towards a T_H1 phenotype (309, 310), while suppressing a T_H2 phenotype, including expression of cell surface markers and cytokine secretion, in an *ex vivo* human PBMC model (311, 312). We saw significant deficiencies in the intracellular production of with IFNγ expression in our flow cytometry assessments, as well as trended towards lower secretion into the supernates in PBMCs from overweight and obese individuals. In addition, we also saw significant increases and trending increases in IL5 secretion into PBMC supernates from overweight and obese individuals, respectively. Taken together these data suggest a shift towards a T_H2 phenotype and away from a T_H1 phenotype. Since leptin is known to do the opposite, perhaps resistance in the leptin signaling pathways in the PBMCs overweight and obese individuals resulted in this dysregulation of the $CD4^+$ T cell phenotypes. Again, these data suggest a possible link between the metabolic alterations associated with higher BMIs, as well as suggest a possible mechanism to help explain the increased morbidity and mortality in pH1N1-infected obese individuals. In addition, leptin signaling induces upregulation of IL6 and TNFα with infection. Importantly, data from our lab show that with an influenza virus challenge in diet-induced obese mice and lean control mice, there was a transient increase in serum leptin concentrations in the lean control mice, while the obese mice displayed a decreased in serum leptin concentrations, which was accompanied by a 42% higher mortality rate, as well as decreased mRNA induction in lungs of the antiviral cytokines IFNα and IFNβ, the proinflammatory cytokines IL6, TNFα, and IL1β,

the chemokines MCP-1 and RANTES, and IL18 and IL10 (11). The data presented in this dissertation show that there were impairments in the upregulation of the proinflammatory cytokines IL6 and TNFα with stimulation from the influenza virus in the obese individuals, which may be caused in part by resistance in the leptin signaling cascade that would normally result in increased expression of IL6 and TNFα in healthy weight individuals. It is interesting to speculate that if effective leptin signaling could be reestablished in overweight and obese individuals through either weight loss or through pharmacological intervention, that perhaps there would an accompanying improvement in the cellular immune response to influenza virus, including expression of markers of activation and function in and secretion of cytokines from $CD4^+$ and $CD8^+$ T cells similar to that of healthy weight individuals.

Interestingly, SOCS3 is also known to inhibit activity of the insulin receptor (317), which would affect the activation and activity of all the downstream intermediates in the insulin signaling pathway. Conversely, mice lacking SOCS3 display remain responsive to insulin, while being protected from diet-induced obesity (318, 319). Although further study is needed to reach firm conclusions from the assessments of T cell glucose metabolism in stimulated and unstimulated T cells from both obese participants with and without type II diabetes, it is interesting to speculate that dysregulation in the leptin signaling pathway augmented the alternations that we saw in the unstimulated T cells from obese participants with type II diabetes. Part of the downstream effects of Akt phosphorylation, which is the intermediate of both the insulin signaling pathway and the CD3/CD28 signaling pathway, is

to increase GLUT1 translation, translocation, and activity in stimulated T cells. Conversely, in the unstimulated state, it is expected that T cells have minimal flux through the glycolytic pathway, while obtaining most of the their energy from mitochondrial oxidative phosphorylation. In addition, in the unstimulated state, T cells should express very little insulin receptor protein on their cell surface, and the decreased activation of the insulin signaling pathway should allow for and promote flux through mitochondrial oxidative phosphorylation. It is possible that the high levels of SOCS3 associated with leptin resistance inhibited the activity of the insulin receptor and therefore of the insulin signaling pathway, resulting in a decreased ability to both upregulate and downregulate flux through glycolysis and mitochondrial oxidative phosphorylation as is necessary in stimulated and unstimulated T cells. In addition, it is interesting to speculate that if T cells from obese participants with type II diabetes are not able to fine-tune flux through differential metabolic pathways, they will have impairments in function and survival which are so necessary in effectively fighting off an influenza infection, and may provide potential mechanism linking the more severe clinical consequences seen in pH1N1-infected patients with type II diabetes.

Implications of Research Findings and Public Health Significance

The data from the three aims of this dissertation have wide-reaching implications for public health, particularly with the dramatic increases in obesity worldwide. In particular, the data presented here are the first to show that overweight impairs the immune response. Overweight- and obesity-associated deficiencies in the both the humoral and

cellular immune responses to influenza likely play a role in the response to other infectious diseases as well. Based on the data from Aim 1, different vaccination or treatment strategies may need to be considered for overweight and obese individuals. One strategy to overcome these deficiencies may be to provide a higher dose of vaccine, similar to the high-dose vaccine that was tested in older individuals, with 4 times the amount of antigen, or to employ a multi-dose vaccination strategy, to ensure that overweight and obese individuals are protected through the duration of flu season. In addition, there is interest in the scientific community in creating a universal influenza vaccine, targeting T cells, which would provide protection against multiple strains of influenza virus over many years. However, based on our data from Aim 2, given that the T cell response to pH1N1 influenza is defective in overweight and obese individuals, vaccines targeting T cells may not be as effective in these populations, and other strategies may need to be explored, such as including an adjuvant in the universal influenza vaccine, assessing the cellular immune response during clinical testing of the universal influenza vaccine, or administering higher or multiple doses to higher risk populations. Finally, the research towards improving the understanding the unique and convergent roles that overweight, obesity, and type II diabetes play in the immune response to infectious diseases are just beginning to be understood, however based on the preliminary data generated in Aim 3, it does appear that this question dose deserve further study. The results presented in this dissertation give us a starting point to guide and inform future studies in this area.

Recommendations for Future Research

In respect to the humoral immune response to influenza, although our data clearly indicate antibody production in response to influenza vaccination declines more rapidly in overweight and obese individuals, our data is from one month and 11 months after vaccination. It will be imperative to ascertain the kinetics of the differential decline in influenza-specific antibody titers over time. For example it would be ideal if we could obtain blood samples once a month for a year following influenza vaccination, that way we would have the pre-vaccination sample and the 12 post-vaccination samples to analyze in order to understand exactly how and when the decline in antibody titers occurs in overweight and obese individuals. These data are essential to determine whether these populations are levels of antibodies that would confer protection from influenza infection throughout the duration of the flu season. Data from the CDC indicate that the flu season can last for 8 months, from October until May, in the US (115). Whether an obese individual vaccinated against influenza in October still has protective levels of influenza-specific antibodies in circulation in May is as of yet unknown. In addition, to looking directly at antibody levels, it will also be important to assess the different subsets of B cells and parameters that affect antibody levels over time. For example, following influenza vaccination or even influenza infection, we could measure activation and numbers of influenza-specific B cells, their differentiation into plasma B cells, and the formation and maintenance of influenza-specific memory B cells. It will also be important to follow-up with study participants to determine whether BMI influences actual rates of laboratory-confirmed influenza in vaccinated

individuals, and in those who develop influenza infection, it will be interesting to assess severity, any complications that occur, and recovery period, to see if those are correlated to BMI as well. Collecting other demographic and health information from our participants, such as dietary and exercise data, would allow for more complex logistical analysis of factors that increase or decrease seroconversion or risk of infection following vaccination.

Regarding the cellular immune response to influenza, we utilized state of the art multi-color flow cytometry to assess intracellular and extracellular markers in dendritic cells, $CD4^+$ T cells, and $CD8^+$ T cells in human samples. Because wanted to gather information on three different cell types and because we used human samples, we were limited in the scope and breadth. If we were able to acquire larger PBMC samples from humans, such as from a blood donation of 50mL, or if we were to use a mouse model, we would be able to obtain more information from our samples. As such, it would interesting to more closely examine the subpopulations of PBMCs, in particular the different subsets of $CD4^+$ T cells, including T_H1, T_H2, and T_H17 $CD4^+$ T cells, as well as regulatory $CD4^+$ T cells. There are different subsets of dendritic cells as well and it would be important to look at numbers, marker expression, and function of myeloid dendritic cells and plasmacytoid dendritic cells.

In addition, as mentioned above, there are a number of mouse studies from our lab that show impaired memory T cell formation and maintenance with obesity. There are also data from the aging literature that show impairments and alterations in memory T cells,

however there are very little data on memory T cells in obese humans and no studies about influenza-specific memory T cells humans. If would be interesting to gather data on the subpopulations of both $CD4^+$ and $CD8^+$ T cells, including numbers, marker expression, and function of central memory T cells, which express both CCR7 and L-selectin, and effector memory T cells which express neither. In addition, levels of circulating cytokines vital for appropriate memory T cell formation and maintenance, including IL7 and IL15. It would be interesting to assess circulating levels of these cytokines, as well as IL7R and IL15R expression on the $CD4^+$ and $CD8^+$ T cells subsets, if possible at multiple timepoints following influenza infection.

Finally, we are just beginning to appreciate that obesity and type II diabetes can have differential and synergistic effects on immune cells and immune function. In addition, to continuing our work in T cell glucose metabolism, it will be important to examine the signaling pathways that are upregulated and downregulated in T cells at both at rest and after activation with obesity and type II diabetes. To examine such signaling pathways we need to look at activation of intermediates on a number of different signaling pathways. Although there are a number of different limitations when working with human cells, as mentioned above, it is still a valuable model to use for its immediate applicability to the human population. One method would be to activate T cells with influenza or another antigen or mitogen, followed by stimulation with insulin, in order to run Western blots to examine presence and activation of signaling intermediates, although the challenge would be obtaining enough protein for the blots. Another method would be to use flow cytometry

to examine presence and activation of signaling intermediates in a similar study, although this method would be limited by commercial availability of antibodies. It would be important to look at expression of the insulin receptor and of GLUT1 on the cell surface of T cells, as well as assessing protein levels of Akt, and phosphorylated Akt both at the Ser473 and the Thr308 positions, in addition, to other markers of activation and function in T cells. In addition, it would be interesting and important to understand how exercise impacts glucose metabolism in T cells, particularly in T cells from adults with type II diabetes. Studies have shown that exercise improves insulin sensitivity, translocation of GLUT4 to the cell surface in muscle tissue, and glucose uptake, even in the absence of any weight loss, so it would appealing to find out if exercise improved glucose uptake and utilization in adults with type II diabetes.

Conclusions

In summary, this dissertation presents compelling evidence that both overweight and obesity impair the humoral and cellular responses to influenza, as well as data that suggest that type II diabetes with obesity alters glucose metabolism in T cells. The dramatic increases in obesity and type II diabetes seem to be continuing, within the US and throughout the world. There are widespread outbreaks of influenza throughout the world each year, and with the increase in global travel, there higher potential for pandemics as well. The data generated from this dissertation provides important information about the mechanisms underlying the increased risk to influenza seen with higher BMIs and will

inform strategies to provide the most effective prevention and treatment for influenza for all individuals.

APPENDIX

CD8$^+$ T CELLS FROM OBESE SHOW IMPAIRMENTS IN ACTIVATION AND FUNCTIONAL MARKERS TO PH1N1, DESPITE INCREASED PBMC PROLIFERATION AT 30 DAYS POST-VACCINATION

Introduction

Reports indicate that obese adults have a greater risk of morbidity and mortality from infection with pH1N1 influenza A virus (6, 41, 275). The CDC recognized obesity as an independent risk factor for morbidity and mortality from influenza in 2009 (274). Vaccination is the most effective method for preventing influenza infection; however there are data to suggest that obesity may alter vaccine effectiveness as well. Vaccinations for hepatitis B and tetanus were found to be less protective in obese individuals (9, 10). Currently, there is little known about how obesity impacts the response to influenza virus infection or vaccination. We were interested in identifying some of the mechanisms that modulate the obesity-associated increase in morbidity and mortality to pH1N1. The research objective of this study was to examine the CD8$^+$ T cell response to pH1N1 influenza in relation to BMI and we hypothesized that obese individuals would display defective CD8$^+$ T cell responses to pH1N1 influenza compared to healthy weight individuals.

Materials and Methods

Participants were recruited as part of an ongoing, prospective observational study focusing on BMI and the response to influenza vaccination at the University of North Carolina Family Medicine Center, an academic outpatient primary care facility in Chapel Hill, NC. Eligible participants included adult patients (≥18 years of age) at the Family Medicine Center scheduled to receive the seasonal TIV. Exclusion criteria included immunosuppression, self-reported use of immunomodulator or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillian-Barre syndrome, or use of theophylline preparations or warfarin (278, 279). At enrollment, informed consent, height, weight, and baseline blood samples were obtained. Participants received one dose of 2010-2011 seasonal TIV [0.5mL Fluzone containing A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008], administered in the deltoid muscle. Participants returned 28-35 days later for a post-vaccination blood draw. PBMCs isolated from whole blood samples obtained from healthy weight (BMI 18.5-24.9) and obese (BMI > 30.0) adults 30 days post-vaccination were used for this study. Information regarding the two groups is detailed in Table A.1.

PBMCs were plated at 1 x 10^6 cells per well in serum-free AIM-V media supplemented with 1% penicillin/streptomycin and 1% glutamine in a 96-well plate. PBMCs were incubated with or without live pH1N1 virus for 72 or 120 hours. Tritium-labeled thymidine was added to some PBMC samples to measure pH1N1-specific proliferation.

Protein transport inhibitor was added to other PBMC samples for the last 6 hours of the incubation. PBMCs were stained with fluorochrome-conjugated antibodies CD3-APC.Cy7, CD8a-eFluor 450, CD69-PerCP, IFNγ-PE and GrB-FITC. All samples were analyzed with a Cyan ADP flow cytometer and analyzed using FlowJo software. Percentages of the different cell populations and expression of intracellular and extracellular proteins were assessed using the Wilcoxon signed rank test. All reported p-values are two-sided and the level of significance was set at 0.05.

Results

PBMCs, which include dendritic cells, lymphocytes, macrophages, and monocytes, were isolated from venous blood samples from volunteers using a Histopaque gradient and cryopreserved using the Mr. Frosty cryopreservation system. Populations of fresh and cryopreserved PBMCS, as well as stimulated and unstimulated PBMCs were compared using flow cytometry. As seen in Figure A.1, the T cell and the dendritic cell populations were almost completely recovered after the cryopreservation and thawing process. As such, we feel confident in the PBMC freezing and thawing protocols.

PBMCs from obese adults produced comparable numbers of $CD8^+$ T cells (Figure A.2 A), but fewer $CD8^+$ T cells expressing the early activation marker CD69 (Figure A.2 B) and fewer activated $CD8^+$ T cells expressing the functional proteins IFNγ (Figure A.2 C) and GrB, (Figure A.2 D) suggesting impairments in both activation and cytolytic function of $CD8^+$ T

cells. Activated $CD8^+$ T cells are necessary both for protection from and response to pH1N1 infection. IFNγ acts to suppress viral replication and to promote macrophage activation, antigen presentation, and B cell function. GrB acts to initiate apoptosis in infected target cells. In addition, proliferation stimulated by pH1N1 was shown to be intact and even increased in PBMCs from obese adults (Figure A.3), suggesting that although pH1N1 generates PBMC proliferation, it is not the $CD8^+$ T cell population that is increasing.

Discussion

The data indicate that 30 days after vaccination, there are obesity-associated defects in $CD8^+$ T cell activation and cytotoxic function, even though pH1N1-specific PBMC proliferation is intact. These data have wide-reaching implications for public health, particularly with the increase in obesity worldwide. In addition, obesity-associated deficiencies in the immune response to influenza likely play a role in the response to other infectious diseases as well. Different vaccination strategies may need to be considered for obese individuals. One strategy to overcome these obesity-associated deficiencies might be to provide a higher dose of vaccine, similar to the high-dose vaccine, which was tested in older individuals, with 4 times the amount of antigen. In addition, there is interest in creating a universal influenza vaccine, targeting T cells, which would provide protection against multiple strains of influenza virus over many years. However, our data suggest that in obese individuals, the T cell response to pH1N1 influenza is defective, so vaccines targeting T cells may not be as effective in obese individuals.

Tables and Figures

Table A.1 BMI and Age of Healthy Weight and Obese Groups

Group	N	Average BMI ± SD	BMI Range	Average Age ± SD	Age Range
Healthy	15	21.0 ± 1.3	18.5-22.6	48.4 ± 11.4	30-63
Obese	30	39.3 ± 6.0	31.1-52.4	48.4 ± 11.2	23-66

Figure A.1 Fresh, Cryopreserved, Stimulated, and Unstimulated PBMC Populations

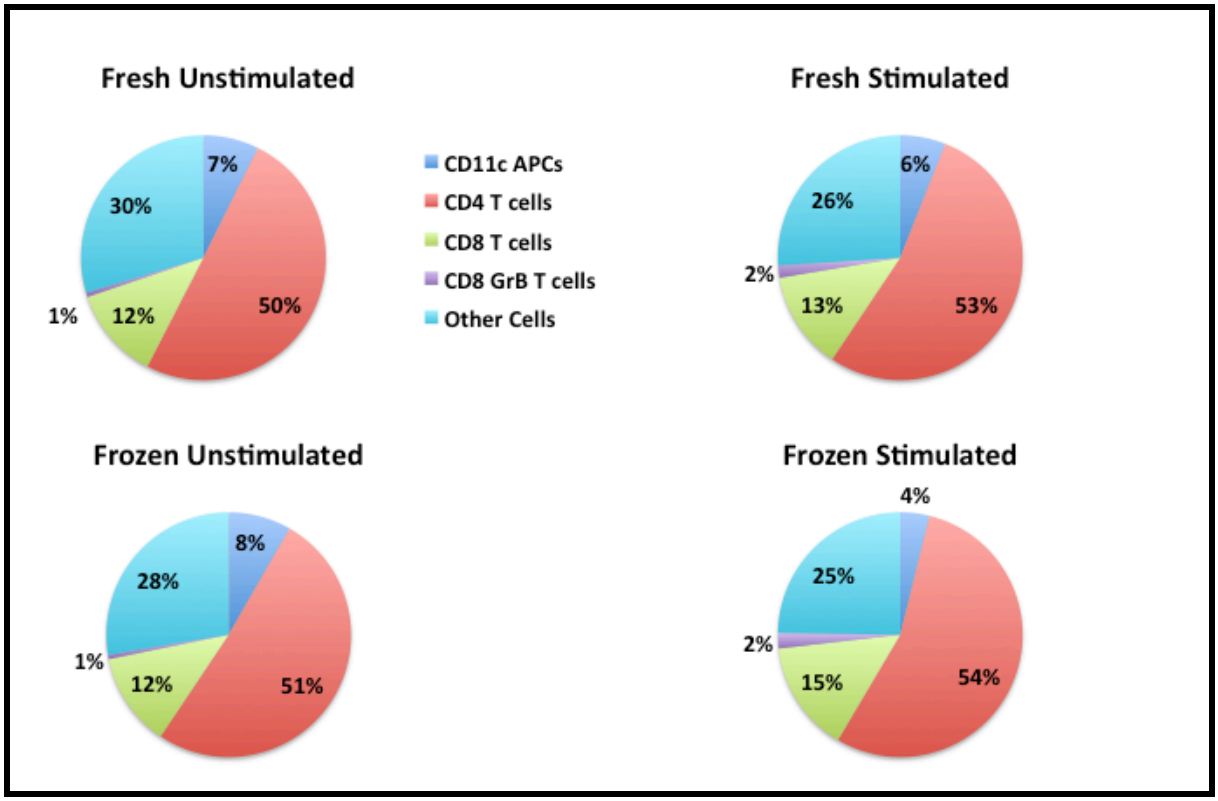


Figure A.1: Representative sample of PBMCs before and after cryopreservation and thawing. PBMCs were isolated from 10mL of blood, and one half of the sample was tested fresh and the other half was cryopreserved and thawed later for testing. Cells were unstimulated or stimulated with influenza vaccine, stained with CD3, CD4, CD8, CD11c, and GrB human antibodies, and analyzed by flow cytometry. Numbers represent percentage of total population.

Figure A.2 $CD3^+CD8^+$ T Cell Populations in Healthy Weight and Obese

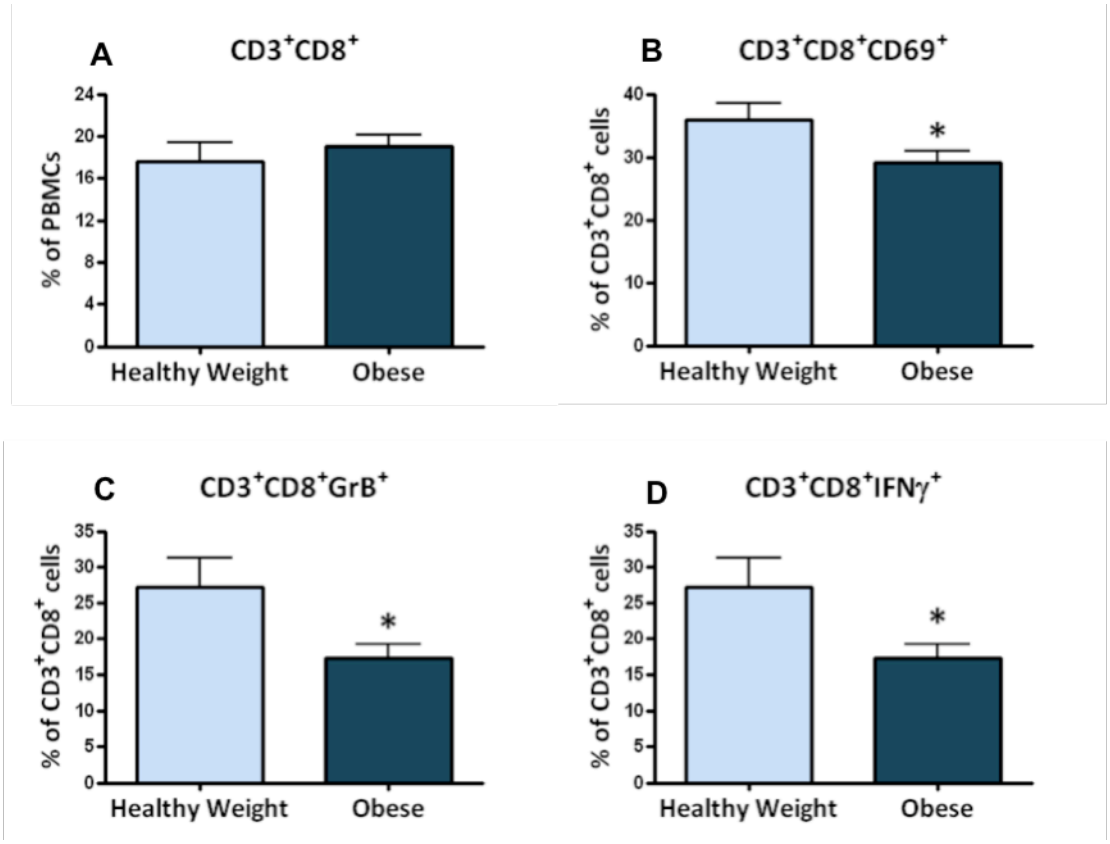


*Figure A.2: (A) $CD3^+CD8^+$ T cells as a percentage of total PBMCs (B) activated $CD3^+CD8^+CD69^+$ T cells as a percentage of total $CD3^+CD8^+$ T cells (C) $CD3^+CD8^+GrB^+$ T cells as a percentage of $CD3^+CD8^+$ T cells (D) $CD3^+CD8^+IFN\gamma^+$ T cells as a percentage of $CD3^+CD8^+$ T cells. Each bar represents the mean +/- SE from 15 healthy weight participants and 30 obese participants. Statistical significance is indicated by * $p< 0.05$.*

Figure A.3 PBMC Proliferation in Healthy Weight and Obese

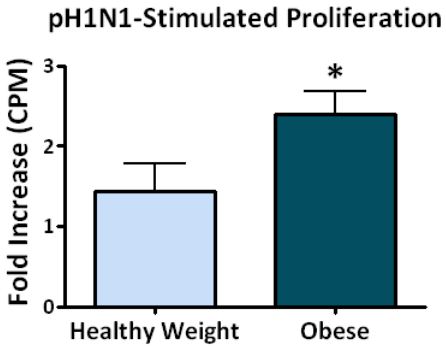


*Figure A.3: Fold increase between pair unstimulated and pH1N1-stimulated PBMCs. Each bar represents the mean +/- SE from 15 healthy weight participants and 30 obese participants. Statistical significance is indicated by * p< 0.05.*

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