METFORMIN IMPROVES $CD8^+$ MEMORY T LYMPHOCYTE FUNCTION IN OBESE INFLUENZA VACCINATED ADULTS

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ABSTRACT

Omar A. Rezk: Metformin improves $CD8^+$ Memory T lymphocyte function in obese influenza vaccinated adults

(Under the direction of Melinda Beck)

Obesity has reached epidemic proportions in the United States over the past few decades and now affects more than one-third of the adult population. In recent years, obesity has been linked to increased risk of morbidity and mortality from influenza infection. The influenza vaccine is the most effective way to prevent infection with the influenza virus, however, despite vaccination, obese individuals have twice the risk of influenza or influenza-like-illness. Obesity has been shown to impair T cell activation and function. What is not known is how obesity causes the dysfunction. Recent studies have demonstrated that T cell function is heavily reliant on cellular metabolism and T cells will switch their metabolic profile in response to extracellular stimuli. Inflamed visceral adiposity, caused by inflammatory cells infiltrating hypertrophic adipose tissue, is a major hallmark of obesity. Visceral adipocytes of obese individuals release high levels of leptin and other pro-inflammatory cytokines into the serum. These changes in the extracellular environment of T cells may influence their metabolic profile and in turn, impair their function. Therefore, we hypothesized that the metabolic drug metformin would improve T cell function. We show that, compared to obese adults, influenza-vaccinated obese adults prescribed metformin have improved CD8+ memory T cell responses to *in vitro* challenge with influenza virus. T cells of obese metformin-treated adults express higher levels of activation and functional markers. These results suggest that metformin may help to restore memory T cell function and act as an immune-enhancing agent for obese individuals at the time of vaccination. Further investigation into the metabolic effects of metformin on T cells is needed to uncover the mechanism(s) for metformin-driven improvements in memory T cell function.

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LIST OF ABBREVIATIONS AND SYMBOLS

Akt	Protein kinase B (serine/threonine protein kinase)
AMPK	Adenosine-mono-phosphate kinase
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BMI	Body mass index
CD4+	Cluster of differentiation 4 – helper T cell marker
CD8+	Cluster of differentiation 8 – cytotoxic T cell marker
ConA	Conacanavalin A
ECAR	Extracellular acidification rate (mpH/min)
Glut1	Glucose transporter 1
H1N1	hemagglutinin 1 neuraminidase 1
HAI	Hemagglutination inhibition assay
IFN-γ	Interferon gamma
IL-2	Interleukin-2 (pro-growth, proliferation cytokine)
IL-7	Interleukin-7 (naïve cell homeostasis cytokine)
IL-10	Interleukin-10 (anti-inflammatory, repressor cytokine)
IL-15	Interleukin-15 (memory cell homeostasis cytokine)
ILI	Influenza-like illness
MHCII	Major histocompatibility complex II ix
mTOR	Mammalian Target of Rapamycin
OCR	Oxygen consumption rate (pmoles/min)
OXPHOS	Oxidative phosphorylation

PBMC	Peripheral blood mononuclear cell
TCR	T cell receptor
T-reg	T regulatory cell
VAT	Visceral adipose tissue

CHAPTER I - BACKGROUND

Obesity

In the United States, and globally, rates of obesity have greatly increased. Obesity is defined using a measure called the body mass index (BMI), which is a numerical relationship between one's weight (kg) and height (m). BMI is reported in kilograms per meter squared (kg/m^2). Over one-third of the United States population is obese (BMI $\geq$ 30 kg/m^2) and over two-thirds are overweight or obese (BMI $\geq$ 25 kg/m^2).[1] The rise of obesity has become an economic strain on our health care system, with $190 billion (21% of total) in healthcare expenditures spent every year on obesity and obesity-related conditions.[2] The causes of this obesity epidemic are numerous and complex, and the consequences are likewise. Genetic, physiologic, environmental, psychological, social, and economic factors all contribute to the obesity epidemic in varying degrees.

The most common cause of obesity is the consumption of excess energy relative to energy expenditure.[3] Over the past few decades, the food environment has changed drastically in a way that promotes overeating. Energy dense foods are now both highly available and affordable. Whereas, in the past, food insecurity and poverty were associated with thinness and being underweight, because of the availability and cheapness of highly processed, calorie dense foods, poverty is often now associated with high levels of obesity.[3] In combination with over consumption of energy dense foods, physical activity has also decreased over the same time frame, with fewer than 50% of US adults engaging in the CDC recommended level of physical activity (30 minutes per day, at least 4 days per week).[3] Obesity has increased indiscriminately

across age, sex, and race, with alarming increases in childhood obesity, predisposing young people to numerous other health complications.[2]

While obesity is generally defined by excess body weight for height, it is more importantly associated with increased abdominal adiposity that can manifest metabolically.[2] Abdominal adiposity is thought to be primarily visceral, as opposed to sub-cutaneous fat, and is associated with metabolic dysregulation[2]. This excess visceral adiposity leads to systemic chronic low grade inflammation that originates from the inflamed adipose tissue. In response to excess lipid stores, visceral adipocytes release inflammatory mediators, such as interleukin-6 (IL-6) and TNF-α, that recruit pro-inflammatory immune cells and disrupt systemic metabolism through reducing insulin sensitivity.[4] Excess fat in visceral adipocytes also signals increased release of leptin, leading to hyperleptinima, concomitantly with reduced release of adiponectin, which has also been associated with a decrease in insulin sensitivity.[4]

Obesity is associated with many other chronic ailments such as type II diabetes (T2D), cardiovascular disease and metabolic syndrome.[5] Metabolic syndrome is the culmination of metabolically related risk factors for cardiovascular disease (CVD) risk and diabetes. Having three of the five following complications results in a diagnosis of metabolic syndrome: hyperglycemia, elevated blood pressure, elevated serum triglyceride levels, low high-density lipoprotein cholesterol levels, and obesity (particularly central adiposity).[6]

Obese individuals have also been found to be more prone to infections.[7,8] In a large retrospective study involving 22,666 consecutive patients undergoing a coronary artery bypass grafting surgery, subjects with a BMI greater than 34 kg/m^2 had 1.8 – 3.7 times the risk of postoperative infection than those of a lower BMI (26 – 26.9 kg/m^2).[8] Obesity has also been found to alter the immune response to acute infection. In a cross-sectional field study of 1129

nine-year-old school children, increased BMI was found to be positively associated with acute respiratory infection, where overweight children had twice the risk of infection.[8] A recent study was conducted looking broadly at respiratory tract infections and the role that obesity plays in their pathologies.[9] They recruited 1455 participants aged 18-70 and invited them to self-report incidents of upper and lower respiratory infections over the span of 18 months. Based on the diary entries collected and analyzed, obesity was found to be associated with both lower (OR: 2.02, 95% CI: 1.35-3.00) and upper (OR:1.55, 95% CI: 1.22-1.96) respiratory tract infections. Through these and other studies[8,10], it has become apparent that obesity contributes to a compromised immune system.

Obesity and immune function

The immune system functions as a highly complex system with many specialized cells and secreted proteins. Crucial to the health of the host is the ability of the immune system to correctly recognize and react to harmful, foreign pathogens. For example, in the case of the influenza A virus, an airborne pathogen that infects millions of people annually worldwide, an efficient immune response could prevent the host from ever experiencing symptoms of the infection. The influenza virus is comprised of a spherical protein that envelopes segmented single stranded viral RNA. The external area of the virus is studded with one of 16 possible hemagglutinin (HA) and 9 possible neuraminidase (NA) glycoprotein spikes, which are used to classify the virus and are often the target of influenza vaccines and antibodies.[11]

Influenza enters the cell by endocytosis following the binding of its hemagglutinin glycoprotein to the sialic acid receptor on the surface of host's cell. The virus releases its RNA into the nucleus of the cell where it is transcribed into mRNA and released into the cytosol to be translated into viral protein. Influenza virus exits the host cell by budding through the membrane.

HA and NA proteins produced by the host cell are associated with lipid rafts on the plasma membrane of host cell. The HA proteins alter the curvature of the membrane, allowing the viral RNA to be packaged inside the envelope proteins. The virus is therefore coated in the host's cellular lipid bilayer and envelope proteins in the lipid raft and buds out of the cell.[12]

When the pathogen first breaches the epithelial cells of the respiratory tract comes into contact with lung resident antigen presenting cells (APCs), consisting of macrophages and dendritic cells. These APCs will phagocytose the virus and process it into smaller protein segments called antigens. Viral antigens are then displayed, bound to MHC class I & II molecules on the outer membrane of APCs, to adaptive immune cells, such as B-cells and T cells. However, for APCs to reach most of the adaptive immune cells, they must migrate to secondary lymphoid tissues, such as lymph nodes or the spleen. Once there, APCs carrying viral antigens induce antibody production (humoral immunity), as well as cytotoxic T cell (CTL) and T helper (Th) cell responses (cellular immunity), initiating the primary adaptive immune response.[12]

Once stimulated adaptive immune cells proliferate. B-cells differentiate into antibody secreting plasma cells and T cells differentiate to have various effector functions (Figure 2). Helper T cells play a critical role in the adaptive immune response to influenza, helping activated B-cells to produce and secrete antibodies and secreting cytokines that prime macrophages to destroy ingested microbes and cytotoxic T cells to kill infected target cells. Both humoral and cellular immunity are important for recovery from acute infection and resistance to reinfection. After infection has cleared many of the antigen-specific adaptive immune cells lose effector function and undergo apoptosis. A small pool of memory B-cell and T cells remain in body, along with circulating influenza specific antibodies.[12]

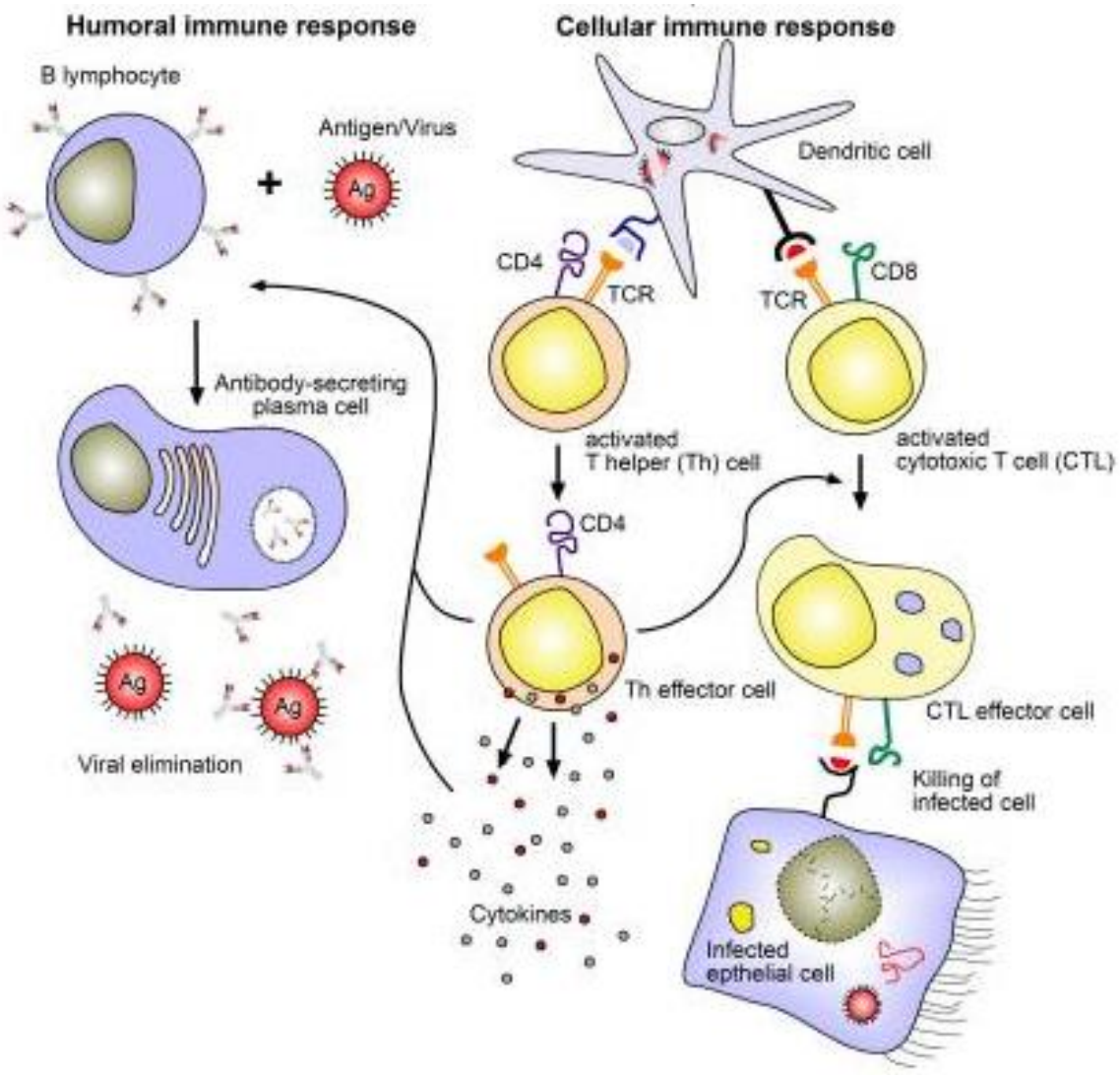

Figure 1. **The humoral and cellular immune response following infection with virus.** From Influenza Report, 2006.

In 2011, a study was published concerning the 2009 H1N1 influenza pandemic which established obesity, for the first time, as an independent risk factor for morbidity and mortality from infection with influenza virus.[1] The researchers analyzed public health surveillance data to assess the association between obesity and death or illness from H1N1 influenza infection in patients 20 years or older. Out of the 534 adults that were assessed in this study 274 (51%) were obese, having a BMI greater than or equal to 30 kg/m^2. This was 2.2 times greater than the

prevalence of obesity in the state of California which was 23% at that time, and was 1.5 times greater than the prevalence of obesity in the United States (33%). Out of the 534 subjects that were analyzed in this study 92 (17%) died, 56 (61%) of which were obese and 28 (30%) of which were extremely obese (BMI>40kg/m^2). Using multivariate analysis to control for other risk factors, such as asthma, age and immunosuppressive conditions, the researchers found that extreme obesity (BMI $\geq$ 40kg/m^2) was associated with death, having an odds ratio of 2.8 with a 95% confidence interval of 1.4-5.8.[1]

Protection from the flu and other viruses is of great public health importance. Influenza is a highly contagious respiratory tract infection. In 1918-19, a deadly pandemic strain of the influenza virus, known as the Spanish Flu, resulted in approximately 21 million deaths worldwide.[13] The recent 2009 influenza pandemic was responsible for 151,700 to 575,400 deaths worldwide.[13] Pandemic flu refers to the introduction of a novel viral strain through antigenic shift due to the combination of multiple influenza viruses. When the resulting strain is dramatically different from the preceding strain, large proportions of the population will not have previously developed immunity against it.[13] These strains are often introduced via zoonotic transfer from animals, such as pigs and birds. This genetic shift dramatically increases the risk of morbidity and mortality from influenza infection.

Besides pandemic strains of influenza, seasonal influenza remains a serious public health threat due to constant changes in protein structure through antigenic drift each year. Seasonal influenza affects all populations because of its relative ease of transmission via droplets from infected persons coughing, sneezing or talking. Typically, in the United States, influenza circulation occurs most prominently from October to May every year. The virus has been known to be especially harmful to very young children, the elderly, pregnant women and persons with

specific chronic medical conditions such as immunodeficiency diseases, asthma and chronic heart or lung diseases and more recently obesity.[1] The flu vaccine is still the single most effective method for protecting against the pathogen.

Obesity and influenza vaccination

Influenza vaccines commonly contain three or four inactivated forms of influenza A and B strains, making the vaccine either trivalent or quadrivalent. The influenza vaccination involves an intramuscular injection that changes from year to year in anticipation of circulating viral strains.[14] The WHO, based on its continuous surveillance, identifies antigenic variants of the influenza virus and analyzes them for inclusion into the yearly vaccine. The selected strains of the virus are then grown in embryonated chicken eggs, extracted from the eggs' allantonic fluid, inactivated, split, and combined to form the final administered vaccine. Once administered, the vaccine stimulates immunological memory development, without the threat of serious symptoms; thereby priming the immune system for a rapid, specific immune response, should exposure to influenza occur.

Vaccination induced immunological memory responses produces a similar immune memory response as previously described following primary infection with influenza. Following vaccination, if re-exposure to the virus, vaccine induced-memory B-cells and T cells elicit a secondary response. Secondary immune responses happen more quickly (within 3-4 days) and are more effective than the primary response. B cells rapidly produce larger amounts of antibody, specific to the virus, called immunoglobulins (primarily IgG), and CD4+ and CD8+ T cells respond by secreting pro-inflammatory cytokines and by targeting and killing infected cells.

In 2007, Smith et al. found that, following infection with influenza virus, diet-induced obese mice had increased mortality, increased lung pathology, and altered immune responses.[15]

Obesity was found to inhibit the ability of the immune system to appropriately respond to influenza infection by reducing expression of antiviral cytokines and reducing natural killer (NK) cell cytotoxicity.[15] NK cells are important innate immune cells that target and kill infected cells by inducing apoptosis. In 2012, Sheridan et al. found that obese adults successfully produced an antibody response to the influenza vaccine 30 days following vaccination.[16] These findings suggest that the B-cells of obese individuals respond properly to the influenza vaccine. In a later study published in 2014 by Sheridan et al., this finding was confirmed.[17] However, one year post vaccination, obese individuals were found to have a significantly lower antibody response compared to lean vaccinated individuals.[16]

In 2017, a study by Neidich et al. demonstrates that flu-vaccinated obese adults were less protected from influenza.[18] Despite vaccination and an antibody response that was not statistically different from the antibody response of lean adults, vaccinated obese individuals had twice the risk of influenza or influenza-like-illness.[18] The antibody response to influenza is characterized using two terms, seroconversion and seroprotection. Seroconversion refers to a 4-fold increase in antibody concentration in the serum (titer) and is associated with a 49% reduction in risk of influenza infection. Seroprotection refers to the subject having an antibody titer that is 40 hemagglutination inhibition (HAI) units or higher. Neither seroconversion nor seroprotection rates were statistically different between healthy weight and obese adults, suggesting the mechanism for increased risk of influenza and influenza-like-illness in obese subjects may be due to poor T cell function.

T cells are known to play an important role in the adaptive immune response against influenza. $CD4^+$ T helper cells are especially important in limiting disease severity by helping to recruit and activate macrophages and cytotoxic CD8+ T cells that destroy infected cells and

assist with viral clearance.[19] In a mouse model, diet-induced obesity was found to impair T cell memory response to the influenza virus.[20] In this study 25% of diet-induced obese mice experienced mortality from an influenza infection, while none of their lean counter parts died from the infection. Obese mice were also found to have one third the number of influenza-specific CD8+ T cells producing IFN-γ and mRNA expression for IFN-γ was 60% less in the lungs of obese mice as compared to lean infected mice.[20] Further studies into the effects of obesity on influenza immunity found that CD4+ and CD8+ T cells of vaccinated obese individuals were defective. T cells of obese individuals expressed lower levels of activation markers (CD28 and CD69) and lower levels of functional markers (IFN-γ and GranB), as compared to lean individuals, in response to ex vivo challenge with live H1N1 virus.[21] This poses an important question that has yet to be answered in full, why do T cells not function properly in obese individuals?

T cell metabolism and obesity

In a study done to characterize the role of T cells in obesity related inflammation, visceral fat from diet induced obese mice was found to contain a higher frequency of CD8+ IFN-γ and Granzyme B secreting T cells than visceral fat from lean mice.[22] Granzyme B is an enzyme secreted by CD8+ cytotoxic T lymphocytes that signals apoptotic cell death in target cells. Levels of granzyme B and IFN-γ are naturally elevated in activated effector T cells in response to infection. In the obesogenic state, similar T cell characteristics are observed in the absence of infection. Consistent with this finding, Yang et al. demonstrates that diet induced obese mice have a reduction in peripheral naive T cells with increased frequency of effector and memory cells when compared to their lean counterparts.[7] In a healthy lean individual the frequency of naïve T cells surveilling the periphery is higher compared to the frequency of effector/memory T

cells.[22] These finding show that in the inflamed adipose environment, T cells are phenotypically different, expressing some of the traits of activated effector T cells and disconcertingly becoming pro-inflammatory without the stimulus of infection. To understand how this occurs, T cell metabolism must be considered.

T cell functional demands are matched with a corresponding metabolic program (Figure 1). The two main metabolic processes used by the cell to generate energy (ATP) are glycolysis and oxidative phosphorylation. Glycolysis is the cytosolic breakdown of glucose into two molecules of pyruvate, generating ATP in the process. Pyruvate generated from glycolysis can continue to be metabolized in the cytosol into lactate using the enzyme LDH (lactate dehydrogenase). In the presence of oxygen, this process is called aerobic glycolysis. Alternatively, pyruvate can be oxidized in the TCA cycle (tricarboxylic acid cycle), generating NAD to be used in the electron transport to generate ATP. Oxidative phosphorylation is a metabolic process that involves the conversion of pyruvate, derived from glucose and amino acids (AAs), as well as fatty acids into acetyl-CoA. Acetyl-CoA is further degraded into carbon dioxide in the TCA cycle in the mitochondria. Degradation of acetyl-CoA yields free electrons which are carried to the electron transport chain on electron carriers, NADH and $FADH_2$. The electron carriers transfer their electrons to the complexes in the electron transport chain, resulting in the movement of protons out of the mitochondrial matrix. The electrochemical potential created by the process fuels the production of ATP in the presence of oxygen.

These metabolic processes are regulated mainly by two important pleiotropic metabolic regulators, mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). These cellular nutrition sensors respond to conditions inside the cell and are influenced by the extracellular environment as well. In T cells, mTOR is activated by phosphorylation with Akt

through a wide range of immunological and metabolic cues.[23] TCR engagement along with costimulation with CD28 activates mTOR through the Akt pathway. Signaling from other molecules such as IL-6, TNF-α, the adipokine, leptin, activates mTOR as well. Activation of mTOR leads to a pro-growth phenotype in the presence of high energy signals. It regulates the transcription of enzymes involved in glucose metabolism, increases protein translation and increases lipid synthesis[24]. It also increases GLUT1 expression on the surface of T cells, increases rates of aerobic glycolysis, and promotes T cell effector differentiation. Some studies have shown that treatment of T cells with rapamycin, an inhibitor of mTOR, prevents T-eff (CD4+ effector T cell) growth and proliferation, providing evidence that mTOR acts to regulate both the metabolism and functional fate of T cells.[24]

Working against mTOR is the complex AMPK. Whereas mTOR promotes a more glycolytic and pro-growth phenotype in the presence of high energy metabolites, AMPK maximizes energy production by catabolic pathways.[24] AMPK is activated by low energy signals in the cell. High levels of AMP (adenosine monophosphate) relative to ATP promotes the phosphorylation and activation of AMPK, leading to increased levels of fatty acid oxidation in T cells through promotion of CPT-1 and inhibition large molecule synthesis. AMPK is also required for T-reg (CD4+ Regulatory T cell) function.[25]

Systemic metabolism can also influence T cell function. Circulating leptin and adiponectin, which are adipokine (cytokines secreted by adipocytes), can greatly enhance or suppress T cell function. Leptin and its receptor have structural and functional similarities to the IL-6 family of cytokines, which are known pro-inflammatory cytokines.[26] Leptin is also reported to increase IFN-γ and IL-2 production in activated T cells. In genetically leptin deficient (ob/ob) mouse models as well as in leptin deficient humans, researchers found reduced T cell numbers

and T cell effector function.[25] Of note, treatment of fasted mice with leptin restored T cell proliferation and inflammatory cytokine production, both *in vivo* to fasted animals and *in vitro* to T cells isolated from fasted mice.[25] Leptin is also required to upregulate the glucose transporter Glut1, allowing metabolic reprograming of activated effector T cells and fueling T cell proliferation and cytokine production.[27] These studies and others have confirmed that leptin is required for proper function of activated T cells and plays an important role in the metabolism of the T cell.

Adiponectin works inversely of leptin on T cells. While the role of adiponectin on T cell metabolism is not clear, adiponectin is known to be strongly immunosuppressive.[8] T cells store adiponectin receptors intracellularly and express adiponectin receptors on the cell surface following stimulation.[25] Adiponectin signaling in T cells helps to control the immune response, leading to decreased cytokine production, decreased proliferation, and apoptosis post antigen stimulation.[28]

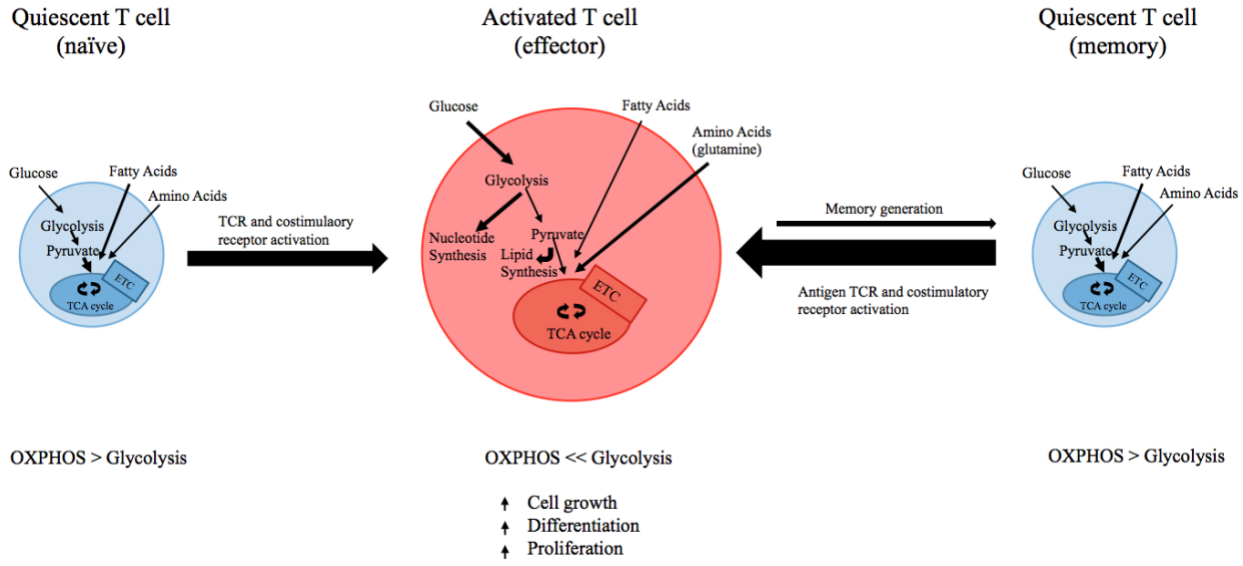

Figure 2. **T-cell metabolism regulates T-cell function**. The resting or memory T cell harvest the majority of its energy using oxidative phosphorylation (left). Activated T cells (right) rely heavily on aerobic glycolysis for energy production. This metabolic reprogramming corresponds with distinct functional demands. From William D. Greene (2017).

For resting naïve and memory T cells the main source of energy is oxidative phosphorylation. The T cell's preference is to funnel glucose through the TCA cycle, oxidizing it and other fuel sources (amino acids and lipids) for more efficient energy production. Oxidative phosphorylation results in the production of large amounts of ATP, which are used to satisfy the high energetic cost of cellular maintenance. Naïve and memory T cells, in their resting state, can exist in secondary lymphoid organs or in circulation for long periods of time until they are activated and as they are not proliferative, they have a low demand for biosynthetic molecules.[29] To maintain a steady state of energy production and self-maintenance, resting T cells rely on a balance between cell survival cytokines such as IL-4, IL-7 and IL-15 and other hormonal signals, like adiponectin.[30] These signals can either be autocrine, paracrine, or endocrine. IL-4 is an autocrine signal that is produced and used by T cells to promote cell survival and size maintenance.[30] IL-7 constantly circulates in the serum and is produced by stromal cells in the bone marrow as well as epithelial cells. Upon ligation with its receptor on T cells, IL-7 promotes homeostasis and survival through activation of the PI3K/Akt/mTOR pathway[30] as well as supporting catabolic metabolism, by promoting mitochondrial biogenesis and fatty acid oxidation.[31] The reliance on these external signals for energy is so great that even if naïve T cells are cultured with high levels of extracellular nutrients, in the absence of IL-4 or IL-7, they will die.[29] Adiponectin also plays an important anti-inflammatory role in the regulation of T cells. In lean states adipocytes produce high amounts of adiponectin and in obese states circulating adiponectin levels are low.[10]

Upon activation, the T cells undergo a metabolic transformation, switching, primarily, to aerobic glycolysis for energy production.[31] T cells require two signals to become activated,

ligation of their T cell receptor (TCR) and subsequent ligation of their costimulatory CD28 molecules. Antigen presenting cells (APCs) expressing MHC molecules on their surface interact with the TCR. When MHC molecules on APCs are carrying antigenic proteins that the TCR can bind to, the TCR becomes engaged and initiates the activation signal. Then, costimulatory molecules on T cells, such as CD28, bind to their ligand on the APC, completing the signal. Following activation, functionality of the T cell shifts from self-maintenance to effector function, cytokine production and proliferation.[24] Because of these new functional demands, T cells need to produce biosynthetic precursors for cell growth and proliferation. T cells start to utilize amino acids for protein synthesis and fatty acids for membrane synthesis rather than for energy production. To keep up with these demands, the metabolic program of the T cell shifts from a primarily oxidative state to a more glycolytic state, where aerobic glycolysis is now a major source of ATP production.

T cells exploit three main signaling pathways, including PKC, AP-1, and NFkB to transduce the stimulatory signal, resulting in activation of the cell's effector functions and production of pro-inflammatory cytokines, like IFN-γ.[32] These important signaling pathways converge on both metabolic and inflammatory pathways and influence functional outcomes.[32] The costimulatory molecule CD28 has a particularly strong convergence with growth factor signaling in T cells. CD28 ligation enhances the PI3k/Akt/mTOR pathway, which is also a direct target of insulin signaling.[31] Activating mTOR promotes a pro-growth phenotype in T cells, enhancing cell survival, growth, and proliferation, which is necessary for activated T cells to protect against infection. After activation the clonal population of effector T cells increases dramatically and T cells rapidly induce glycolysis.[29] The glycolytic pathway is fed by up-regulation of GLUT1 surface expression, which it mediated by mTOR activation.[25] The influx of

glucose into the T cell, along with antigen stimulation, signals the induction of glycolytic enzymes such as lactate dehydrogenase (LDHA), hexokinase (HK) and phosphofructokinase (PFKFB3).[25,33]

Through these processes, it has been shown that changes in systemic and cellular metabolism are immediately linked to T cell activation and function.[24] The presence of increased circulating nutrients and leptin in the obesogenic state may be promoting the activation of mTOR in resting T cells, similar to the aberrant activation of T cells around inflamed adipocytes.[34] Activation of mTOR shifts the cells to become more glycolytic, similar to the metabolic program of an activated T cell. We have preliminary evidence suggesting that T cells from obese individuals are more glycolytic than those of lean individuals. The presence of excess nutrients in the blood and in the adipose tissue of obese individuals, along with the pro-growth and pro-inflammatory molecules, results in the aberrant activation of immune cells.[34] This means that T cells that would naturally be resting under normal conditions are instead in a pro-inflammatory state, even without being activated by antigen stimulation. Altering T cell metabolism to maintain a constant pro-growth phenotype could be responsible for immunological dysfunction in response to viral infection. There is sufficient evidence that the metabolic dysfunction associated with obesity is the cause of a reduced T cell response in obese individuals. However, the question still remains: how can the T cell response against influenza virus be improved in obese individuals?

Metformin

Metformin is an important drug treatment for T2D and has been for decades. It is recommended by the ADA (American Diabetic Association) as the first line of defense for newly diagnosed type 2 diabetic patients. Metformin reduces blood sugar levels in hyperglycemic

patients by decreasing hepatic glucose production and increasing peripheral glucose uptake. Metformin acts by activating AMPK in a range of different cell types in the body including - hepatocytes, skeletal muscle cells, endothelial cells, pancreatic beta cells, peripheral blood mononuclear cells (PBMCs) and platelets.[35] Mechanistically, metformin is known to inhibit complex I of the electron transport chain in the mitochondria, thereby increasing cytosolic levels of AMP and activating AMPK.[35] As previously described, AMPK has a host of cellular metabolic effects, including inhibition of mTOR.

Metformin has been considered for its cardio-protective effects and for its role in cancer treatments. More recently, the effects of metformin on immune cell metabolism and function have been studied. Macrophages have been found to be directly affected by metformin.[36] In-vitro culture of macrophages from obese mice with metformin was found to increase levels of p-AMPK and reduce production of inflammatory cytokines.[36] Diaz et al. found that in-vivo and in-vitro use of metformin was associated with increased levels of p-AMPK in B-cells and higher antibody titers.[37]

Metformin has also been shown to act directly on T cells to activate AMPK in both $CD4^+$ and $CD8^+$ T cells.[38,39] Administration of metformin in a murine asthma model was found to increase $CD4^+$ T-reg numbers and decrease numbers of $CD4^+$ T-effector cells. T-regs were found to have higher expression of p-AMPK.[38] Lipid oxidation, as a result of AMPK activation, was upregulated in T-regs of metformin treated mice. T-effector cells are selectively inhibited by increased lipid oxidation while T-regs require lipid oxidation for function. These data suggest that activation of AMPK with metformin can change the fate of T cell populations through alterations in T cell metabolism.

Metformin has also been shown to enhance CD8+ memory T cells in mice.[39] Pearce et al. found that murine T cells cultured with metformin in the absence of IL-2 were found to have elevated levels of beta-oxidation.[39] The study also demonstrates that *in vivo* metformin administration increased AMPK activation and enhanced $CD8^+$ T cell memory formation in response to an experimental cancer vaccine. The mechanism by which this improved vaccine response occurred is not entirely clear, but the study suggests that regulation of fatty acid oxidation is partly responsible for the improvement in memory T cell formation. Consistent with this finding, inhibiting mTOR, an inhibitory target of AMPK, during the memory formation phase improved the functional qualities of memory T cells.[40] These data are important because the goal of vaccines is to generate $CD8^+$ memory T cells that can quickly respond to infection.

It is important to note that Zarrouk et al. found that metformin plays a role in the metabolic reprogramming of activated T cells but can do so in an AMPK independent manor.[41] This is not the first report of metformin acting to inhibit mTOR independent of AMPK signaling.[42] Zarrouk et al. reported that metformin treated T cells had increased levels of AMPK but suggests that metformin can act on the T cell independent of AMPK. Isolated T cells from T cell specific deletion of AMPK knockout mice ($AMPK\alpha1^{null}$) and wild-type mice were treated with metformin. T cells from knockout mice and wild-type mice were both found to exhibit lower levels of mTOR activity, suggesting that metformin's action on mTOR can be independent of AMPK activation. Metformin-treated, antigen receptor-activated T cells also showed reduced glucose uptake compared to control antigen-activated cells.[41]

Given how T cell metabolism drives T cell function, metformin, through its activation of AMPK and inhibition of mTOR, may influence T cell metabolism, and therefore restore T cell

function. **My hypothesize is that metformin will improve the memory T cell response and antibody response to the influenza virus in obese vaccinated individuals.**

CHAPTER II – JOURNAL MANUSCRIPT[1]

Overview

Obesity has reached epidemic proportions in the United States over the past few decades and now affects more than one-third of the adult population. In recent years, obesity has been linked to increased risk of morbidity and mortality from influenza infection. The influenza vaccine is the most effective way to prevent infection with the influenza virus, however, despite vaccination, obese individuals have twice the risk of influenza or influenza-like-illness. Obesity has been shown to impair T cell activation and function. What is not known is how obesity causes the dysfunction. Recent studies have demonstrated that T cell function is heavily reliant on cellular metabolism and T cells will switch their metabolic profile in response to extracellular stimuli. Inflamed visceral adiposity, caused by inflammatory cells infiltrating hypertrophic adipose tissue, is a major hallmark of obesity. Visceral adipocytes of obese individuals release high levels of leptin and other pro-inflammatory cytokines into the serum. These changes in the extracellular environment of T cells may influence their metabolic profile and in turn, impair their function. Therefore, we hypothesized that the metabolic drug metformin would improve T cell function. We show that, compared to obese adults, influenza-vaccinated obese adults prescribed metformin have improved CD8+ memory T cell responses to *in vitro* challenge with influenza virus. T cells of obese metformin-treated adults express higher levels of activation and functional markers. These results suggest that metformin may help to restore memory T cell

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function and act as an immunoenhancing agent for obese individuals at the time of vaccination. Further investigation into the metabolic effects of metformin on T cells is needed to uncover the mechanism(s) for metformin-driven improvements in memory T cell function.

Introduction

Obesity is now of epidemic proportions in the United States, reaching a prevalence of greater than 36.5% in adults and greater than 17% in children. The causes of obesity are numerous and complex and its consequences are likewise. Most commonly associated with having a BMI greater than 30kg/m^2 is increased visceral adiposity that can manifest metabolically and cause low grade chronic inflammation.[4] Obesity is associated with many chronic conditions such as type II diabetes (T2D), cardiovascular disease and metabolic syndrome.[5] Obesity has also been associated with increased risk of postoperative infection, acute respiratory infection, and influenza infection.[1,8,9]

In 2011, a study was published concerning the 2009 H1N1 influenza pandemic which established obesity, for the first time, as an independent risk factor for increased morbidity and mortality from infection with influenza virus.[1] This finding was of great public health concern, because over two thirds of the U.S. population is overweight or obese, because seasonal influenza remains a public health threat.[43] The influenza vaccine is the single most effective method for protecting against infection with the pathogen. The vaccine stimulates immunological memory development, priming the immune system for a rapid, specific immune response, should exposure to influenza occur.

However, in 2012, Sheridan et al. found that obese adults successfully produced an antibody response to the influenza vaccine 30 days following vacination.[16] These findings

suggest that the B-cells of obese individuals respond properly to the influenza vaccine, effectively producing antibodies against the influenza virus. However, at one year post vaccination, obese adults had a steeper decline in antibody response compared to lean adults.[16] In 2017, a study by Neidich et al. demonstrated that despite vaccination, obese individuals had twice the risk of influenza or influenza-like-illness.[18] The influenza-specific antibody response of obese adults was not statistically different from the response of lean adults, suggesting the mechanism for increased risk of influenza and influenza-like-illness in obese subjects may be, instead, due to poor T cell function.[18]

Further studies into the effects of obesity on influenza confirmed that T cells of obese mice and humans are impaired.[19–21] Of note, CD4+ and CD8+ T cells of obese individuals expressed lower levels of activation markers (CD28 and CD69) and lower levels of functional markers (IFN-γ and GranB), as compared to lean individuals, in response to ex vivo challenge with live H1N1 virus.[21] These findings and others lead to further investigation of T cell activation and function.

T cell immunological demands are matched with a corresponding metabolic program.[31] These metabolic processes are regulated mainly by two important pleiotropic metabolic regulators, mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). For resting naïve and memory T cells the main source of energy production is oxidative phosphorylation. However, upon activation, the T cell undergoes a metabolic transformation, switching, primarily, to aerobic glycolysis for energy production.[31]

Well understood was the classical T cell activation through TCR engagement and costimulation with CD28 but more recently we have started to understand that T cells respond to other extracellular cues, including nutritional and metabolic signals.[25] Leptin and adiponectin are

directly associated with T cell inflammatory status.[8,25,28] Obesity is associated with high levels of leptin, free fatty acids, IL-6, and TNF-α which are known modulators of pro-inflammatory pathways in T cells.[25] These signals overlap on metabolic pathways as well, influencing the activation, differentiation and function of T cells.[32] The target for these pro-inflammatory signals is mTOR activation, which leads to increased anabolic metabolism, namely glycolytic pathways.

AMPK is a known inhibitor of mTOR and upregulates oxidation in T cells.[31] Metformin is a metabolic drug that is most widely prescribed for type II diabetics, to help reduce blood glucose levels through activation of AMPK. Metformin has been studied in multiple immune cells, including B-cells, T cells and macrophages and has been shown to have anti-inflammatory effects.[36,37] Metformin acts directly on T cells to activate AMPK and inhibit mTOR.[39,41] Metformin has also been shown to enhance CD8+ memory T cell formation in mice post vaccination and improve B-cell function in obese individuals.[39]

These recent findings suggest that metformin could have a therapeutic role in protecting against influenza infection through modulation of T cell metabolism. This study examines the effects of metformin on T cell activation and function, as well as antibody levels, in vaccinated obese individuals. **We hypothesized that metformin will improve the memory T cell response and antibody response to the influenza virus in obese vaccinated individuals.**

Methods & Materials

Study Design & Subjects

Study participants were chosen from a large clinical research study carried out at the University of North Carolina at Chapel Hill Family Medicine Center. All subjects were adult patients (≥18 years of age) who received the annual trivalent inactivated influenza vaccine (TIV). Participants were excluded on the basis of immunosuppression, self-reported use of

immunosuppressive drugs, acute febrile illness, history of hypersensitivity of any influenza vaccine components, history of Guillian-Barre syndrome, or use of theophylline preparations or warfarin.[21] All procedures were approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill.

At enrollment, informed consent, demographic characteristics, height, weight, and a blood sample were obtained from each participant. Additional relevant information was obtained, with consent, from participants' online medical records. A single dose of the TIV was given to each participant via intramuscular injection and a blood draw was obtained during the day of vaccination and again 30 days post vaccination. Blood was processed into serum and PBMCs as previously described.[16]

A total of 3,304 participants were enrolled between 2009-2017. A group of 77 participants, comprised of 40 obese non-diabetic (OB) subjects and 37 obese metformin-treated diabetic (OB+MET) subjects, were drawn from the last 3 years of the larger study (2014-2017). All samples are under the age of 60 years. Race was dichotomized into African American or Caucasian. PBMCs and serum samples obtained 30 days post vaccination were analyzed in this study. Demographics of each subject are included in Table 1 and Table 2.

Cell Isolations & Stimulation.

Frozen PBMC samples were taken from the -80°C liquid nitrogen tanks and thawed in a 37°C water bath. Cells were washed, then plated in a 96 well flat bottom tissue-culture treated plate. Cells were cultured for 66 hours under three conditions, unstimulated, stimulated with 20µL of stock H1N1 virus in media, or with 1µg of ConA in media at 37°C in 5% CO_2. Cells were cultured for an additional 6 hours in media containing GolgiPlug (BD Biosciences).

PBMC Staining and Flow Cytometry

PBMCs were stained with five fluorochrome-conjugated human antibodies: anti-CD3 (FTIC), anti-CD4 (V450), anti-CD8a (APC), anti-CD69 (BV786), and anti-GranzymeB (PE). PBMCs were also stained with a viability dye, Zombie-Yellow (Biolegend). Extracellular staining was done using a FACS buffer (2% FBS). Permeablization was done using CtyoFix/CytoPerm (BD Bioscience) and intracellular staining was done using PermWash (BD Bioscience) buffer. Sample data was acquired using the AttuneNxT flow cytometer and were analyzed using FlowJo v10. The gating strategy is provided in Figure 3.

Influenza Specific Antibody Determination

The HAI titers were determined for all subjects using the WHOs (World Health Organization) guidelines and procedures.[44] The HAI assay is an extremely reliable test and is the WHO test of choice for influenza surveillance.

Identification of Intracellular Protein: Western Blot

Intracellular protein levels of p-S6 S235/236, p-AMPK T172, and AMPK were determined using western blot assay. Whole PBMC pellets were lysed on ice for 20 min in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 0.5% Na deoxycholate, 0.1% SDS, 0.1%NP40) containing SPP, BGP, Na3VO4(phosphatase inhibitors), and Halt (protease inhibitor cocktail).

Protein content was determined by Bradford assay. Protein (30ug) was suspended in Laemmli buffer, and boiled for 5 minutes then run out on a 4-16% gradient SDS-PAGE gel. Followed by transferr to nitrocellulose membrane in Towbin buffer (25mM Tris, 192mM Glycine pH 8.3 20% methanol), with ponceau S solution for 1 min.

Blots were washed 3 times or 5min in TBST (150mM NaCl, 50mM Tris pH7.2 .1%tween20), blocked with 5% BSA/TBST at room temp for 1 hour, then washed 3 times for 5min TBST.

Blots were the incubated at 4C overnight in specific antibodies at 1:1000 dilution in 5% BSA/TBST. Blots were then washed 3 times for 5min TBST, followed by incubation with 1:10000 dilution of goat anti rabbit/mouse (licor [P/N 925-32211], [P/N 925-68070]) 5%BSA/TBST at room temp for 1 hour. Protein was detected using LI-COR Odyssey imaging system. All blots were run in technical quadruplicates and densitometry was determined using image studio lite software.

Statistical Analysis.

Data presented were analyzed using Two-way ANOVA with Sidak's multiple comparisons test and parametric unpaired T-tests on Graphpad Prism v7.

Results

OB and OB+MET Group show no significant differences in the number of CD4+ or CD8+ T cells. The number of CD4+ and CD8+ T cells in both groups were not significantly different in unstimulated cultures and remained the same upon stimulation. Data not shown.

CD4+ T cells from OB and OB+MET subjects show no significant differences in expression of activation marker CD69. CD69 expression is indicative of activation status, where T cells that are activated will express higher levels of CD69. CD4+ T cells from the OB and OB+MET group did not express significantly different levels of CD69. However, a trend was observed in the difference between the OB and OB+MET group in the number ($p=0.0975$) and percent ($p=0.0728$) of CD4+ T cells expressing CD69. Individual subjects in the OB+MET group expressed the highest levels of CD69 in the unstimulated influenza stimulated cultures.

When comparing the median fluorescence intensity (MFI) (Figure 4), there is no significant difference between the OB and the OB+MET group (p=0.8523).

CD4+ T cells of OB subjects have a significantly greater fold change in expression of CD69. The fold change was calculated by dividing the percent of CD4+CD69+ T cell in the unstimulated cultures by the percent of CD4+CD69+ T cells in the influenza stimulated culture for each group. The fold change of the percent of CD4+CD69+ T cells from unstimulated to influenza stimulated in the OB group is significantly greater than the fold change seen in the OB+MET group (p=0.0204) as well as with the fold change in numbers (p=0.0256) (Figure 5).

The number and frequency of CD8+GranB+ T cells is not influenced by metformin treatment. Granzyme B is an enzyme produced by T cells that signals programmed cell death in infected cells. The number of CD8+GranB+ T cells in the OB+MET group was not significantly increased compared to the OB group in both the unstimulated (p=0.1077) and influenza stimulated (p=0.0503) condition. Although OB+MET subjects had a higher percent of CD8+ T cells that expressed GranzymeB in both the unstimulated (p=0.3874) and influenza stimulated (p=0.3353) conditions, this finding is not significant (Figure 6).

CD8+ Memory T cells from obese metformin-treated diabetic adults express a higher frequency of CD69+GranB+ T cells than obese non-diabetic adults. In response to exposure with live H1N1 A/California/7/2009 virus, obese metformin-treated diabetic adults had significantly higher percentages of CD8+ T cells expressing both the activation marker (CD69) and the functional marker (GranB). The percent of CD8+CD69+ T cells in the OB+MET was not

significantly different as compared to the OB group (p=0.2421). The unstimulated cultures showed no difference in the percent of CD8+CD69+ or CD8+CD69+GranB+ T cells (Figure 7).

Influenza specific antibody concentrations post-vaccination trend higher in the metformin treated individuals compared to obese non-diabetics. The A/California/7/2009 H1N1 antibody titers increased in both groups post vaccination, regardless of metformin treatment status. No differences were seen in rates of seroconversion, defined as a four-fold or greater increase in antibody titer following vaccination. Although, post vaccination, OB+MET subjects had a trend towards an increase in mean antibody titer compared to that of OB subjects, this finding was not significant (p=0.0776) (Figure 8).

Preliminary data to suggest higher levels of phosphorylated AMPK and phosphorylated downstream activation marker of mTOR expressed in PBMCs of OB+MET subjects. Western blot analysis was done on whole PBMCs from 4 OB and 6 OB+MET subjects (Figure 9) (Table 4). Because of high levels of protein degradation in some samples, data from only 2 OB and 2 OB+MET subjects are presenedt. Expression of pAMPK was found to be higher in the two OB+MET subjects as compared to the two OB subjects. Expression of p-S6, a downstream activation marker of mTOR, was also found to be higher in the OB+MET group as compared to the OB group.

Discussion

Previous studies in our lab suggest that the adaptive immune response of obese mice and humans is impaired in response to influenza vaccination and infection. The impairment is not

seen across all immune cells however. Obese individuals were found to have a successful antibody response 30 days post vaccination, suggesting that B-cells are responding appropriately to the influenza vaccination. However, despite vaccination, obese individuals are two times as likely to have influenza or influenza like illness. Further studies pointed to T cells in obese individuals being the source of impaired immune response. T cells from obese individuals were found to express lower levels of activation markers CD69 and CD28 and lower levels of functional markers IFN-γ and Granzyme B. Impairments in T cell function were also found in diet-induced obesity studies in mice.

Recently, T cell metabolism has been found to have a direct relationship with T cell activation, differentiation and function. In a resting state, T cells are highly oxidative, using OXPHOS as the primary source for energy metabolism. Upon antigen stimulated activation, T cells undergo a metabolic shift and become highly glycolytic. Activated T cells use mainly aerobic glycolysis for energy metabolism (Figure 2). We have preliminary data to suggest that T cells of obese individuals are more glycolytic, having a higher utilization of glucose per unit time and having higher expression of glycolytic markers such as hexokinase-2 and Glut-1. These data and the current body of research suggest that if the metabolism of the T cell is impaired, then so too is its function. Therefore, restoring metabolism may restore T cell function and improve the adaptive immune response to influenza vaccination.

Metformin, a metabolic drug used to treat patients with type-II diabetes, has been found to affect T cell metabolism directly by activating AMPK and inhibiting mTOR. Activation of AMPK and inhibition of mTOR through administration of metformin could be a key target for restoring the metabolism and function of T cells. Here we demonstrate that obese metformin-

treated diabetic (OB+MET) subjects have improved CD8+ memory T cell responses compared to obese non-diabetic (OB) subjects not taking metformin.

In response to stimulation with A/California/7/2009 influenza virus, OB+MET subjects exhibited significantly higher levels ($p = 0.0433$) of CD8+ T cells expressing both CD69 and granzyme B (CD8+CD69+GranB+). This finding reinforces that administration of metformin at time of vaccination enhances memory T cell generation and results in greater protective immunity.[39] Furthermore, CD8+ T cells of OB+MET subjects expressed higher overall levels of granzyme B than did OB subjects. While there were no significant differences in the overall independent expression of CD69 and granzyme B in influenza stimulated CD8+ T cells, trends were observed in the OB+MET group. Influenza stimulated CD8+ T cells of OB+MET subjects expressed non-significant increases in CD69 expression ($p=0.2421$) and granzyme B expression ($p=0.0503$). When considering the effects of metformin use on CD4+ T cells, no significant differences were seen in expression of CD69. However, interestingly, a trend, similar to CD8+ T cell, CD4+ T cells from OB+MET subjects display non-significantly higher percentages ($p = 0.0728$) and numbers ($p=0.0975$) of CD69 expression.

Further analysis uncovered a significant difference in the fold change of CD4+CD69+ T cells between the unstimulated and influenza stimulated conditions between OB and OB+MET groups. OB subjects had a significantly higher fold change in the number ($p = 0.0256$) and percent ($p = 0.0204$) of CD4+CD69+ T cells. Small changes in the denominator could have large effects on the fold change. Therefore, these findings, are likely observed because higher levels of CD69 expression are observed in the CD4+ T cells of OB+MET subjects prior to influenza stimulation.

CD4+ T cells are made up of a diverse set of cell subtypes that have specialized metabolism and function including Th1, Th2, Th17, Tfh and T-reg subsets. Metformin treatment could be influencing the activation and/or function of any one of these subtypes. T-regs are of particular relevance because they are regulated primarily by AMPK.[24] Stimulation of AMPK *in vivo* with the AMPK activator metformin has been found to increase T-reg numbers.[24]

Influenza specific antibody concentrations HAI titers were also assessed. No significant differences between the OB group and the OB+MET group were observed. However, the OB+MET group did have a non-significant increase in the mean antibody titer as compared to the OB group ($p = 0.0776$). Interestingly this is in contrast with to Diaz et al. 2017, in which metformin use was found to increase antibody titer. However, the researchers report the dosage patients were taking (1000 mgs of MET twice/day), the duration of treatment ($\geq$ 3 years), and they report that none of their MET treated participants had any side effects or had to stop MET treatment before completion of the study.[37]

Interestingly, the highest expression of CD69 and GranB, as well as the highest antibody concentrations are always observed in the metformin treated group. This trend suggests that metformin may be having an effect on immunity but that these effects are dampened by the large variation seen in the antibody concentration and CD4+ and CD8+ T cell response of OB+MET subjects. For most of the results, the only information we have on OB+MET subjects regarding their metformin status is that they have been prescribed metformin after being diagnosed with T2D. This does not confirm whether or not subjects are taking metformin as prescribed. Further, no information about length of treatment nor the dosage of the prescription was available. Lastly, it is also entirely possible that genetic differences exist with regard to T cell responsiveness amongst metformin users.[45,46]

Further assays were conducted to discover the effects of metformin on the protein expression of two important cellular metabolic regulators, AMPK and mTOR. As expected, the subjects in the OB+MET group expressed higher levels of phosphorylated AMPK, the activated form of the protein, than subjects in the OB group. However, unexpectedly, subjects in the OB+MET group also expressed higher levels of p-S6, a downstream marker for mTOR activation. Because of small sample size (n=4), there is not enough information to make any conclusions, however, it is an interesting finding that requires further examination.

This study offers the first reports of metformin-induced activational and functional differences in human memory T cells. While much of the data shown here does not meet significance level ($p < 0.05$), the trends suggest important differences between the activation and function of CD4+ and CD8+ T cells of OB and OB+MET individuals. Further investigation into the metabolic effects of metformin on T cells is needed to uncover the mechanism(s) for metformin-driven improvements in memory T cell function.

Table 1. Demographic information for obese metformin-treated diabetic patients

Subject #	Sex	Age	Race	BMI	Diabetes	Metformin	Assays		
							Flow	HAI	Western
1	Female	30.9	African American	32.4	Type 2	YES	✓	✓	
2	Female	31.6	African American	33.1			✓	✓	
3	Female	42.0	African American	42.9			✓		
4	Female	43.7	African American	37.2			✓	✓	
5	Female	46.4	African American	59.5				✓	
6	Female	50.6	African American	31.4			✓	✓	
7	Female	50.8	African American	41.4			✓	✓	
8	Female	51.1	African American	34.6			✓	✓	
9	Female	51.9	African American	40.4			✓	✓	
10	Female	52.8	African American	40.2				✓	
11	Female	53.0	African American	37.2			✓	✓	
12	Female	54.0	African American	36.8				✓	
13	Female	55.0	African American	37.7			✓	✓	
14	Female	55.0	African American	37.0			✓	✓	
15	Female	55.6	African American	34.6			✓	✓	
16	Female	56.1	African American	36.8			✓	✓	
17	Female	60.8	African American	30.4			✓	✓	
18	Female	61.7	African American	34.3				✓	
19	Female	63.7	African American	39.1				✓	
20	Female	38.4	Caucasian	49.3				✓	
21	Female	44.9	Caucasian	30.3				✓	
22	Female	51.3	Caucasian	43.8			✓	✓	
23	Female	52.0	Caucasian	37.1				✓	✓
24	Female	52.6	Caucasian	32.4			✓	✓	
25	Female	60.2	Caucasian	52.0			✓	✓	
26	Female	63.2	Caucasian	33.0				✓	
27	Female	64.0	Caucasian	50.8				✓	
28	Male	45.3	African American	42.2			✓	✓	
29	Male	46.2	African American	41.0			✓	✓	
30	Male	60.0	African American	31.3			✓	✓	
31	Male	61.0	African American	30.7			✓	✓	
32	Male	43.8	Caucasian	41.0			✓	✓	
33	Male	44.8	Caucasian	41.0				✓	
34	Male	48.2	Caucasian	30.5			✓	✓	
35	Male	60.6	Caucasian	36.4				✓	✓
36	Male	64.1	Caucasian	39.1			✓	✓	
37	Male	65.1	Caucasian	38.0				✓	

Table 2. Demographic information for obese non-diabetic (OB) group

Subject #	Sex	Age	Race	BMI	Diabetes	Metformin	Flow	HAI	Western
1	Female	29.4	African American	30.4	No	NO		✓	
2	Female	32.8	African American	35.4	No			✓	
3	Female	27.1	African American	35.1	No		✓		
4	Female	41.8	African American	43.4	No		✓		
5	Female	43.0	African American	37.7	No			✓	
6	Female	47.4	African American	61.1	Type 2		✓	✓	
7	Female	53.2	African American	31.0	No			✓	
8	Female	57.8	African American	40.3	No			✓	
9	Female	51.0	African American	34.0	No		✓	✓	
10	Female	55.0	African American	40.8	No			✓	
11	Female	53.2	African American	39.9	No			✓	
12	Female	53.0	African American	34.7	No		✓	✓	
13	Female	60.0	African American	37.4	No			✓	
14	Female	58.8	African American	37.9	No			✓	
15	Female	57.0	African American	36.6	No			✓	
16	Female	56.6	African American	36.0	No		✓	✓	
17	Female	57.9	African American	34.0	No			✓	
18	Female	57.7	African American	31.0	No			✓	
19	Female	61.8	African American	32.9	No		✓	✓	
20	Female	63.2	African American	41.7	Type 2		✓	✓	
21	Female	33.6	Caucasian	48.3	No			✓	
22	Female	48.1	Caucasian	30.7	No			✓	
23	Female	49.5	Caucasian	41.0	No		✓	✓	
24	Female	53.0	Caucasian	37.3	No			✓	
25	Female	53.0	Caucasian	32.3	No			✓	
26	Female	59.4	Caucasian	33.8	No		✓		
27	Female	66.0	Caucasian	53.0	No			✓	
28	Female	63.5	Caucasian	32.0	No		✓	✓	
29	Female	63.0	Caucasian	50.4	No			✓	
30	Male	56.5	African American	41.8	No			✓	
31	Male	53.5	African American	40.4	No			✓	
32	Male	60.7	African American	34.2	No		✓	✓	
33	Male	57.7	African American	32.7	No		✓	✓	
34	Male	45.9	Caucasian	41.6	No		✓	✓	✓
35	Male	49.0	Caucasian	42.6	No		✓	✓	✓
36	Male	55.1	Caucasian	32.1	No		✓		
37	Male	49.8	Caucasian	30.8	No		✓	✓	
38	Male	59.6	Caucasian	33.9	No		✓	✓	
39	Male	64.7	Caucasian	39.3	No		✓	✓	
40	Male	65.2	Caucasian	35.8	No		✓	✓	

Table 3. Total Sample Subject Demographic

	Obese Non-Diabetic	Obese Metformin-Treated Diabetic	Total
Participants	40	37	77
BMI	37.9 ± 6.6	38.3 ± 6.6	
BMI Range	30.4 - 61.1	30.3 - 59.5	
Age	53.1 ± 9.6	52.2 ± 8.7	
Age Range	27.1 - 66.0	30.9 - 65.2	
Sex			
Female	29 (72.5%)	27 (73.0%)	
Male	11 (27.5%)	10 (27.0%)	
Race			
African American	24 (60.0%)	23 (62.2%)	
Caucasian	16(40.0%)	14 (37.8%)	
Type 2 Diabetes	2 (5.0%)	37 (100.0%)	
Metformin	0 (0%)	37 (100.0%)	

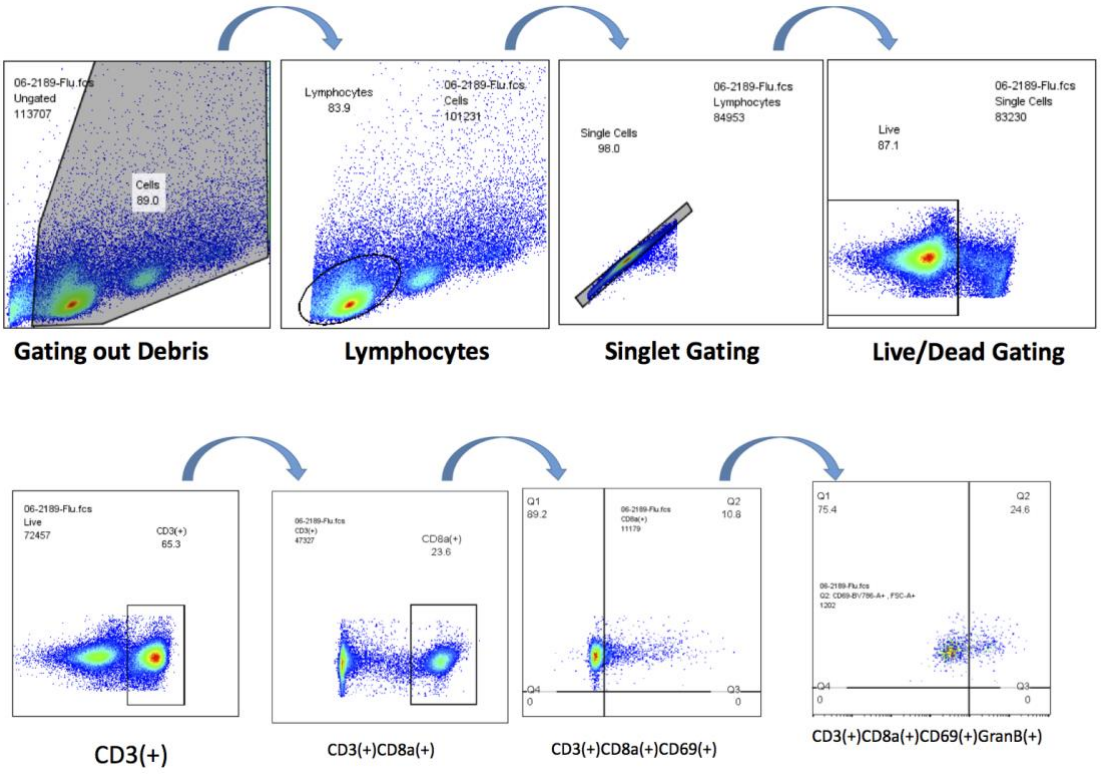

Figure 3. Complete gating strategy for CD8+ T cells. Each subsequent chart represents the gated proportion of the one before it in the gating lineage. This gating tree ends with CD3+CD8a+CD69+GranB+ T cells in the upper right quadrant of the last chart.

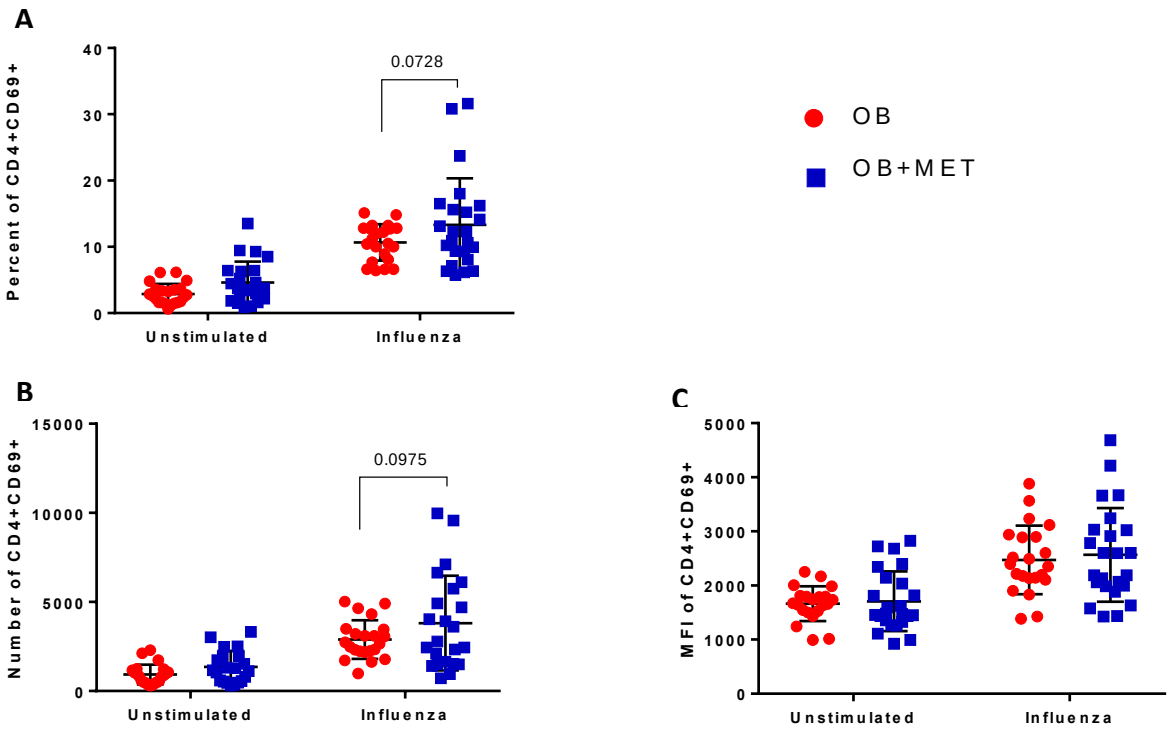

Figure 4. Number, percent and MFI of CD4+CD69+ T cells for OB and OB+MET group. PBMCs from OB (n=22) and OB+MET (n=24) subjects were cultured in RPMI buffer, unstimulated and infected with live H1N1 virus. The percent (p=0.0728) and number (p=0.0975)of CD4+CD69+ T cells from OB+MET subjects trends higher than the CD4+CD69+ T cells of OB (A-B). No difference was seen between the MFI of CD4+CD69+ T cells of OB and OB+MET subjects (C). The unstimulated cultures showed no significant differences (A-C). Data points are individual subjects plotted with mean ± SD. Two-way Anova with Sidak's multiple comparisons test.

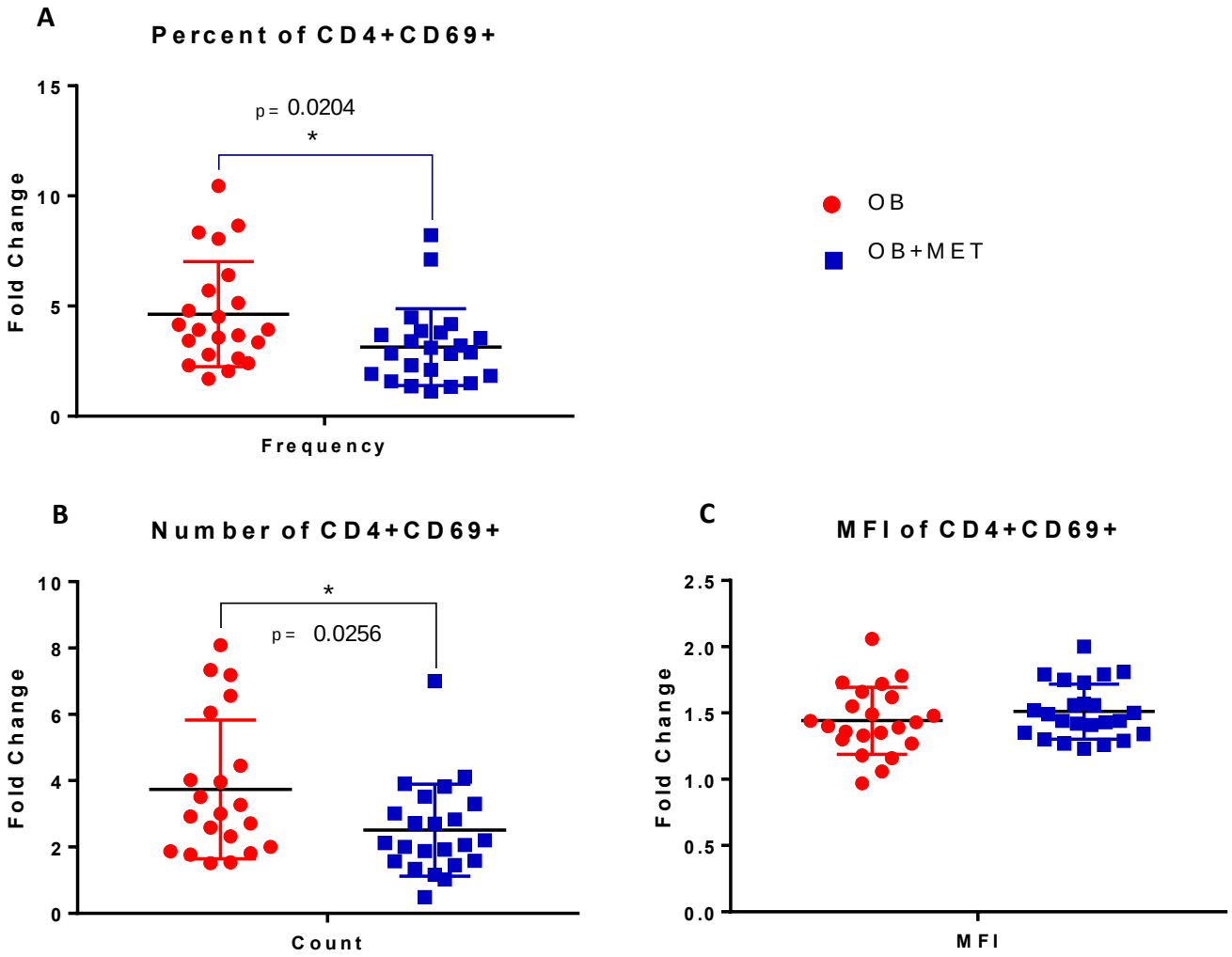

Figure 5. Fold change of number, percent and MFI of CD4+CD69+ T cells for OB and OB+MET group. Significant differences were observed in the fold change of the number and percentages of CD4+CD69+ in OB and OB+MET subjects. OB subjects expressed a significantly higher fold increase in both number (0.0256) and percent (0.0204) but not MFI (p=0.3214) of CD4+CD69+ T cells (A-C). The unstimulated cultures showed no significant differences (A-C). Data were as individual dots with mean ± SD. Parametric unpaired t-test.

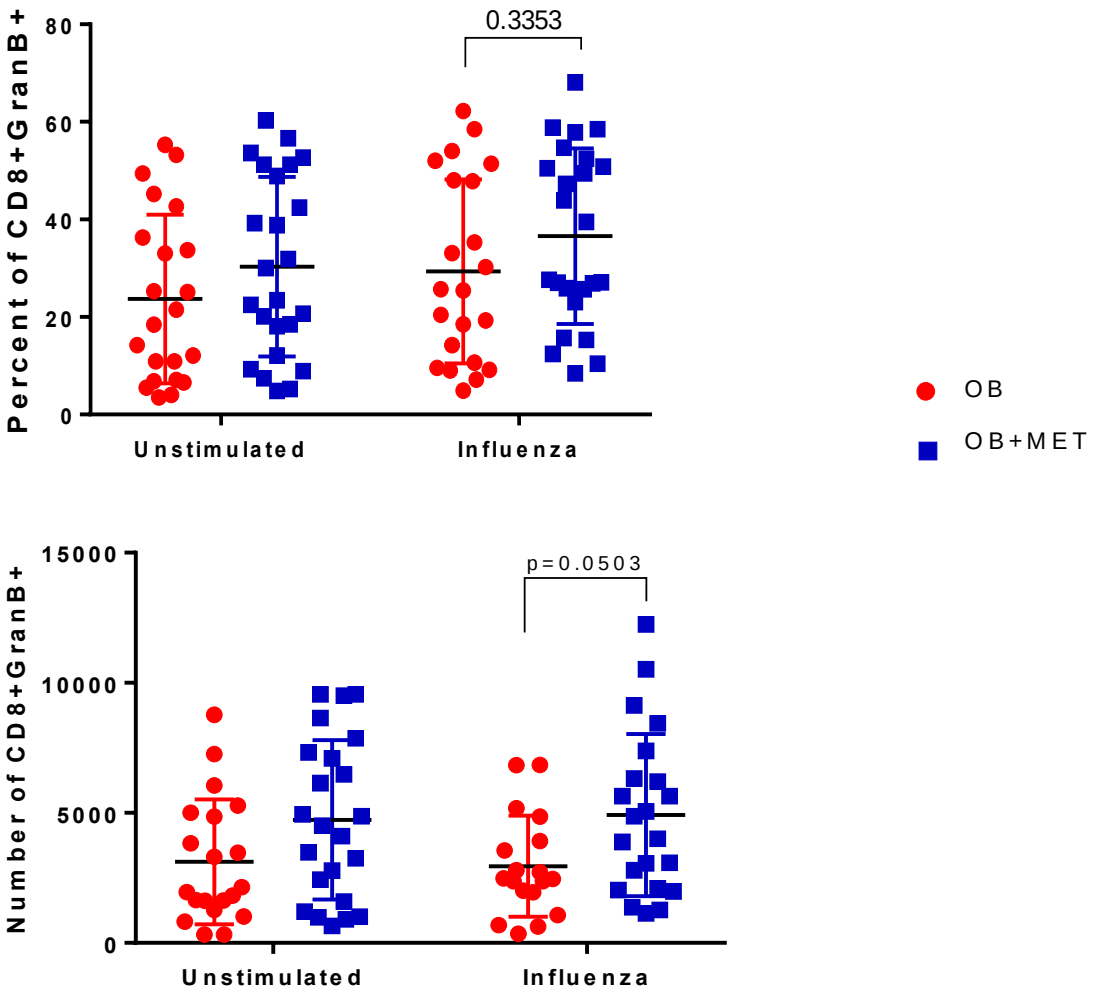

Figure 6. The number and percent of CD8+GranB+ T cell, OB and OB+MET subjects. No significant differences were observed in the number (p=0.0503) or percent (p=0.3353) of CD8+GranB+ T cells. The unstimulated cultures showed no significant differences. Data points are individual subjects plotted with mean ± SD. Two-way Anova with Sidak's multiple comparisons test

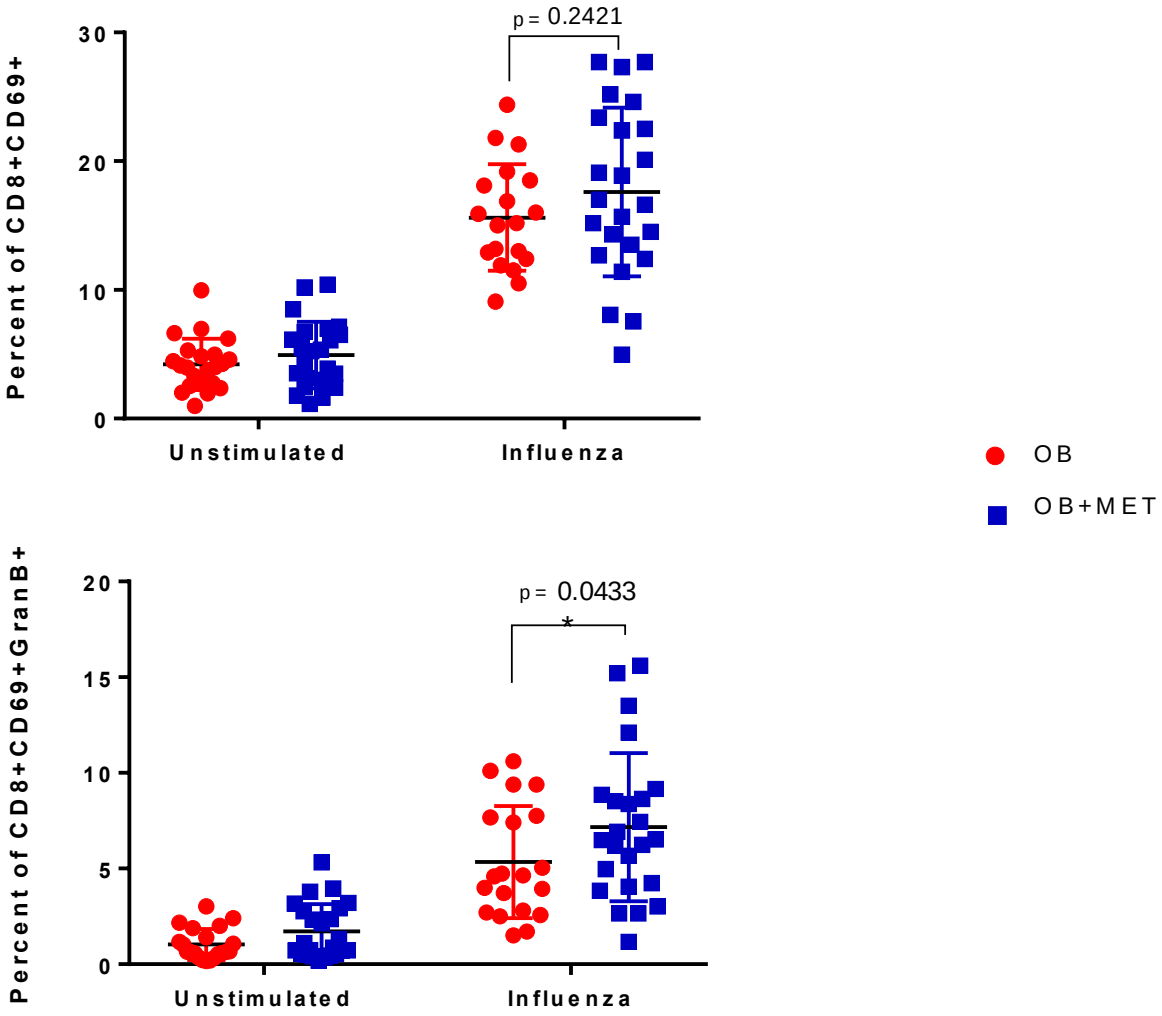

Figure 7. The percent of CD8+CD69+ and CD8+CD69+GranB+ T cells, OB and OB+MET subjects. No significant differences were observed in the percent of CD8+CD69+ T cells (p=0.2421) in OB and OB+MET subjects. OB+MET subjects expressed significantly increased percentages of CD8+CD69+GranB+ T cells (p=0.0433). The unstimulated cultures showed no significant differences. Data points are individual subjects plotted with mean ± SD. Two-way Anova with Sidak's multiple comparisons test

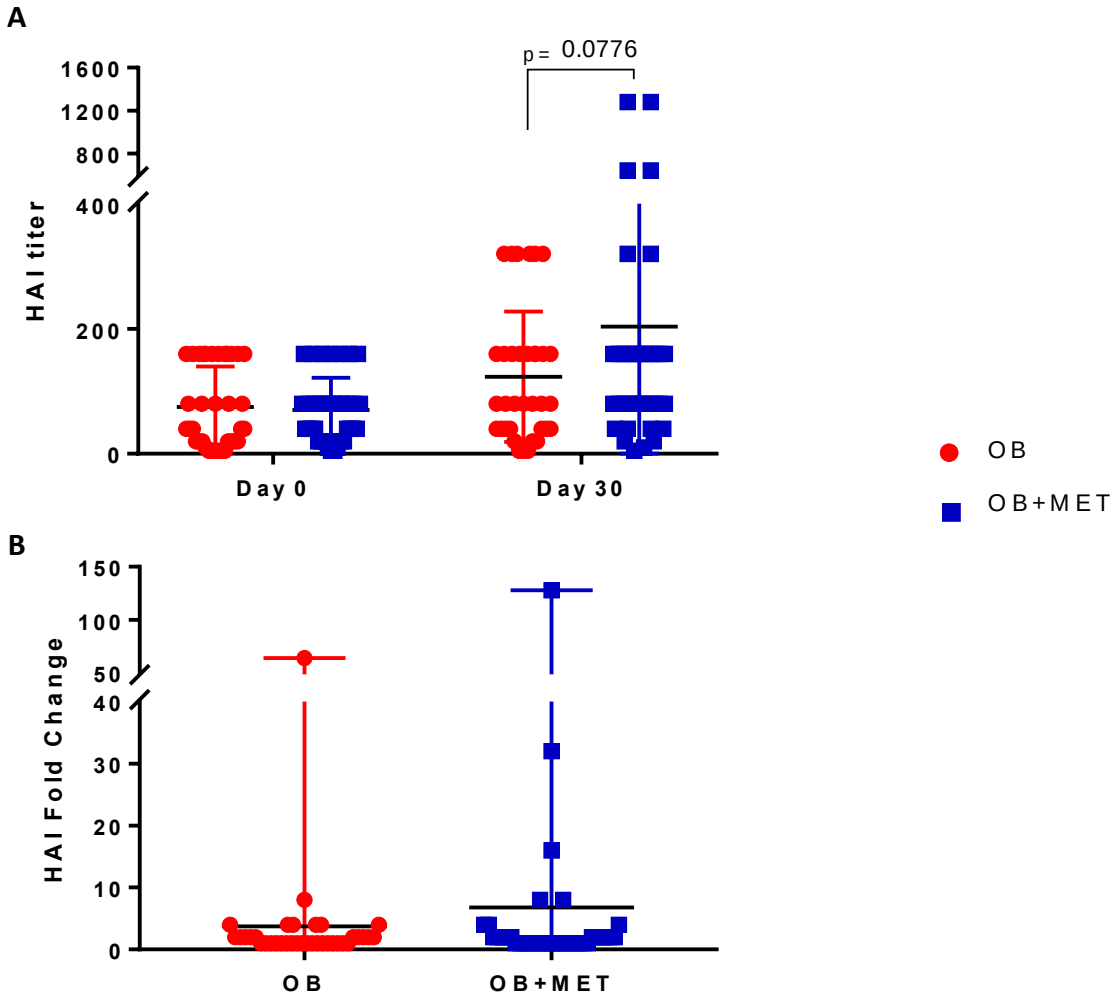

Figure 8. Influenza specific HAI antibody titers and fold change for A/California/7/2009, OB and OB+MET subjects. HAI titers increased from pre- to post-vaccination in both OB (n=36) and OB+MET (n=36) subjects. No significant differences were observed in the HAI titers between the OB and OB+MET (p=0.0776). Data points are individual subjects plotted with mean ± SD. Two-way Anova with Sidak's multiple comparisons test. There was no significant difference between OB and OB-MET subjects in the fold change of the antibody titers pre- and post-vaccination (0.4627). Data points are individual subjects plotted with mean ± range. Parametric unpaired t-test.

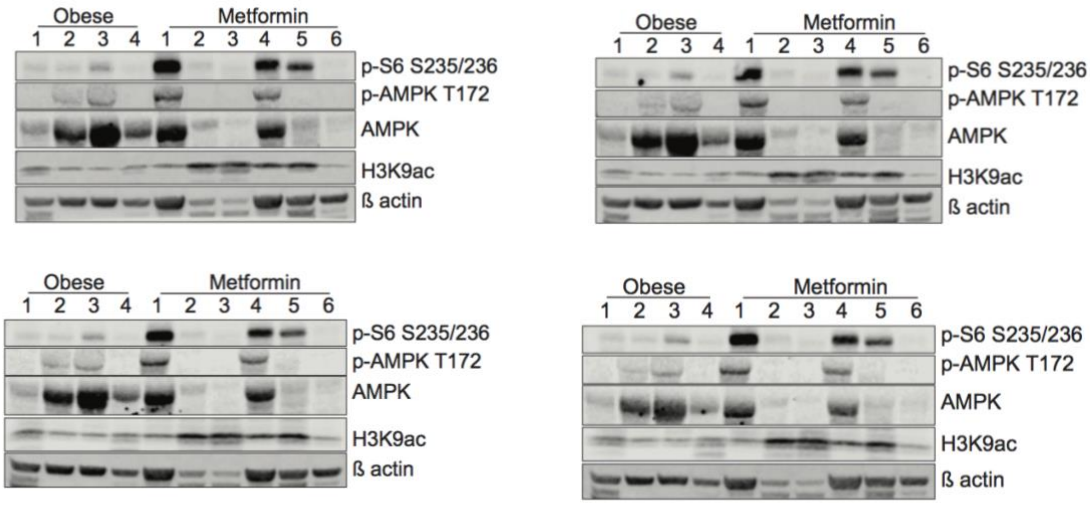

Figure 9. AMPK, p-AMPK and p-S6 protein levels in PBMCs of OB and OB+MET subjects. Western blot analysis on PBMCs of OB (obese non-diabetic) (n=2) and OB+MET (obese metformin treated diabetic) (n=2) was done in quadruplicate on four individual gels. Tests revealed higher levels of p-AMPK and p-S6 in OB+MET group. Small sample size limits statistical power

Table 4. Protein levels of p-S6 and p-AMPK normalized to beta actin

p s6/b actin

	gel 1	gel 2	gel 3	gel 4	average
ob 2	0.638469	0.847486	0.474473	0.41396	0.593597
ob 3	1.361531	1.152514	1.525527	1.58604	1.406403
m 1	11.06157	6.959167	11.86413	11.98413	**10.46725**
m 4	5.336508	5.34244	6.36908	5.715333	**5.69084**

p ampk/b actin

	gel 1	gel 2	gel 3	gel 4	average
ob 2	0.897279	0.738239	0.925168	0.62043	0.795279
ob 3	1.102721	1.261761	1.074832	1.37957	1.204721
m 1	1.693211	4.078643	2.228951	3.729606	**2.932603**
m 4	1.879823	4.115368	1.875329	2.924678	**2.6988**

CHAPTER III – SIGNIFICANCE & FUTURE DIRECTIONS

The CDC estimates that during the 2016–2017 influenza season, the influenza virus infected 30.9 million people, resulted in 14.5 million visits to a health care provider, and hospitalized 600,000 people. These numbers make it clear that seasonal influenza still poses a great threat to the entire population. However, the burden falls disproportionately on those who are obese.[1] Given the growing proportion of obese children and adults in the United States, it is critical that we understand how obesity-driven impairments in T cell immunity to influenza infection can be rectified.

This work is the first to show differences in the activation and functional status of T cells in response to metformin treatment in humans. CD8+ T cells from vaccinated obese metformin-treated diabetic patients showed improved memory response to *in vitro* stimulation with influenza virus. CD8+ T cells of obese metformin-treated diabetic (OB+MET) subjects expressed significantly increased levels of CD69+GranB+ cells. Similarly, although not significant, analogous trends were observed in the overall expression of CD69 and GranB in CD8+ T cells and in expression of CD69 in CD4+ T cells. OB+MET subject also had a non-significant increase in their influenza specific antibody concentration compared to the obese non-diabetic (OB) group.

Interestingly, the fold change between unstimulated and influenza stimulated conditions was significantly increased in obese non-diabetic (OB) subjects. This is likely due to the diversity of CD4+ T cell subtypes. Each subtype has distinct functional properties along with

varying metabolic profiles. Metformin, through regulation of CD4+ T cell metabolism, could be driving up the number of T-regs by activation of AMPK.[24]

These findings suggest that metformin could have an enhancing effect on adaptive immunity against influenza infection. The mechanisms by which this happens is not clear. However, one proposed mechanism is that metformin could directly be impacting T cell metabolism by activating AMPK and inhibiting mTOR. T cell immunity is known to be driven by metabolism and metformin has been shown to alter T cell metabolism.[41] Metformin has been shown to be effective in the treatment of cancer and has anti-inflammatory effects that may help alleviate some of the stresses associated with obesity-associated inflamed visceral adipose tissue.[36] These findings, along with ours, suggests that metformin is a promising target for immunoenhancing therapies.

The major strength of this study is that it provides the first look into the effects of metformin on T cell mediated immunity to influenza infection. We show that administration of metformin could enhance the CD8+ memory T cell response in vaccinated obese adults. Further, the two study groups are evenly matched having very similar mean age and BMI with an evenly distributed sex and race across the two study groups. Finally, the data is strengthened by a large and diverse sample size (n=77).

This study also had several limitations. First, and most importantly, is the lack of detail about metformin use in metformin-treated subjects. There was no available information about duration of use, dosages, or whether or not participants were in-fact taking the drug. Little information was available on the participants' blood glucose levels. Secondly, high variability within metformin-treated group could have been due to any of those factors that we do not have information about, making it difficult to draw conclusions. However, even with wide variability

in the metformin-treated group, we were able to observe a significant difference in the percent of CD8+CD69+GranB+ T cells. Finally, this study does not provide a clear link to how metformin may be affecting the metabolism of memory T cells.

The results of this study and its limitations pose some interesting questions moving forward. First, does metformin have a different effect on different people or was the variability noted above a product of dosage or misuse? To answer this question, an HPLC-MS assay could be done to determine level of metformin and metformin metabolites in the serum of metformin-treated subjects. This would confirm if participants who are prescribed metformin are in-fact taking their prescribed dose. Blood glucose and HbA1c levels could also be assessed more thoroughly to determine whether or not metformin is helping to control participants/ hyperglycemia.

Second, to help illuminate the mechanism by which metformin is acting, a mitochondrial stress test could be done to determine oxygen consumption rates (OCR) and extracellular acidification rates (ECAR). This assay would provide critical information about the glycolytic and oxidation rates of T cells, which is a key characteristic of their functional status. Also, AMPK and mTOR are important regulators in T cell metabolism and function and are direct targets of metformin. Using a western blot assay to determine intracellular protein levels would help to reveal the mechanism by which metformin is acting.

The effect of metformin on T cells, seen in this study, warrants further investigation into mechanism that drives memory T cell improvement. Together with future research, these findings may help to alleviate the burden of influenza on obese adults and help provide a tailored preventative treatment to individuals who respond well to metformin.

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