EFFECTS OF METFORMIN ON MITOCHONDRIA, METABOLISM, AND FUNCTION OF CD4+ T CELL SUBSETS IN OBESITY

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A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Nutrition in the Gillings School of Global Public Health.

Chapel Hill
2024

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ABSTRACT

Devika Rajeev: Effects of Metformin on Mitochondria, Metabolism, and Function of CD4+ T Cell Subsets in Obesity
(Under the direction of Nancie J. MacIver)

Obesity, characterized by inflammation, leads to poor response to select viral infections. Our lab has identified that T cells, critical players of infection response, have increased glucose oxidation, which can be normalized with metformin treatment. Metformin mechanisms are presumably mediated through mitochondrial electron transport inhibition. To investigate mitochondrial mechanisms of metformin-mediated changes in T cell metabolism and function, CD4+ T cells were isolated from diet-induced obese mice and differentiated in vitro toward T effector cells (Teff: Th1 or Th17) or regulatory T cells (Treg) in the presence or absence of metformin. Metformin alters T cell mitochondrial metabolism and function differentially in anti-inflammatory Treg cells versus proinflammatory Teff cells, significantly decreasing mitochondrial membrane potential and downstream complex I activity in Treg cells and altering phosphorylation of AMP kinase in Th17 cells. Further elucidating mechanisms by which metformin alters T cell responses may provide novel therapeutic approaches to obesity-associated dysfunction.
To my grandparents, you are my pillars.
ACKNOWLEDGEMENTS

I would like to extend my deep and sincere gratitude to Dr. Nancie MacIver for allowing me to pursue this invaluable opportunity. She has taught me the importance of resilience, molding me into a better person and scientist. I am immensely grateful to all the members of the MacIver lab for the skills, both personal and professional, that I have accrued over these last few years. I would especially like to extend my gratitude to Dr. Yazan Alwarawrah for teaching me to think critically, while patiently addressing all my questions. Thank you to Dr. Kaitlin Kiernan for helping me lay the groundwork and emulating the laboratory team player I strive to be. Thank you to Amanda Nichols for her unwavering kindness and Caitlin Molloy for her guidance. To my friends and family, thank you for cheering me on through my successes and challenges. To my parents, thank you for your love and encouragement—you’ve kept me grounded through it all.
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<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin-converting enzyme 2</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>EAE</td>
<td>Autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
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<tr>
<td>HFD</td>
<td>High-fat diet</td>
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<tr>
<td>IFNγ</td>
<td>Interferon-gamma</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>Interleukin-10</td>
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<tr>
<td>IL-17</td>
<td>Interleukin-17</td>
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<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
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<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
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<tr>
<td>P-AMPK</td>
<td>Phosphorylated AMPK</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NC</td>
<td>Normal chow</td>
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<tr>
<td>Teff</td>
<td>CD4+ effector T cell</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TMRE</td>
<td>Tetramethyl</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>Term</td>
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<tr>
<td>Treg</td>
<td>CD4+ regulatory T cell</td>
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<td>WHO</td>
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CHAPTER ONE: INTRODUCTION

Obesity

Definitions, Prevalence, and Mortality

The prevalence of obesity has continued to rise over the last few decades, reaching epidemic proportions, and currently affecting 42.4% of adults and 19.3% of children in the United States alone.\textsuperscript{1-3} Obesity, defined as the condition of excessive adiposity, with body mass index (BMI) of 30 kg/m\textsuperscript{2} or greater, causes inflammation of multiple organs, including the liver, pancreas, and adipose tissue, contributing to chronic metabolic diseases such as hepatic steatosis, insulin resistance leading type 2 diabetes mellitus, and cardiovascular disease.\textsuperscript{4,5} These obesity-related disorders are among the leading causes of preventable death.\textsuperscript{6} In addition to cardiometabolic disease, obesity is also associated with impaired immune function, characterized by inflammation, and leading to poor response to infection\textsuperscript{7-10} and impaired vaccine response.\textsuperscript{10-12} Additionally, there is evidence that weight loss alone may be insufficient to reverse the immune dysfunction caused by obesity.\textsuperscript{13-15} For these reasons, it is critically important to identify alternative therapeutic approaches to improve obesity-associated inflammation and impaired immune responses.

\textsuperscript{1} This chapter is modified from the following manuscript: “Metformin as a therapeutic agent for obesity-associated immune dysfunction” by Devika Rajeev and Nancie J. MacIver, Journal of Nutrition, 2024 (submitted and currently under review)
Obesity and Immune Dysfunction

Obesity is associated with increased adipose tissue volume, leading to increased production of pro-inflammatory adipocytokines and changes to the composition and function of the local immune milieu. This promotes recruitment of pro-inflammatory innate immune cells (macrophages, neutrophils, and mast cells) and adaptive immune cells (CD8+ cytotoxic T cells and CD4+ T effector cells (Teff)), leading to the further release of pro-inflammatory cytokines. There is a notable polarization of circulating macrophages from the anti-inflammatory M2 phenotype to the proinflammatory M1 phenotype in obese adipose tissue, subsequently stimulating circulating inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), the latter of which induces the liver to release C-reactive protein, a hallmark of systemic inflammation. Simultaneously, there is a decrease in anti-inflammatory innate immune cells (natural killer T cells, type 2 innate lymphoid cells, and eosinophils) and adaptive immune cells, (CD4+ regulatory T cells (Treg)). The resulting imbalance of pro-inflammatory and anti-inflammatory factors creates the local and systemic low-grade chronic inflammation seen in obesity.

Obesity and Infectious Disease

Obesity is a risk factor for increased morbidity and mortality in patients infected with select viruses, including influenza and SARS-CoV-2, the virus causing COVID-19. Multiple studies have now shown that individuals with obesity have an increased risk of poor outcomes from COVID-19 including increased risk of hospital admission, intensive care unit admission, mechanical ventilation, and death. Moreover, a recent cross-country study of 142 countries by Arulanandam et al. demonstrated a significant positive correlation between COVID-19
mortality and the prevalence of adult obesity. Likewise, individuals with obesity have been found to have increased risk of poor outcomes following both seasonal influenza and pandemic H1N1 influenza. The shift to excessive proinflammatory conditions seen in obesity may augment symptoms of COVID-19 and influenza and lead to poor outcomes such as respiratory distress, pneumonia, and organ failure.

The characteristic inflammation of obesity also leads to diminished vaccine efficacy. A systemic review of fifteen observational studies by Fu et al. found that the humoral response to COVID-19 vaccination decreased with increasing BMI. This finding was supported in a longitudinal study by van der Klauuw et al., which showed that humoral immunity towards COVID-19 waned following vaccination at a greater rate in patients with severe obesity compared to patients with normal BMI. A clinical study by Neidich et al. showed that adults with obesity were twice as likely to develop influenza or influenza-like illness, despite vaccination and with an influenza-specific antibody titer equivalent to healthy weight adults, underscoring the importance of T cells in that setting. These findings are critical because they demonstrate an increased risk of breakthrough infection and associated complications in individuals with obesity.

**Anti-Diabetic Drug: Metformin**

*Clinical Effects and Usage*

With the goal of targeting immune dysfunction in obesity, the pleiotropic effects of the antidiabetic drug metformin make it a candidate for adjuvant therapy for viral infection, autoimmune disease, and tumor immunity. Metformin (1,1-dimethylbiguanide hydrochloride) is a synthetic biguanide that was approved as an antidiabetic drug in 1994 by the U.S. Food and
Drug Administration. It is an organic hydrophilic cation with a pKa of 11.5 at physiological pH, requiring cation transporters, such as organic cation transporters (OCTs), for cellular distribution. Metformin is administered orally, and approximately 70% of the drug is absorbed by the small intestine within the first six hours of treatment. Following intestinal absorption, the drug is widely distributed to the large intestine, liver, and kidneys. It remains unmetabolized and is excreted in the urine, unchanged. According to the World Health Organization (WHO), metformin is considered an essential medicine, as it is the most widely prescribed drug for the treatment of type 2 diabetes. It is also commonly prescribed for treatment of obesity and related pathologies, such as insulin resistance, polycystic ovarian disease, non-alcoholic fatty liver disease, and hypertension. Metformin allows for fasting and post-prandial glycemic control through decreased intestinal absorption, inhibition of hepatic glucose production, and increased efficacy of peripheral glucose usage and clearance. Although not commonly prescribed for these reasons, additional clinical effects of metformin include decreased risk and improved prognosis of cardiovascular disease, cancer, dementia, autoimmune disease, and infection.

Metabolic Mechanism: Inhibition of Mitochondrial Function

The antidiabetic effects of metformin are presumed to be mediated through actions on the mitochondria. Over the last two decades, several studies in hepatocytes have elucidated that metformin inhibits complex I of the electron transport chain. This results in a decrease in ATP production and subsequent activation of 5’ AMP-activated protein kinase (AMPK)-dependent signaling. AMPK promotes catabolic mechanisms that increase ATP production, while inhibiting anabolic mechanisms that decrease ATP utilization, to restore energy balance.
There is, however, controversy over whether metformin enters the mitochondria to interact with complex I directly or instead regulates complex I activity through alternate cascade mechanisms that remain to be elucidated.\textsuperscript{53,54} The physiological relevance of \textit{in vitro} findings that metformin directly interacts with complex I in the mitochondria has been brought into question by multiple critics, such as He and Wondisford.\textsuperscript{55} These critics argue that the observed \textit{in vitro} effects of metformin on complex I activity are dose-dependent and that direct complex I inhibition has only been reported in studies using supra-pharmacologic doses.\textsuperscript{44,50} Others indicate that both mechanisms may be feasible, but cell-dependent.\textsuperscript{48} For instance, in cell types with cation transporters, the accumulation of metformin inside of the mitochondria may be possible, whereas in cells without identifiable cation transporters, metformin may utilize a signaling cascade which acts on complex I and/or AMPK and activates AMPK-dependent\textsuperscript{56,57} or AMPK-independent pathways (\textbf{Figure 1}).\textsuperscript{58,59}
Figure 1. Potential mechanisms of metformin effects on cellular metabolism include inhibition of complex I and/or activation of AMPK to act in AMPK-dependent or AMPK-independent pathways. Created with Biorender.

Metformin Effects on Immune Cells

Metformin has been noted to have immune-modulatory functions, stimulating or suppressing the immune system based on disease state. However, the mechanism of action of metformin on immune cells remains to be fully elucidated. In some immune cells, transporters have been identified for internalization of metformin. Post-internalization, metformin inhibits complex I and acts through AMPK-dependent and AMPK-independent mechanisms to mitigate the inflammatory conditions of obesity. In an in vitro study, Bhansali et al. demonstrated expression of OCT, an important transporter for metformin uptake, in peripheral blood mononuclear cells (PBMCs) from patients with type 2 diabetes and showed that treatment of PBMCs with metformin led to increased mitophagy. These findings were supported in a cross-
sectional study by de Maranon et al., showing that metformin treatment of patients with type 2 diabetes promoted PBMC expression of electron transport chain proteins, decreased reactive oxygen species, restored activation of AMPK, and enhanced mitophagy. In the same study, treatment with metformin also decreased serum levels of TNF and IL-6. As we will discuss here, there is evidence that metformin decreases obesity-associated inflammation and offers beneficial effects on obesity-associated immune dysfunction in the context of infection.

**Metformin Effects on Viral Immunity**

Interestingly, metformin has a long history as an anti-microbial drug and was used against influenza as early as the 1940-50s, well before its mechanism of action was understood. Since then, metformin has also been used against parainfluenza, cowpox, tuberculosis, HIV, hepatitis C, and Zika virus. Metformin acts on both innate and adaptive immune cells to diminish infectious disease risk in patients with obesity. In a mouse macrophage cell line using lipopolysaccharide treatment to model infection response, cells treated with metformin had reduced chemokine expression via AMPK signaling, increased M2 polarization, and increased expression of the anti-inflammatory cytokine interleukin-10. One potential mechanism by which metformin may influence immune response to infection is through AMPK-dependent regulation of mammalian target of rapamycin (mTOR). Additional studies have found that physiologically relevant doses of metformin can restore functional AMPK-dependent T cell changes and normalize levels of proinflammatory cytokines in obese patients with viral infection. In a mouse model of viral hepatitis, metformin attenuated liver injury and decreased Teff cell inflammatory cytokines through regulation of mTOR and mitochondrial function. Clinical studies have supported these findings, indicating that metformin may be a promising hepatoprotective tool in viral hepatitis in
select patient populations. Studies over the last several years have demonstrated that patients taking metformin have improved clinical outcomes following infection with COVID-19 or influenza. A retrospective cohort study of 3551 patients with obesity found that obese patients with a history of metformin treatment had lower mortality to influenza compared to obese patients without history of metformin use. Additional observational studies have shown that patients taking metformin have reduced disease severity and mortality to COVID-19. One particularly large systematic review and meta-analysis of 28 studies with a total of 2,910,462 individuals with COVID-19 showed 34% lower mortality and 27% lower hospitalization rate in patients taking metformin. SARS-CoV-2 enters human cells through binding to angiotensin-converting enzyme 2 (ACE2). Metformin counteracts this mechanism by phosphorylating ACE2 and subsequently changing its conformation to prevent binding with the virus. As seen with other viral infections, metformin also attenuates COVID-19 through modulation of cytokine storm and decreasing levels of the pro-inflammatory cytokines TNF and IL-6, while increasing levels of anti-inflammatory IL-10.

T cells are critical for the immune response to select viral infections, such as influenza and SARS-CoV-2, as they eliminate infected lung endothelial cells, decrease tissue-injury, and signal B cells to trigger a humoral immune response. In a mouse model of obesity and influenza, our lab has shown that obese mice treated with metformin had improved survival to influenza, similar to levels of lean control mice infected with influenza (Figure 2A). In that same study, metformin was found to directly influence T cell function and metabolism in culture, and obese mice treated with metformin systemically had normalization of T cell metabolism, similar to lean controls (Figure 2B).
Figure 2. Systemic metformin improves survival to influenza and decreases CD4+ T cell oxidation following influenza infection. C57BL/6 mice were placed in on low-fat chow (lean), diet-induced obese mice fed HFD (obese) for 18 weeks, or obese mice fed HFD for 18 weeks and treated with metformin in drinking water for the last 6 weeks (obese +metformin). Following 18 weeks on diet with and without metformin, all mice were infected intranasally with H1N1 influenza virus PR8. (A) Survival was monitored, showing a significant increase in survival of obese mice treated with metformin (n=24) compared to obese mice without treatment (n=19); *p<0.05. (B) CD4+ T cells were isolated from spleens, and oxygen consumption rate (OCR) was measured using Seahorse flux analyzer and Agilent MitoStress test. Treatment of obese mice with metformin significantly reduced OCR in CD4+ T cells. n=5 mice per group; *p<0.05.

Adapted from Alwarawrah et al., Int J Obes, 2020

Study Goals

Understanding the effect of metformin on T cell metabolism and function in obesity and viral infection is significant. Therefore, the goals of this project were as follows: 1) test if metformin differentially affected the metabolism and function of CD4+ T cell subsets (Teff versus Treg) from lean versus obese mice and 2) elucidate the mitochondrial mechanisms by which metformin directly alters T cell metabolism and function.
CHAPTER TWO: METFORMIN REGULATES T CELL METABOLISM AND FUNCTION IN A SUBSET-SPECIFIC MANNER

Introduction

CD4+ T cells are critical as they orchestrate the overall immune response by secreting cytokines that influence all other immune cell responses. CD4+ T cells can differentiate into Teff subsets, Th1 and Th17, as well as Treg cells. Teff cells are responsible for the production of inflammatory cytokines, including interferon-gamma (IFN$\gamma$) from Th1 cells and interleukin-17 (IL-17) from Th17 cells. In contrast, Treg cells are characterized by expression of the transcription factor Foxp3 and secrete anti-inflammatory cytokines, interleukin-10 (IL-10) and transforming growth factor beta (TGF-$\beta$) (Figure 3).

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2 This chapter has previously appeared in the BSPH Honors thesis: Rajeev D. The Effect of Metformin on the Metabolism and Function of CD4+ T Helper Subsets in Obesity. Apr 28 2023. doi:10.17615/6sfp-pf11
Figure 3. CD4+ T cell differentiation to proinflammatory, effector T cell subsets (Th1 and Th17) and anti-inflammatory, regulatory T cell subset (Treg). CD4+ T cells differentiated into Th17 cells with IL-6 and TGF-β treatment express the transcription factor RORγt and produce proinflammatory cytokines, IL-17 and IL-22. CD4+ T cells differentiated into Th1 cells with IL-12 treatment express the transcription factor Tbet and produce proinflammatory cytokines, IFNγ and TNF. CD4+ T cells differentiated into Treg cells with TGF-β treatment express the transcription factor Foxp3 and produce anti-inflammatory cytokines, IL-10 and TGF-β. Created with Biorender.

A normal Teff:Treg balance allows for a robust immune response, while preventing progression towards autoimmunity or chronic inflammation. However, in obesity, there is an increase in adipose tissue mass as well as the release of adipokines and other inflammatory signals. Adipokines signal for an increase in proinflammatory cells, including an increase in proinflammatory Teff cells, and a proportional reduction in anti-inflammatory Treg cells. T cell
metabolism and function are highly linked. In normal weight humans and mice, resting CD4+ T cells take up glucose, amino acids, and lipids and metabolize these fuels in the mitochondria to generate ATP for immune surveillance. Upon activation, Teff cells shift their metabolic phenotype towards glycolytic metabolism with increased glutamine and glucose uptake and increased lactate production, whereas Treg cells upregulate lipid oxidation to fuel immune surveillance. Many studies have shown that changes in T cell metabolism can impact T cell function. Our lab has found that activated bulk CD4+ T cells under obesogenic conditions exhibit a distinct metabolic phenotype characterized by increased glucose uptake and mitochondrial oxidation. This altered metabolic phenotype of CD4+ T cells in obesity may mechanistically explain T cell dysfunction in obesity, which has been elucidated as a critical contributor to the dysfunctional response to infections (Figure 4).

Figure 4. Metabolic phenotypes of CD4+ T helper cells are altered by activation and obesity. Teff cells (Th1 and Th17) upregulate glycolytic metabolism (ECAR), whereas Treg cells upregulate lipid oxidation and increase oxidative metabolism (OCR). Bulk activated obese T cells upregulate glucose uptake and oxidative metabolism (OCR). Adapted from Greene and MacIver, Front. Immunol, 2020.
The purpose of this chapter is to determine if metformin can normalize T cell metabolism and thereby improve T cell function in obesity. We hypothesized that increased glucose oxidation of Teff cells in obesity may be targeted and reversed with the metabolic drug metformin. As previous studies have shown that metformin treatment causes a shift towards anti-inflammatory conditions, we further hypothesized that CD4+ T cell subsets would exhibit decreased production of pro-inflammatory cytokines, IFNγ and IL-17, and increased anti-inflammatory Treg proportions following metformin treatment. To explore these hypotheses, we set up the following studies (Figure 5).

Figure 5. Experimental setup exploring metformin effect on CD4+ T cell subset metabolism and function. Created with Biorender.

Methods

Animals

Eighteen-week-old diet-induced obese (obese) C57BL/6J and age-matched wildtype (lean) C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and
allowed 2–3 weeks of acclimation during which they were maintained on high-fat diet (HFD, 60 kcal% fat, Research Diets, New Brunswick, NJ) or low-fat normal chow diet (NC, 10 kcal% fat, LabDiet, St. Louis, MO), respectively. Mice were group housed (up to 5 per cage), maintained at ambient temperature, and given ad libitum access to food and water. Mouse weights were collected weekly. Obese mice and age-matched controls were utilized at approximately 21–22 weeks of age, which was equivalent to 18–19 weeks on HFD. All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of North Carolina at Chapel Hill.

*T cell isolation, activation, and differentiation*

Mice were euthanized using CO₂ inhalation. Spleens were mashed and strained in PBS, washed, and resuspended. CD4⁺ T cells were isolated from splenocytes using the StemCell CD4 T cell isolation kit (StemCell technologies, Vancouver, BC, Canada). CD4⁺ T cells were differentiated toward Th1, Th17, and Treg cell subsets as previously described by Espinosa et al. and plated on 6-well tissue culture plates pre-coated with 25 μg/mL anti-hamster rabbit antibody (MPI). Briefly, isolated CD4⁺ T cells were cultured for 72 hours with 2.5 μg/mL soluble anti-CD3 (Biolegend, San Diego, CA) and 2.5 μg/mL anti-CD28 (Biolegend). The following cytokines were added to each subset—Th1: 10 ng/ml IL-12 (R&D Systems), 10 μg/ml anti–IL-4 (eBioscience); Th17: 20 ng/mL IL-6 (Biolegend), 2.5 ng/ml human TGF-β (R&D Systems), 10 μg/mL anti-IFNγ, 10 ng/ml IL-23; and Treg: 6 ng/ml human TGF-β.
**Flow Cytometry**

For the identification of Treg cells the following antibodies were used: PE/Cy7 rat anti-mouse Foxp3 (Biolegend). Treg cells were plated at 200,000 cells per well and fixed and permeabilized using the Foxp3 Transcription Factor Staining Buffer kit (eBioscience) and stained for Foxp3 following the manufacturer instructions. For the identification of Teff cells (Th1 and Th17) and evaluation of their function, the following antibodies were used: PE/Cy7 rat anti-mouse IFNγ (Biolegend) and APC rat anti-mouse IL-17A (eBioscience). Th1 and Th17 cells, plated at 200,000 cells per well, were stimulated for 4 hours in complete media containing Golgi Plug (2 μg/mL) (BD Biosciences, San Diego, CA), PMA (50 ng/mL) (Sigma-Aldrich, St. Louis, MO), and ionomycin (1 μg/mL) (Sigma-Aldrich), then permeabilized and fixed with Cytofix/Cytoperm kit (BD Biosciences) and stained for IFNγ, IL-17A following the manufacturer’s protocol. All samples were acquired on an Accuri C6 flow cytometer (BD Biosciences), and data was analyzed using FlowJo (BD Biosciences). To determine glucose uptake by flow cytometry, cells were incubated for 30min in RPMI 1640 glucose-free media containing 0.5% heat inactivated FBS and 100 μM 2NBDG (Thermo Fisher, Waltham, MA), after which the cells were washed with FACS buffer. To determine fatty acid uptake, cells were incubated in PBS containing 0.1% fatty acid free BSA and 5 μM BODIPY™ 500/510 C1, C12 (Thermo Fisher) for 15 min and then washed with FACS buffer.

**Metabolic Flux Assays**

CD4+ T cell subsets (Treg, Th1, and Th17) were washed with Seahorse XF RPMI 1640 media (Agilent, Santa Clara, CA) and plated at a density of 250,000 cells/well (50 μL) in a Seahorse XFe96 plate (Agilent) pre-coated with Cell-Tak (Corning, Corning, NY). After
spinning down the plate at 200 rpm for 1 min, the plate was incubated for 30 min in a humidified 37°C incubator in the absence of CO2. Seahorse XF RPMI 1640 media (130 μL) was added, and the plate was incubated for an additional 20 min. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe96 Analyzer (Agilent).

**Statistical Analysis**

All statistical analysis was performed using GraphPad Prism version 9.0.0 (GraphPad Software, Inc., La Jolla, CA) and statistical significance was determined as p-values less than 0.05.

**Results**

Previous work from our lab demonstrated that in response to metformin treatment, bulk CD4+ T cells upregulated glucose uptake but did not change fatty acid uptake.\(^{15}\) We therefore measured nutrient uptake in CD4+ T cell subsets, from lean versus obese mice, in response to metformin. Following 18 weeks on diet, spleens were dissected from lean and obese mice, and CD4+ T cells were isolated from splenocytes and differentiated into Th1, Th17, and Treg subsets in the presence and absence of metformin. The fluorescent tracers, BODIPY and 2NBDG, allowed us to monitor fatty acid and glucose uptake, respectively, into living cells. No significant change was seen in glucose uptake of Th1 (Figure 6A), Th17 (Figure 6B), or Treg (Figure 6C) cells after metformin treatment. Likewise, no significant change was seen in fatty acid uptake of Th1 (Figure 6D), Th17 (Figure 6E), or Treg (Figure 6F) cells after metformin treatment. This
indicates that metformin does not change fuel uptake in differentiated CD4+ T cell subsets in vitro.

Figure 6. Nutrient uptake is unaffected by metformin treatment in CD4+ T helper subsets. C57BL/6J male mice were fed low-fat normal chow or high-fat diet (HFD) for 18 weeks. CD4+ T cells were isolated from spleens of lean and HFD-induced obese (obese) mice and differentiated to Teff (Th1 and Th17) and Treg subsets, and glucose and fatty acid uptake were measured by flow cytometry. Proportion of Th1 subset (A), Th17 subset (B), Treg subset (C) that uptake BODIPY were measured via flow cytometry to characterize lipid uptake. Proportion of Th1 subset (D), Th17 subset (E), Treg subset (F) that uptake 2NBDG were measured via flow cytometry to characterize glucose uptake. Statistical analysis was conducted using one-way ANOVA. n= 2.
We next examined the metabolic flux of Teff and Treg cells differentiated from lean and obese mice in the presence or absence of metformin. To do so, we used metabolic flux analysis to measure oxygen consumption rate (OCR, a surrogate for oxidative phosphorylation), and extracellular acidification rate (ECAR; a measure of lactate production and a surrogate for glycolytic metabolism). The OCR:ECAR ratio is used to discern the energy produced through oxidative phosphorylation versus glycolytic metabolism. In the proinflammatory Th1 subset, metformin treatment resulted in a significant decrease in oxidative metabolism in both the lean and obese setting, demonstrated via a striking decrease in OCR (Figure 7A). However, there was no effect of metformin on glycolytic metabolism, as demonstrated by unchanged ECAR (Figure 7B). These results led to a significant decrease in the ratio of oxidative to glycolytic metabolism in Th1 cells from both lean and obese mice differentiated in the presence of metformin (Figure 7C). In the proinflammatory Th17 subset, metformin treatment also resulted in a significant decrease in oxidative metabolism in both the lean and obese setting (Figure 7D) and no significant change in glycolytic metabolism (Figure 7E), which also resulted in a significant decrease in the ratio of oxidative to glycolytic metabolism (Figure 7F). Interestingly, the anti-inflammatory Treg subset responded differently to metformin. While Treg cells differentiated in the presence of metformin showed a significant decrease in oxidative metabolism (Figure 7G), there was also a trend increase in glycolytic metabolism in Treg cells from lean mice and a significant increase in glycolytic metabolism in Treg cells from obese mice (Figure 7H). The overall metabolic effect of metformin on Treg cells was still a significant decrease in the OCR:ECAR ratio (Figure 7I), indicating the increase in glycolytic metabolism did not completely counteract the decrease in oxidative metabolism. Altogether, these results indicated
that metformin may be acting on the anti-inflammatory Treg subset in a different way than it acts on the proinflammatory, Teff subsets.
Figure 7. CD4+ T helper cells oxidative metabolism is significantly downregulated by metformin treatment, while Treg glycolytic metabolism is specifically upregulated by
**metformin treatment.** C57BL/6J male mice were fed low-fat normal chow or high-fat diet (HFD) for 18 weeks. CD4+ T cells were isolated from spleens of lean and HFD- induced obese (obese) mice, differentiated to Teff and Treg subsets, and metabolic flux analysis was performed. Basal oxygen consumption (OCR; mean±standard error), basal extracellular acidification rate (ECAR; mean±standard error), and OCR:ECAR ratio (mean±standard error) of each subset was measured. Th1 cells treated with metformin exhibited a significant decrease in OCR (A) and no change in ECAR (B), which indicated a significant decrease in OCR:ECAR ratio (C) compared to untreated Th1 cells. Th17 cells treated with metformin exhibited a significant decrease in OCR (D) and no change in ECAR (E), which caused a significant decrease in OCR: ECAR ratio (F) compared to untreated Th17 cells. Treg cells treated with metformin exhibited significant decrease in OCR (G), lean Treg cells treated with metformin exhibited a trend increase in ECAR, and obese Treg cells treated with metformin exhibited a significant increase in ECAR (H), which overall caused a significant decrease in OCR: ECAR ratio (I) compared to untreated Treg cells. Statistical analysis was conducted using one-way ANOVA. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; n= 2. Each plotted point represents a technical replicate (n=2; 4 experimental replicates per mouse).

Given that metformin caused changes to the metabolic phenotype of CD4+ T cells from lean and obese mice, we next assessed the effects of metformin on the function of CD4+ T helper subsets in obesity. To do so, we used intracellular flow cytometry to measure production of proinflammatory cytokines, IFNγ and IL-17, of the Teff subsets Th1 and Th17, respectively, and we measured Foxp3 transcription factor expression in Treg cells. Proinflammatory Teff cells treated with metformin exhibited decreased functionality, as measured by a trend decrease in IFNγ production from Th1 cells (Figure 8A) and a trend decrease in IL-17 production from Th17 cells (Figure 8B). However, metformin did not alter Foxp3 expression in Treg cells from either lean or obese mice (Figure 8C). This demonstrates that metformin acts on Treg cells in a different manner than Teff subsets and indicates that metformin treatment may promote an overall anti-inflammatory effect on CD4+ T cells.
Figure 8. CD4+ effector T cell functionality is selectively targeted by metformin treatment, while CD4+ regulatory T cell proportions increase with metformin treatment. C57BL/6J male mice were fed low-fat normal chow or high-fat diet (HFD) for 18 weeks. CD4+ T cells were isolated from spleens of lean and HFD-induced obese (obese) mice, differentiated to Teff and Treg subsets, and cytokine or Foxp3 transcription factor was measured by intracellular flow cytometry. Proportion of Th1 subset that produce IFNγ cytokine (A) and proportion of Th17 subset that produce IL-17 (B) was measured to characterize trends in functionality via flow cytometry (n = 1). The proportion of Treg subset that expresses Foxp3 transcription factor (C) was measured to characterize functionality via flow cytometry. Statistical analysis was conducted using one-way ANOVA. n = 1-2.

Discussion

Overall, this work provides evidence that metformin targets oxidative metabolism of all CD4+ T cell subsets studied, while preferentially targeting the function of proinflammatory effector T cells, resulting in an overall anti-inflammatory effect (Figure 9).
Figure 9. Metformin normalizes obesity-associated inflammation in T cells. Obesity is associated with increased adipose tissue volume, leading to increased production of pro-inflammatory adipocytokines and recruitment of pro-inflammatory immune cells leading to the further release of inflammatory cytokines, IFNγ and IL-17, from Th1 and Th17 cells. The resulting imbalance of pro-inflammatory and anti-inflammatory factors creates the local and systemic low-grade chronic inflammation seen in obesity. Treatment with metformin upregulates production of anti-inflammatory cytokines, IL-10, from Treg cells. Created with Biorender.

These differences in how metformin alters Teff versus Treg cells may help us understand how T cell dysfunction can be reversed in specific immune cell subsets. By downregulating Teff function specifically, metformin may be able to mitigate the proinflammatory cytokine storm seen in severe infections of COVID-19 and influenza. Moreover, as obesity is associated with chronic inflammation, treatment with metformin could serve a dual-purpose in treating patients with obesity following COVID-19 or influenza infection.
CHAPTER THREE: IDENTIFYING MITOCHONDRIAL MECHANISMS BY WHICH METFORMIN REGULATES T CELL METABOLISM AND FUNCTION

Introduction

It is now clear that obesity alters T cell metabolism and function in a subset-specific manner. First, we have seen that metformin decreases oxidative metabolism in both Teff and Treg cells. Moreover, we have demonstrated that metformin downregulates pro-inflammatory cytokine production from Teff cells. However, the mechanisms through which metformin alters T cell metabolism and function remain unknown. As discussed in Chapter 1, metformin has been shown to alter cellular metabolism in liver and other metabolic cells and tissues through direct or indirect inhibition of mitochondrial complex I of the electron transport chain and downstream phosphorylation of the metabolic regulator AMPK. Other proposed mechanisms are complex I-independent activation of AMPK and AMPK-independent transcriptional regulation. Since metformin’s mechanisms vary based on cell-type, it is unknown if metformin alters complex I and/or AMPK activity in T cells and if there are subset-specific differences.

The purpose of this chapter is to identify mitochondrial mechanisms by which metformin alters T cell metabolism and function. As we have demonstrated subset-specific action of metformin on metabolism and function, we hypothesized that metformin may utilize a different mechanism to act on anti-inflammatory CD4+ Treg cells compared to proinflammatory CD4+ Teff cells. We also hypothesized that mitochondrial membrane potential, complex I activity, and AMPK activity would be altered following metformin treatment in a subset-specific manner (Figure 10).
Figure 10. Hypothesized mechanisms by which metformin alters CD4+ T cell metabolism and function. Metformin may inhibit complex I activity and/or alter downstream AMPK activity to decrease oxidative metabolism in CD4+ T cells. Created with Biorender.

Methods

Animals

Eighteen-week-old diet-induced obese (obese) C57BL/6J and age-matched wildtype (lean) C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and allowed 2–3 weeks of acclimation during which they were maintained on high-fat diet (HFD, 60 kcal% fat, Research Diets, New Brunswick, NJ) or low-fat normal chow diet (NC, 10 kcal% fat, LabDiet, St. Louis, MO), respectively. Mice were group housed (up to 5 per cage), maintained at

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ambient temperature, and given ad libitum access to food and water. Mouse weights were collected weekly. Obese mice and age-matched controls were utilized at approximately 21–22 weeks of age, which was equivalent to 18–19 weeks on HFD. All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of North Carolina at Chapel Hill.

*T cell isolation, activation, and differentiation*

Mice were euthanized using CO₂ inhalation. Spleens were mashed and strained in PBS, washed, and resuspended. CD4+ T cells were isolated from splenocytes using the StemCell CD4 T cell isolation kit (StemCell technologies, Vancouver, BC, Canada). CD4+ T cells were differentiated toward Th17 and Treg cell subsets as previously described by Espinosa et al. and plated on 6-well tissue culture plates pre-coated with 25 μg/mL anti-hamster rabbit antibody (MPI). Briefly, isolated CD4+ T cells were cultured for 72 hours with 2.5 μg/mL soluble anti-CD3 (Biolegend, San Diego, CA) and 2.5 μg/mL anti-CD28 (Biolegend). The following cytokines were added to each subset—Th17: 20 ng/mL IL-6 (Biolegend), 2.5 ng/ml human TGF-β (R&D Systems), 10 μg/mL anti-IFNγ, 10 ng/ml IL-23; and Treg: 6 ng/ml human TGF-β.

*Mitochondrial Flow*

To measure mitochondrial mass, 50,000 cells were plated in a V-bottom 96-well plate and stained with mitotracker green (50nM final concentration) for 30 minutes at 37°C. To measure mitochondrial membrane potential, cells were stained with tetramethylrhodamine
(TMRE; 100nM) for 30 minutes at 37°C. All samples were acquired on an Accuri C6 flow cytometer (BD Biosciences) and data was analyzed using FlowJo (BD Biosciences).

**Complex I Activity Assay**

Complex I Activity was measured as described in manufacturer’s protocol (ab109721, Abcam). Briefly, cell pellets were lysed with 1 part detergent and 4 parts PBS. The pellets were then spun down for 20 minutes at high speed and the supernatants collected. Protein concentration was determined by colorimetric protein assay. Protein was diluted to 250µg/mL in lysis buffer and 5x sample buffer. 200 µL of sample and buffer were loaded into wells in duplicates to complex I antibody pre-coated 96-well plate and incubated on a shaker at RT for 3 hours. Wells were washed 3X using 300uL wash buffer. 200 µL of solution was added to each well and complex I activity was measured via colorimetric complex I activity assay.

**Western Blot**

Cell pellets were lysed with RIPA buffer plus protease inhibitors and phosphatase inhibitors (PhosStop) for 20-40 minutes on ice, vortexing every 5 minutes. Lysates were then centrifuged for 30 minutes at high speed and the supernatants collected. Protein concentration was determined by colorimetric protein assay. Protein was diluted to 2µg/µL in lysis buffer and 5x sample buffer. 20uL of protein was loaded onto an 8-15% gel, with 1-2uL ladder on either side of samples. Gel was run for 40 minutes at 200V. Gel was transferred onto a PVDF membrane (BioRad) using the BioRad transfer case. Membrane was blocked 5 minutes in EveryBlot blocking buffer (BioRad) in a shaker at RT. Membrane was then blotted with antibodies to either phosphorylated-AMPK (P-AMPK; Cell Signaling), total AMPK (Cell
Signaling), or actin (Cell Signaling) for 1 hour at room temperature, up to overnight at 4°C. Blots were imaged on BioRad gel imager and analyzed using BioRad ImageLab software.

**Results**

To determine the effects of metformin on T cell mitochondria, we measured mitochondrial mass and mitochondrial membrane potential. Following 18 weeks on low-fat or high-fat diet, spleens were dissected from lean and obese mice, and CD4+ T cells were isolated from splenocytes and differentiated into Th17 and Treg subsets. Following differentiation, cells were treated with metformin for 24 hours, and mitochondrial mass and membrane potential were measured flow cytometrically using mitotrackr green and TMRE staining. No significant change was seen in mitochondrial mass of Th17 or Treg cells from lean or obese mice following metformin treatment (**Figure 11A-B**). Interestingly, metformin caused a significant decrease in mitochondrial membrane potential in Treg cells from both lean and obese mice but not in Th17 cells (**Figure 11C-D**), indicating that metformin alters the mitochondrial dynamics of Treg cells. These results also indicate that the mechanisms by which metformin acts on Teff and Treg cells may vary by subtype.
Figure 11. Metformin downregulates mitochondrial membrane potential of Treg cells. CD4+ T cells from lean and obese mice were differentiated in culture toward Th17 and Treg cells, as described, and incubated with 2 mM of metformin for 24 hours. Mitochondrial mass was measured using mitotracker green and mitochondrial membrane potential was measured using TMRE staining and analyzed by flow cytometry. n=3 mice/group; *p<0.05.
Given that metformin treatment altered mitochondrial membrane potential in Treg cells, but not Th17 cells, we next assessed the effect of metformin on mitochondrial complex I activity. Following differentiation to Th17 or Treg subsets, cells were treated with metformin for 6 hours and complex I activity was measured. Cells were lysed, complex I was captured on an antibody coated plate, and NADH oxidation was measured over time as a readout of complex I activity. No significant change was seen in complex I activity of Th17 cells from lean or obese mice following treatment with metformin (Figure 12A). Likewise, no significant change was seen in complex I activity of Treg cells from lean mice; however, Treg cells from obese mice exhibited significantly less complex I activity following metformin treatment (Figure 12B). These data indicate that metformin has differential effects on complex I activity in anti-inflammatory Treg versus proinflammatory Teff cells.
Figure 12. Metformin inhibits mitochondrial complex I activity in Treg cells from obese mice. CD4+ T cells from lean and obese mice were differentiated in culture toward Th17 and Treg cells, as described, and incubated with 2 mM of metformin for 6 hours. Cells were then lysed, complex I was captured on antibody coated microtiter plate, and complex I activity was measured by monitoring the NADH oxidation over time. n=4 mice/group; **p<0.01.

Inhibition of complex I by metformin in metabolic cells is thought to generate an energy deficiency that activates the metabolic regulator AMPK. Therefore, we next assessed the activation of AMPK (as measured by phosphorylation). Differentiated Th17 and Treg cells were treated with metformin for 0, 2, and 6 hours, and phosphorylation of AMPK was measured via immunoblotting, to determine if metformin induced AMPK activity. Metformin did not cause a significant change in the phosphorylation of AMPK in Th17 cells from lean mice, but Th17 cells from obese mice exhibited a significant decrease in AMPK phosphorylation following metformin treatment (Figure 13A). No significant change was seen in phosphorylation of AMPK in Treg cells from lean or obese mice (Figure 13B).
Figure 13. Metformin alters AMPK activity in Th17 cells from obese mice. CD4+ T cells from lean and obese mice were differentiated in culture toward Th17 or Treg, incubated with 2 mM of metformin for 0, 2, and 6 hours, and expression of Phosphorylated-AMPK (P-AMPK), total AMPK, and actin were measured via immunoblot. n=2-3 mice/group; *p<0.05, **p<0.01.

Discussion

Overall, these data provide evidence that metformin utilizes different mitochondrial mechanisms in anti-inflammatory Treg cells compared to proinflammatory Th17 cells. While we see no change in complex I activity in Th17 cells, we observe decreased AMPK phosphorylation
following treatment of metformin. This indicates that metformin acts on Th17 cells in a complex-I independent, AMPK-dependent manner. In Treg cells from obese mice, metformin inhibits complex I activity to decrease oxidative metabolism. However, it seems that metformin does not have a significant effect on AMPK activity in Treg cells at 2 hours of treatment, whereas there is a trend increase in AMPK activity in Treg cells from obese mice at 6 hours of treatment ($p=0.108$). We can conclude that metformin directly inhibits complex I activity in Treg cells and directly inhibits AMPK activity in Th17 cells, affirming that there are subset-specific differences (Figure 14).

Figure 14. Metformin acts via different mechanisms to target mitochondrial dysfunction in obese Th17 and Treg cells. Created with Biorender.
CHAPTER FOUR: DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSION

Discussion

In recent years, influenza and coronavirus pandemics have highlighted the increased risk of morbidity and mortality experienced by individuals with obesity when faced with infection.\textsuperscript{28,92} Multiple studies have identified that T cells are key players that have impaired response to infections in obesity.\textsuperscript{88,93} As prevalence of obesity is steadily increasing, it is important to elucidate targeted treatment approaches to normalize obesity-associated T cell dysfunction.\textsuperscript{94} Our lab has previously demonstrated that obesity alters T cell response to influenza infection (Figure 2). Interestingly, the anti-diabetic drug metformin targets mitochondrial oxidation and regulates the balance of regulatory and effector T cells (Treg/Teff) to normalize obesity-associated immune dysfunction. However, there are significant gaps in knowledge regarding subset-specific differences on T cell metabolism and function and the mechanisms by which metformin directly acts on T cells. This \textit{in vitro} work provided a novel opportunity to investigate the subset-specific effects that metformin has on T cell mitochondria, metabolism, and function.

\textit{Metformin targets proinflammatory Teff cells and promotes anti-inflammatory Treg cells to assuage obesity-associated immune dysfunction}

To investigate the effect of metformin on obesity-associated T cell metabolism, we used the high-fat diet induced obese mouse model. To investigate the effects of metformin on pro-
inflammatory Teff subsets, Th1 and Th17 cells, versus anti-inflammatory Treg cells, we measured nutrient uptake, metabolic flux, Teff cytokine production, and Treg proportions. While nutrient uptake was unaffected (Figure 6), metformin treatment led to significantly decreased oxidative metabolism (Figure 7) in all T cells. Our results are similar to our previous study, where we saw decreased OCR and OCR:ECAR ratio in bulk CD4+ T cells from lean and obese mice when treated with metformin in vitro. Here, we found that decrease in OCR and OCR:ECAR ratio in Teff cells (Figure 7) was also linked to decreased pro-inflammatory IFNγ and IL-17 production (Figure 8), supporting the idea that T cell metabolism and function are tightly linked. Additionally, we observed a significant decrease in OCR with an increase in ECAR in Treg cells (Figure 7). This switch from oxidative to glycolytic metabolism was also linked to increased Treg proportions (Figure 8), showing that metformin has an anti-inflammatory effect.

Our results are consistent with other studies exploring the importance of metformin in the regulation of inflammatory response. Isoda et al. showed that metformin inhibited the production of proinflammatory cytokines, IL-6 and IL-8, from vasculature to act as a cardio-protective agent. In a review, Bai and Chen provided extensive support for metformin’s inflammation-inhibitor effects in immune cells and diseases. They identified that metformin abated inflammation in renal disease and neurodegenerative disease by downregulating pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF. Martin et al. supported this with evidence of metformin in influenza and SARS-CoV-2 infection and vaccine response. They showed that metformin can downregulate Th17 mediated inflammation, promote CD8+ memory cell formation, and induce anti-inflammatory M2 macrophage polarization. Given this evidence from
the literature along with our data, we can conclude that metformin modulates T cell dysfunction in obesity by specifically targeting Teff functionality and upregulating Treg cells.

Metformin targets pro-inflammatory Th17 cells via different pathway than anti-inflammatory Treg cells

Based on our findings that metformin can directly target CD4+ T cell function and oxidative metabolism in a subset-specific manner in vitro, we sought to identify the mitochondrial mechanisms that metformin uses to target these cells. We found that metformin has different effects on mitochondria in Treg cells versus Th17 cells. In Treg cells, metformin polarizes the mitochondrial membrane (Figure 11), directly inhibits complex I activity (Figure 12), and uses a signaling cascade to phosphorylate and activate AMPK (Figure 13); In Th17 cells, metformin acts directly on AMPK, but does not alter mitochondrial membrane potential or complex I activity (Figure 13).

Metformin is widely presumed to act in metabolic cells via inhibition of complex I of the electron transport chain to suppress mitochondrial ATP production and activate AMPK. However, studies have shown that metformin treatment can be complex I-dependent but AMPK-independent or complex I-independent but AMPK-dependent. Kelly et al. demonstrated that metformin treatment of LPS-activated macrophages directly inhibited complex I activity, without acting on AMPK, to provide an anti-inflammatory effect. In an autoimmune insulitis mouse model, Duan et al. showed that metformin treatment inhibited Teff differentiation, while supporting Treg development in vitro in a dose-dependent, AMPK-dependent manner. Sun et al. demonstrated similar Th17/Treg responses in mice with experimental autoimmune encephalomyelitis (EAE) and attributed the metformin protective effects to suppression of the
AMPK antagonist, mTOR.\textsuperscript{101} A review by Lin et al. evaluated numerous studies to conclude that metformin can act on mTOR with or without influencing AMPK activity in immune cells.\textsuperscript{102} As such, our data on metformin’s mechanism of action on Th17 cells supports the need for continued research evaluating the role of mTOR regulation following metformin treatment of Teff cells. While several studies have demonstrated that the mechanism targeted by metformin is cell and pathology dependent,\textsuperscript{60} our study provides novel evidence that the mechanism of action of metformin is subset-specific in T cells.

**Limitations and Future Investigations**

*Limitations*

There are limitations to our study that must be acknowledged. For these experiments we used male obese mice, consistent with our previous study on targeting oxidative metabolism in bulk T cells. We did not use female obese mice, as they demonstrate resistance to weight gain and progression of obesity-associated inflammation on high-fat diet.\textsuperscript{103} For that reason, our results may not be generalizable to both males and females. Additionally, the studies shown above were only done in splenic CD4 T cells, and effects of metformin on other T cell types such as CD8+ T cells and gd T cells may be different. Lastly, to identify the mechanistic effects of metformin on T cell mitochondria, we used a supra-pharmacologic concentration of 2 mM. For physiological relevance, future studies need to be conducted using pharmacologic doses (<1 mM).
Future Investigations

To complete the picture of metformin’s mechanism of action on Teff versus Treg cells, further studies need to assess mTOR expression and activation, as mTOR is a downstream target of AMPK activation and has also been implicated in AMPK-independent pathways. Examination of additional downstream targets of AMPK, such as acetyl-CoA carboxylase (ACC) may also give insight into the metabolic shifts from anabolic to catabolic pathways following metformin treatment. While we now understand the effects of metformin on CD4+ T helper subsets, we need to further explore CD8+ T cells. Lastly, given the pandemics of recent years and the critical role of T cells in the immune response to viral infection, in vivo studies investigating the physiological effects of metformin on obesity should be conducted. The effects of metformin on CD4+ T cell subsets and CD8+ T cells will be explored in a mouse model of influenza infection.

Conclusion

In summary, we provide evidence that the anti-diabetic drug metformin can target obesity-associated T cell inflammation and dysfunction using subset-specific mechanisms to improve immune response. As T cells are key players in the immune response to infection, there is a critical need to further explore T cell dysfunction in obesity. Our future studies will focus on adding to the mechanistic understanding of metformin action on T cells in obesity, as well as in response to infection. Given the ever-rising risk of obesity and the wide-spread prevalence of seasonal and pandemic viral infections, such as influenza and coronavirus, it is crucial to identify novel approaches to target subset-specific T cell infection response.
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