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The Immunological Response to Chronic Stress in Obese Mice

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The Immunological Response to Chronic Stress in Obese Mice

Matthew Corey Parsley

Thesis to be submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements of the degree:

Master of Science in Exercise Physiology

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Key Words: Obesity, chronic stress, depression, inflammation, Oxidative stress, immune, febuxostat, xanthine oxidase

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Abstract

The Immunological Response to Chronic Stress in Obese Mice

Matthew C. Parsley

Obesity and psychological stress are major risk factors for cardiovascular disease and have a positive correlation with vascular dysfunction. This functional deficit has been linked to a pro-inflammatory milieu, exacerbated by an upregulation of pro-inflammatory cytokines. Febuxostat, a xanthine oxidase inhibitor, has been shown to rescue vascular impairment in these models. The purpose of this study was to examine the individual and combined effects of obesity and chronic stress on neuroimmune cell populations, and assess if febuxostat is able mediate immune alterations.

Male mice (n=48) were assigned into either normal chow (lean) and high-fat diet (obese) groups. The lean animals were then separated into one of four subgroups (n=6/group): 1) lean control, 2) lean control + febuxostat, 3) lean unpredictable chronic mild stress (UCMS), 4) lean UCMS + febuxostat. The obese counterparts were also separated into four subgroups: 1) obese control, 2) obese control + febuxostat, 3) obese UCMS, 4) obese UCMS + febuxostat. Febuxostat (50mg/L) was delivered in drinking water. Stressed mice were subjected to the UCMS paradigm which included cage tilt, no bedding, damp bedding, bath, social stress, and altered light-dark cycles for 7 hours a day, 5 days a week for 8 weeks. Coat scores were assessed to confirm the implementation of the UCMS protocol. Immediately following terminal procedures, brains were collected and processed for flow cytometry, to identify immune cell populations.

Obese animals showed a significant increase in body mass ($p<0.05$) and UCMS groups showed a significant increase in coat scores ($p<0.05$). Compared to lean controls, obese controls showed a significant decrease in total white blood cells, B-cells, M2 macrophages, and T-cells, and a significant increase in helper-T and cytotoxic-T cells ($p<0.05$). Obese+UCMS showed a significant increase in total white blood cells and significant decrease in M2 macrophages ($p<0.05$) compared to obese controls. Delivery of febuxostat did not significantly affect immune cell populations.

These data suggest that the acquisition of obesity and/or subjection to chronic stress leads to negative alterations in immune cell subsets.

Dedication

To my pretty lady – I love you, Granny. To my friends – thank you for teaching me unconditional love. To my mom – I adore you immensely. To my special fella – thank you for taking a chance on me. To Yara, Mani, and Sally – you three are perfect.

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My graduate teaching assistantship provided a clinical complement to my basic science research, and I was fortunate enough to be guided by Dr. Ryan and Danny. The two of them provided me with so much knowledge in leadership and gave me the chance to improve my teaching abilities. To say I am thankful for the friendship I have built with the two of them is a true understatement, as they are truly angels.

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Abbreviations

XO Xanthine Oxidase

ROS Reactive Oxygen Species

BMI Body Mass Index

WAT White Adipose Tissue

BAT Brown Adipose Tissue

IL- Interleukin-

TNF- α Tumor Necrosis Factor- α

INF- γ Interferon- γ

PRR Pattern Recognition Receptors

MAMP Microbe-Associated Molecular Patterns

NK Cell Natural Killer Cell

MHC Major Histocompatibility Complex

TCR T-Cell Receptors

UCMS Unpredictable Chronic Mild Stress

DHEA Dehydroepiandrosterone

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Chapter 1

Introduction

1.1 Background and Significance

Clinical obesity is an accumulation of excessive adipose tissue, and is defined as a Body Mass Index (BMI) of 30kg/m^2 or higher in adults, and a BMI $\geq 95^{\text{th}}$ percentile in children.^{13,16,18} Studies have identified that 30-40% of adults and 15-20% of children in the United States are classified as obese, with percentages increasing by 2-3 fold over the 40 years **(Figure 1-1)**.^{1,2,13,14,17} Though interventions have been implemented, there have not been any substantial methodologies to deter the ongoing obesity trends; this is especially concerning due to the global prevalence of obesity.^{2,3,4} Obesity has been shown to have a strong correlation with additional disease states, often being a contributing factor for diabetes mellitus, cardiovascular disease, and systemic inflammation.^{2,5,6,7,8,9,10}

Figure 1-1 shows the prevalence of obesity among US citizens from 1972-2014. Blue line with diamond markers are adults ages 20-47, red line with box markers are ages 2-19.¹⁴

A hallmark of obesity is an overaccumulation of endocrinologically-active adipose tissue, which acts as a reservoir for immune cells.¹¹ The composition of the adipose tissue has a significant correlation with its immunological activity, with obesity correlating with disproportionate increases in white adipose tissue (WAT), and a subsequent accumulation of immune cells, including macrophages.^{20,22} Macrophages are a primary cell type in the body's innate immune system, acting as the primary line of defense against a foreign entity.¹⁹ The functional characteristics of the macrophages are dependent on their environment, which affects its phenotype; M1 macrophages are characterized as pro-inflammatory cells, while M2 macrophages are anti-inflammatory.²² While inflammation is an important step of the immune response, chronic activation of pro-inflammatory immune cells increases the production of pro-inflammatory cytokines, which are chemical messengers that, in excess, can exacerbate the potential of developing co-morbidities.^{21,22}

Similarly, to obesity, psychological disorders have been recognized as a risk factor for the propagation of other diseases. According to the National Institute of Mental Health, more than 17 million adults have suffered a depressive episode, and approximately 10-20% of adults in the U.S. are expected to suffer from major depressive disorder, a form of chronic stress, at some point in their lives.^{23,24} Chronic stress increases neuroendocrine activation, with stress hormones like cortisol being activated in synergy with the sympathetic nervous system **(Figure 1-2)**.²⁵ As this occurs, systemic inflammation manifests, with activated immune cells secreting pro-inflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and interferon- γ (INF- γ).^{25,26,28} The chronic release of cytokines results in a positive feedback loop; continued inflammation

decreases immune cells' sensitivity to glucocorticoids, which would normally deactivate the inflammatory response.^{27,29} The persisting inflammation caused by chronic stress or obesity has negative systemic implications, including potential damage to the brain and catalyzing vascular dysfunction. Overaccumulation of immune cells increases the development of arterial plaques; combined with TNF- α acting as an antagonist of nitric oxide (NO) production, a potent vasodilator, the potential for vascular damage is greatly compounded.^{28,36,37} Persistence of stress leads to subsequent insulin resistance, a decreased sensitivity to insulin which results in mitochondrial dysfunction, oxidative stress, and an exacerbation of inflammation.⁸⁵ This pro-inflammatory milieu increases the long-term potential of developing cardiovascular disease or having a stroke.^{38,39}

Figure 1-2 shows an overview of inflammatory activation and subsequent effects resulting from psychosocial stress.²⁵

A mutual consequence of chronic stress and obesity is a surge of oxidative stress, resulting from the increase in pro-inflammatory cytokines.³⁰ Oxidative stress is the result of free radicals or reactive oxygen species (ROS) accumulating in the system, and are distinguished by their unpaired electrons **(Figure 1-3)**.¹⁵ Normally scavenged by antioxidants, an increase in ROS can lead to intermolecular reactions, ultimately causing tissue and vascular damage.^{15,31}

Figure 1-3 shows the reduction of molecular oxygen, forming free radicals.¹⁵

Along with uric acid, ROS are a major byproduct in the catalysis of xanthine, a process mediated by xanthine oxidase (XO).³² This pathway is overstimulated in obese models, leading to an influx of ROS production and subsequent tissue damage.^{31,32} Independent of obesity, xanthine+xanthine oxidase supplementation has been utilized as a model for oxidative stress induction; this model has been shown to lead to an increase in anxious and depressive symptoms, as well as insulin resistance.³³ Effects of XO have been shown to be attenuated by pharmacological intervention of febuxostat, which acts as an inhibitor of uric acid metabolism by exerting opposing action to the catalyst, XO.³⁵

Febuxostat, in turn, has been shown to inhibit ROS production and improve endothelial function (**Figure 1-4**).^{34,130}

Figure 1-4 shows **A**) effects of oxidation of hypoxanthine to uric acid and superoxide by xanthine oxidase (XO), **B**) inhibition of XO by febuxostat.¹³⁰

1.2 Purpose and Specific Aims

The purpose of this study was to examine the individual and combined effects obesity and chronic stress have on the prevalence of immune cell populations. This was done to better understand the pathways leading to vascular dysfunction. Further, the study used the chronic delivery of febuxostat in drinking water, to combat the potential dysfunction associated with the production of superoxides by way of the xanthine oxidase pathway. This was implemented to assess if febuxostat has the ability to attenuate increases in the pro-inflammatory milieu observed with the delivery of stressors. We hypothesized that obesity, chronic stress, and a combination of obesity and chronic stress would lead to an increase in pro-inflammatory immune cells and subsequent release of pro-inflammatory cytokines. Additionally, we hypothesized that the animals given febuxostat would have a decrease in pro-inflammatory agents, and an increase in anti-inflammatory cells.

Specific Aim 1: To determine the effects of chronic febuxostat treatment on brain immune cell populations in lean and obese mice.

Hypothesis: The chronic delivery of febuxostat will decrease the exacerbated pro-inflammatory milieu that is seen in obese models, giving a more anti-inflammatory phenotype, similar to lean controls.

Specific Aim 2: To determine the effects of chronic stress on the brain's immune cell populations in lean and obese mice.

Hypothesis 2: Eight weeks of unpredictable chronic mild stress will increase the prevalence of pro-inflammatory cells, including CD8⁺ T-cells and the M1 macrophage phenotype and obesity will exacerbate these effects.

Specific Aim 3: To determine the effects of chronic febuxostat treatment on brain immune cell populations in lean and obese mice that have been chronically stressed.

Hypothesis 3.1: Chronic delivery of febuxostat on lean animals that have been chronically stressed will attenuate the shift in the pro-inflammatory milieu, thereby increasing the ratio of CD4⁺ to CD8⁺ cells and decreasing the M1 to M2 macrophage ratio.

Hypothesis 3.2: The chronic delivery of febuxostat will reverse the pro-inflammatory state, resulting from a combination of obesity and chronic psychological stress. This would cause an anti-inflammatory shift, overall, leading to an increase in anti-inflammatory cells such as CD4⁺ T-cells and the M2 macrophage, with an additional decrease in the pro-inflammatory CD8⁺ T-cells and the M1 phenotype.

Chapter 2

Literature Review

2.1. The Immune System

The immune system is the body's intricate defense used to ward off potential pathogens; entities that have been internalized and are recognized as "foreign". When a pathogen is detected, the immune system utilizes a diverse array of cell types and molecular pathways to eliminate the invasive target, thereby reducing harm to the host. The immune system is generally divided into two branches: the innate immune system and the adaptive immune system.

2.1.1. The Innate Immune System

When a pathogen protrudes beyond the physical barriers of the skin and mucosa, the cellular component of the innate immune system coordinates a rapid response.⁴¹ Led by monocyte-originating macrophages, the immune cells serve a dual role in the inflammatory response: phagocytosis of foreign invaders and cytokine release.^{41,42,43} Phagocytosis is the engulfment of the invader, with intracellular enzymes nullifying the pathogen, allowing for subsequent antigen presentation to the adaptive immune system.⁴² Macrophages utilize toll-like receptors and scavenger receptors, collectively referred to as pattern recognition receptors (PRR), which allow them to recognize the microbe-associated molecular patterns (MAMP) of pathogens, leading to engulfment and phagocytosis.^{42,43,44,48}

The immunological hallmark of the Central Nervous System is the presence of microglia: cells that serve as the brain's resident macrophage population, that are housed on the internal side of the blood brain barrier.^{49,50} Like peripheral macrophages, microglia exhibit both the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype.

When activated, microglia utilize phagocytosis as a means of attack. Additionally, the phagocytic capability of microglia, in conjunction with astrocytes, is used for clearing neuronal synapses and debris.^{40,50} Failure to appropriately dispose of the neural debris as a result of microglial dysfunction or knockout has been shown to be associated with downstream synapse dysfunction, thereby drifting from homeostasis.^{40,51}

Activated macrophages initiate the inflammatory pathway. Upon phagocytizing an invader, macrophages release pro-inflammatory cytokines, chemical messengers that alert local and systemic tissues that an immune response is underway.^{42,43,48} TNF- α and IL-6 are examples of pro-inflammatory cytokines that serve as catalysts for downstream inflammatory consequences, initiating signals that lead to swelling, fever, and additional immune cell recruitment.^{41,43} This leads to the translocation of additional innate-associated immune cells, including neutrophils and natural killer (NK) cells. Neutrophils primarily attack using phagocytosis, releasing enzymes that are deadly to both pathogens and the neutrophils themselves, while NK cells form a synapse with their targets and exocytose specialized secretory lysosomes, eliminating the pathogen with their cytotoxic contents.^{41,43,47}

2.1.2. The Adaptive Immune System

Macrophages serve as a major player in immune system crosstalk; engaging in a role shared with dendritic cells, macrophages act as antigen-presenting cells. These innate immune cells engulf pathogens, and present the remaining antigen using their surface-bound Major Histocompatibility Complex (MHC), which interacts with extracellular T-cell Receptors (TCR) **(Figure 2-1)**.^{42,52} This is the marked beginning of the activation of the antigen-specific adaptive immune system.

The adaptive immune system is comprised of two chief cell types: T-cells and B-cells. T-cells are further differentiated into CD4⁺ or Helper T-cells and CD8⁺ or Cytotoxic T-cells. The distinct T-cell subsets allow the system to engage in a specialized attack. Specifically, CD4⁺ T-cells are “the quarterback of the immune system”. Once activated, CD4⁺ T-cells differentiate into effector and memory cells. Effector CD4⁺ T-cells release cytokines to increase both the activation and the rate of division of B-cells and CD8⁺ T-cells, allowing the system to mount a stronger immune response against the pathogen.⁵⁶ Conversely, activated CD8⁺ T-cells have a more aggressive nature. The CD8⁺ T-cells directly attack pathogens, including virally-infected or mutated cells.⁵⁶ Utilizing cytotoxic enzymes, including perforin and granzyme, CD8⁺ T-cells neutralize pathogens that express a specific antigen.⁵⁷ Compared to the phagocytic cells of the innate immune system, T-cells have a greater deal of specificity, when it comes to targeting pathogens.^{42,47,56}

CD4⁺ and CD8⁺ T-cells contribute neural support, as well, providing an extra degree of surveillance within the Blood Brain Barrier. While they are neuroprotective in nature, it is of note that the cells are not necessarily always in a state primed to fight; a decrease in resting catalytic potential has been observed in CD8⁺ cells, likely to serve as a protective measure.⁵³ The role of T-cells in neuroimmunity is multifaceted, and grossly ignored until recent years. T-cells have demonstrated an anti-inflammatory role, by way of IL-10 secretion, leading to a decrease in microglial-associated inflammation.⁵⁴ T-cell subsets have also been used in knockout studies, assessing contributions to the balance between autoimmune protection and disease.⁵⁵

T-cells are highly coordinated with B-cells, forming the adaptive immune system. The surface of B-cells contains antibodies, that express a heavy and light chain, with a highly-specific variable region.⁵⁸ B-cells can either come into direct contact with pathogens or be presented an antigen by a phagocytic cell from the innate immune system.^{52,58} Once stimulated, B-cells further differentiate into their effector forms, known as plasma cells. Plasma cells produce an abundance of antibodies that aid phagocytic and CD8⁺ T-cells in eliminating the threat.⁵⁶ Both T-cells and B-cells differentiate into an effector form and a memory subset. The effector lineage is responsible for the ongoing attack against the pathogen; the memory cells are clones created to continually survey the system, allowing the immune response against subsequent encounters with the pathogen to occur more quickly.^{42,52,56,58}

Figure 2-1 presentation of antigen from Major Histocompatibility Complex region of antigen presenting cell to the unique T-cell receptor for T-cell priming.⁵²

2.2. Psychological Stress

2.2.1. Definition and Prevalence of Chronic Stress

Stress is an umbrella term, often used to describe a physiological reaction to a stressor, which is defined as an event that is perceived to drive the system away from homeostasis.⁶¹ Stressors may be routine or unpredictable, and elicit a stress mediator response, led by a molecular messenger such as a corticosteroid. This leads to an adaptive change, within the system.⁶⁰ Individual instances of stress are common and can be easily adapted to, but chronic stressors can lead to overstimulation and cause a systemic deviation from baseline.⁶¹

Chronicity is a key factor when it comes to the implementation of stress models. It has been shown that a chronic stress model has the potential to cause depressive symptoms in rodents.⁶² One of the most widely accepted models is the unpredictable chronic mild stress (UCMS) protocol, which has been shown to sufficiently provide appropriate, exogenous stress to cause a depressive phenotype.⁶³ In addition to its chronic approach, the UCMS protocol's unpredictable composition avoids habituation, which would ultimately blunt the stress response; this is also modified with the intensity of the stressors.⁶⁴

The validity of the implementation of a stress protocol is attained with behavior testing, which uses an array of tasks to assess the acquisition of stress and depression-related symptoms. One of the most prevalent symptoms is the induction of anhedonia, a key component of depression that is associated with a loss in pleasurable sensation.⁶⁸ The determination of coat scores, a measure of an animal's cleanliness, or lack thereof,

is a commonly-used test to aid in determining stress – animals who show increases in stress are likely to have worse coat scores, indicating a lack of personal cleaning.^{63,69} Behavior testing methods are often done in a comprehensive fashion, utilizing multiple procedures to sufficiently determine the success of the protocol.

The negative impacts of chronic stress have the potential to translate systemically. In both a rodent and a human model, cardiovascular health has been shown to be an altered variable in the depression cascades. UCMS implementation has been shown to lead to vascular dysfunction in rodents and depression has been identified as a risk factor for cardiovascular disease in humans.^{66,67} Preliminary signs, such as increases in aggression and sleep disturbances, or decreases in sex drive, cognitive memory, sucrose preference, and food intake are measurable indicators of depression.^{70,71} Treatment of the depression can, notably, reverse many of these negative alterations.^{62,63,65} It is especially important to continue with translational studies, as an estimated 10-20% of American adults will experience major depressive disorder, indicating a surge of chronic stress.^{23,24}

2.2.2. Consequences of Stress on the Hypothalamic-Pituitary-Adrenal Axis

The Hypothalamic-Pituitary-Adrenal (HPA) Axis is a neuroendocrine network that is stimulated from perceived stress, in conjunction with sympathetic nervous system activity.^{72,74,75} This results in the activation of the hypothalamus, a neuroendocrine tissue that releases corticotropin-releasing hormone (CRH). CRH elicits downstream activation of the pituitary gland, causing the release of adrenocorticotropin hormone (ACTH). This ultimately results in the production of glucocorticoids, which are secreted by the adrenal glands.⁷⁶ This response functions to increase blood glucose levels to provide the system

with energy, while simultaneously inhibiting the release of pro-inflammatory cytokines such as TNF- α , IL-6, and IFN- γ and upregulating the release of anti-inflammatory cytokines IL-4 and IL-10.⁷² The hypothalamus detects increasing levels of cortisol, and in a negative feedback fashion, terminates the cascade – additionally, dehydroepiandrosterone (DHEA) is secreted to act as an antagonist to the glucocorticoids.⁵⁹ This negative feedback loop allows the system to appropriately adjust to a perceived stress, and return to homeostasis.^{59,75,77}

While acute stress is a part of the everyday response, chronic exposure to stress can have adverse neural, systemic, and immune consequences.⁷⁴ When the negative feedback loop fails to appropriately reduce levels of glucocorticoids, hypercortisolism occurs. This chronic increase in cortisol has been shown to stunt the release of Growth Hormone and its subsequent actions, as well as DHEA and testosterone.⁷⁶ Due to cortisol's immunosuppressive effects, both the actions and proliferation of the components of the innate and adaptive immune systems can be blunted, potentially leading to an immunocompromised state.⁵⁹ Chronic stress can also invoke another serious phenomenon known as hypocortisolism.⁷³ The term encompasses absolute (i.e. overall low levels) relative (i.e. lower than normal in a given situation) cortisol levels, as well as overall ability to bind; though, exact repercussions have not been thoroughly defined.^{73,77} In opposition to the immune response in hypercortisolism, a cortisol deficiency will lead to an influx of pro-inflammatory cytokines, subsequent recruitment of immune cells, leading to the possibility of tissue damage, metabolic dysregulation, autoimmune disease acquisition, and vascular damage.^{59,73,78,79}

2.3. The Obese Phenotype

2.3.1. Collective Trends

Clinical obesity is a state of excess adiposity that acts as a risk factor for a number of negative physiological consequences – this includes, but is not limited to, cardiovascular disease, metabolic disorders, and musculoskeletal impairments. This fat accumulation has the potential to lead to systemic dysfunction, and is especially of concern when it is accumulated in areas closer to visceral organs.¹⁰² While potentially a result of genetic or endocrine issues, obesity is often acquired as a result of malnutrition by means of excessive energy consumption and a lack of structured, planned physical activity.¹⁰³ Studies have identified that nearly two thirds of the U.S. adult population is classified as overweight, with approximately 50% of them being considered obese.^{1,2,81} The onset of an obese phenotype has been shown to cause an increase in both endocrine activity and systemic inflammation.⁹⁰

2.3.2. Adipokines

The endocrine-active adipose tissue regularly secretes tissue-specific cytokines, dubbed adipokines, with the two most well-researched being leptin and adiponectin.^{93,94,95,96} Having significant influences on appetite regulation and substrate metabolism, respectively, these adipokines play a huge role in establishing homeostasis in a physiological system.^{97,98} Interestingly, environmental alterations can have adverse effects on the functionality of these adipokines.⁹⁹

Under normal circumstances, adiponectin has the potential to aid in energy conservation and attenuate insulin resistance.^{93,95,98,100,101} Adiponectin can bind receptors

AdipoR1 and AdipoR2, creating a phosphorylation cascade that mediates insulin sensitization.^{93,94} The molecule's dual role of being anti-inflammatory and insulin-sensitizing makes it a critical adipokine.^{93,94,95} However, a systemic deviation, such as excess adiposity, leads to a decrease in the concentration of circulating adiponectin, altering metabolism as a result of increased insulin resistance.⁹⁶ This result is further exacerbated by an increase in another cytokine named resistin, an adipocyte-derived molecule whose concentration has an inverse correlation with insulin resistance.¹⁰⁴ In contrast to the anti-inflammatory adiponectin, leptin has been shown to exhibit pro-inflammatory properties.^{93,94,95} Leptin normally signals to the hypothalamus that the body has sufficient energy stored; the development of obesity leads to hypothalamic leptin resistance, and an inevitable increase in the adipokine.^{96,97} This influx of leptin reinforces hypothalamic resistance, while also contributing to a pro-inflammatory environment.^{93,94,95}

2.3.3. Consequences of Obesity

The establishment of systemic homeostasis is highly dependent on the physiological environment. Under normal conditions, many systems work in synergy to attain a homeostatic phenotype. This balance is a result of a specific coordination between both central and peripheral branches. Chapelot and Charlot specified six vital systems that contribute to homeostasis (**Figure 2-2**).⁸⁰

Adipose tissue is a vital structure that has been well-documented for serving thermogenic and metabolic purposes; while it is commonly known as a means of storing energy, adipose tissue is also recognized as an endocrine organ. By releasing hormones and adipokines, including leptin and adiponectin, adipose tissue has a significant influence on metabolic homeostasis.^{82,83} However, while it is necessary for survival,

excessive amounts of the lipid-rich tissue can lead to negative consequences. The accumulation of adipose tissue, by way of adipocyte hypertrophy and adipocyte hyperplasia, leads to the onset of obesity.^{82,84}

Excessive adiposity has the potential to induce insulin resistance. Insulin is a key metabolic mediator that is released from pancreatic β -cells, in response to a rise in blood glucose.^{81,85,86} Insulin's cell receptor is an $\alpha_2\beta_2$ disulfide-linked heterodimer, a transmembrane protein with an extracellular insulin binding site.⁸⁶ Insulin causes a conformational change to its respective receptor, leading to autophosphorylation and subsequent phosphorylation of IRS1/2 and further downstream molecules.^{85,86} This cascade leads to the translocation of the GLUT-channels, which allow glucose to move from the blood, into the cell; this cascade is disrupted in the obese phenotype.^{81,87} The ongoing systemic inflammation can weaken insulin's ability to bind to a receptor, and can lead to dephosphorylation of intermediates. The shift in the environment is largely attributed to an imbalance of pro and anti-inflammatory cytokines, resulting from the accumulation of fat, causing a state of systemic inflammation.⁸⁷ The obese phenotype has been shown to lead to an increase in myeloid-derived M1 macrophages; unlike their M2 counterparts, M1 macrophages exacerbate inflammation, releasing cytokines that continue to propagate a systemic immune response.^{83,88,89,90,91} This translates to a subsequent increase in the adaptive immune response, especially CD8⁺ T-cells. As a result, anti-inflammatory molecules, such as adiponectin and IL-10, are overpowered.⁹²

Figure 2-2 showcases the complex interplay of a multitude of systems providing feedback to attain systemic homeostasis. The six specified contributors are: 1) afferent network of hormones and neurons, 2) the hypothalamus, 3) the brain stem, 4) the reward system, 5) the prefrontal cortex, 6) the sympathetic nervous system.⁸⁰

2.4. Oxidative Stress

2.4.1. Physiological Mechanisms

Oxidative stress is the physiological imbalance between ROS/free radicals and antioxidants, within a system. ROS are derivatives of oxygen that contain unpaired electrons: common examples include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot-}$), and nitric oxide ($NO^{\cdot-}$).¹²³ ROS are a common byproduct of aerobic metabolism; during oxidative phosphorylation, the creation of the proton gradient assists in the production of ATP.^{121,122,123} However, the system will have occasional errors, resulting in an incomplete transfer of available electrons within the electron transport chain. In addition, they can be a byproduct of enzymatic pathways, including the NADPH oxidase and XO pathways.^{122,123} While an excess of ROS can be damaging, these compounds assist in maintaining homeostasis; one such example being the generation of nitric oxide to stimulate vasodilation.

In a homeostatic environment, excess ROS are nullified by antioxidants, including vitamins A, C, E, and also glutathione.^{121,122,123} Additionally, select enzymes scavenge for free radicals, to prevent excessive accumulation. Indeed, proteins including catalase, superoxide dismutase, and glutathione peroxidase work to prevent an overabundance of free radicals.¹²¹⁻¹²⁴ When an unbalanced system allows them to accumulate, free radicals can cause severe cellular and tissue damage. With a goal of capturing an electron, free radicals can commence lipid peroxidation; the double bond of an unsaturated fatty acid of the plasma membrane is often the target, leading to the potential of damaging the cell membrane.¹²¹⁻¹²⁴ In addition, ROS can engage with proteins. These radicals form covalent bonds with proteins, aiming to steal an electron. This binding process has the potential to disturb both the active site or the overall conformation of the protein, and may

render it useless.^{122,123} In a similar manner, ROS can lead to DNA damage; this can lead to mutation of the DNA, or overall breaking of the strands.¹²¹⁻¹²⁴

2.4.2. Xanthine Oxidase and Febuxostat Attenuation

XO is a major enzymatic source of ROS. The starting material of the pathway is hypoxanthine, a derivative of adenosine. XO catalyzes the reaction in a two-step process, resulting in uric acid and the creation of free radicals.^{122,123} Indeed, cardiovascular dysfunction has been linked to the uric acid pathway, with XO being the primary problem.¹²⁷ From a pharmacological standpoint, delivery of anti-inflammatory and anti-hypertension drugs can increase vascular reactivity and reduce vascular stiffening, respectively.¹²⁸ XO's activity can be reversibly inhibited by the drug febuxostat, decreasing the end production of ROS.^{35,126} Febuxostat acts by blocking the conversion of hypoxanthine to xanthine and xanthine to uric acid. As a therapeutic agent, febuxostat delivery has been shown to attenuate atherosclerosis in mice, decreasing ROS production and returning vascular dilatory capacity.³⁴ Febuxostat has been shown to be particularly effective in attenuating XO activity in pathological states, and decreases ROS production in vascular endothelium.¹²⁹

Figure 2-3 Febuxostat causes reversible inhibition of xanthine oxidase action.

Chapter 3

Methods

3.1. Animal Models

Mice (n=24), aged 16 weeks, arrived at the West Virginia University Health Sciences Center from the Jackson Laboratory, and were fed a standard diet to retain a lean phenotype. An additional 24 mice, aged 6 weeks, arrived from the Jackson Laboratory, and were placed on a high-fat diet (60 kcal% fat) to induce obesity (Research Diets, New Brunswick, NJ, D12492). Half of the animals in each cohort were chronically delivered febuxostat in their water bottles. The lean animals were then separated into one of four groups: 1) lean control, 2) lean control + febuxostat, 3) lean UCMS, 4) lean UCMS + febuxostat. The obese counterparts were also separated into four groups: 1) obese control, 2) obese control + febuxostat, 3) obese UCMS, 4) obese UCMS + febuxostat. All mice received chow and water ad libitum, and protocols received approval from the West Virginia University Health Sciences Center Animal Care and Use Committee prior to experimentation.

3.2. UCMS Protocol

The UCMS Protocol was developed to produce depression-like behaviors in rodents. This approach is the most appropriate model for clinical depression in rodents and mimics human-like depression behaviors, including anhedonia and learned helplessness. During the UCMS protocol, mice were singly-housed, and randomly exposed to the following mild environmental stressors for 7 hours each day, 5 days per week, over the 8-week course:

1. Damp bedding – 10 oz. of water was added to each standard cage

2. Bath – all bedding was removed and ~0.5 inches of water was added to the empty cage. Water temperature was at room temperature, ~24°C
3. Cage tilt – cage was tilted to 45° without bedding
4. Social stress – each rat was switched into a cage of a neighboring rat
5. No bedding – all bedding was removed from the cage
6. Alteration of light/dark cycles – turning the lights off/on in random increments for a scheduled period

3.3 Coat Scores

To validate the unpredictable chronic mild stress paradigm, animal coat scores were assessed at 8 anatomical sites (head, neck, back, stomach, tail, forelimbs, hindlimbs, and genitals). Scoring was binary with 0 meaning “clean”, and 1 equating to “dirty”. The sum of the 8 sites equaled the coat score.

3.4. Flow Cytometry

3.4.1. Spleen Processing

Following terminal procedures, the spleen was removed and placed in a weigh boat containing 3mL of Roswell Park Memorial Institute medium (RPMI) and placed on ice. Using two glass microscope slides, the spleen was thoroughly crushed, and filtered through a 70µm strainer, into a 50mL conical. The sample was then spun down at 1500 RPM for 5 minutes, and the resultant supernatant was poured off. The remaining pellet was re-suspended in 3mL of tris-buffered ammonium chloride, vortexed, and placed on ice for 10 minutes, while red blood cell lysis occurred. The total volume of the solution

was then doubled by adding RPMI, and re-centrifuged at 1500 RPM for 5 minutes. The supernatant was then discarded, and the remaining leukocytes were re-suspended in 5 mL of RPMI. Samples were left on ice until the other tissues were prepared.

3.4.2. Brain Homogenization

After terminal procedures, the brain was removed and transferred to a weigh boat containing 2mL of ice cold RPMI, and placed on ice. Using a clean razor blade, the brains were minced into a fine consistency, and transferred to a gentleMACS C tube. Next, 300 μ L of DNase and 150 μ L of collagenase were added into each tube, and the total volume was brought up to 5mL with additional RPMI. Tubes were then transferred to and started on the gentleMACS Program 37C_ABDK_01 for 30 minutes.

3.4.3. Brain Immune Cell Isolation

Following the incubation, the resultant solution was filtered using a 70 μ m strainer, into a clean 50mL conical. Using RPMI, the total volume was brought up to 10mL. The samples were then centrifuged at 1500 RPM for 5 minutes at 4°C. Following the centrifugation, the supernatant liquid was removed from the remaining pellet. The pellet was then re-suspended in 4mL of 70% isotonic percoll. In a clean 15mL conical, 7 mL of 30% percoll was added, with the newly-suspended pellet being added as an underlay. The conical was centrifuged at 500xg for 20 minutes at 22°C with an acceleration of 4 and break of 0. This centrifugation resulted in the establishment of a gradient, with immune cells residing in a foam-like appearance in the middle. The cells were extracted

using a pipette with a beveled tip, transferred to a 15mL conical, and placed on ice. The tubes were brought up to 9mL with RPMI and spun down at 400xg for 5 minutes at 4°C. The resultant supernatants were discarded, and the remaining pellets were suspended in 1mL of RPMI.

3.4.4. Cell Counting

Cells were diluted to count on the hemocytometer, with a 1:2 dilution used for the brain (10μL of sample + 10μL of dye) and a 1:100 dilution used for the spleen (1μL of sample + 99μL of dye). Samples were loaded on the hemocytometer and counted under the microscope. The 16-block quadrants were counted and used to estimate the number of cells/mL of sample:

$$\frac{\text{Sum of \# of cells counted}}{\text{\# of quadrants counted}} * \text{dilution factor} * 10^4 = \text{\# of cells / 1 mL}$$

$$\frac{\text{\# of desired cells}}{x} = \frac{\text{\# of cells}}{1 \text{ mL}} \rightarrow x = \frac{\text{\# of desired cells}}{\text{\# of cells}}$$

Figure 3-1 Stained spleen cells magnified under microscope

3.4.5. Cell Staining

After volumes were calculated, with respect to the desired number of cells, single stain controls and experimental samples were added to a 96-well plate. Calculated volumes were added to each well, and each sample was washed by adding 100 μ L of phosphate-buffered saline with azide (PBS-Az), followed by centrifugation at 1500 RPM, at 4°C, for 5 minutes. The resulting supernatant was disposed of and the wash was repeated with 200 μ L of PBS-Az. The cells were then blocked with mouse Fc Block (CD16/CD32, BD Biosciences) and incubated on ice for 15 minutes. Cells then underwent surface staining with fluorescently-tagged antibodies: CD45-BB700 (BioLegend), CD11b-PE-Cy5 (BioLegend), Ly-6C-PE-CF594 (BD Biosciences), Ly-6G-APC-Vio770 (Miltenyi

Biotech), CD11c-BV480 (BD Biosciences), CD206-PE-Cy7 (BioLegend), CD4-FITC (Miltenyi Biotec), CD3e-PE (Miltenyi Biotec), CD8a-APC (Miltenyi Biotec), and CD45R-Alexa Fluor 700 (Thermo Fisher Scientific). Following a 30 minute incubation, the cells were washed 3x as described above, using 200 μ L of PBS-Az. After the final wash, 100 μ L of 2% paraformaldehyde was added to each well, for overnight fixing of the cells.

Antibodies

Antibody markers were utilized to identify specific immunological cell types within the tissues (**Table 3-1**).

Table 3-1: Antibody-to-cell type pairings

Antibody	Cell Type
CD45 ⁺	General White Blood Cells
(CD45 ⁺)CD3 ⁺	General T-Cells
(CD45 ⁺ CD3 ⁺)CD4 ⁺	Helper-T Cells
(CD45 ⁺ CD3 ⁺)CD8 ⁺	Cytotoxic-T Cells
(CD45 ⁺)CD45R ⁺	B-Cells
(CD45 ⁺)CD11c ⁺ CD206 ⁻	M1 Macrophages (pro-inflammatory)
(CD45 ⁺)CD206 ⁺ CD11c ⁻	M2 Macrophages (anti-inflammatory)

3.4.6. BD LSRFortessa

Following a 12-hour minimum membrane fixation, samples were transferred from their respective wells to test tubes, using 300 μ L of PBS-Az for each well. The samples were run on the BD LSRFortessa, which utilizes a spectrum of lasers to excite the fluorescent tags. By utilizing a blank control and an array of single stain controls, the LSRFortessa was able to measure the wavelengths of the excited fluorescent markers, determining the profile of cell type percentages in the samples.

3.5. Data analysis

3.5.1. Gating strategies

After the samples were run on the LSRFortessa, data files were produced, which were used to quantify the percentages of immune cell populations. **Figure 3-2** shows the gating strategy used to discriminate between cell types. The FCS Express 6 Flow Research Edition software was utilized for analysis. Beginning with the inclusion of all cells, gates were able to be created to exclude cells based on their fluorescent tag. This allowed for the discrimination of each cell type of interest.

Figure 3-2 Gating strategy to determine cell populations obtained from LSRFortessa using FCS Express 6 Flow Research Edition software.

3.5.2. Data and Statistical analysis

All data are presented as mean $\pm$ SEM. Normality was evaluated by the Kolmogorov–Smirnov test. Anthropometric assessments were analyzed by analysis of variance (ANOVA) for UCMS condition, as well as by analysis of co-variance (ANCOVA) for exposure by obesity. The distribution of immune populations were examined based on specific aims. A One-way ANOVA was used to compare the differences between lean and obese groups in the presence and absence of febuxostat, and a Tukey post-hoc test to determine differences between groups. Similarly, comparisons between control and UCMS groups (by lean or obese group) with or without febuxostat was examined by two-way ANOVA with a Tukey post-hoc. In all cases, $p \leq 0.05$ was taken to reflect statistical significance.

Chapter 4

Results

Results

Animal Characteristics

Implementation of the high-fat diet resulted in significant increases in body mass between lean and obese animals. The addition of the UCMS protocol to the obese mice resulted in a significant decrease in end mass that was not seen in any of the other groups (Table 4-1).

Table 4-1: Animal Characteristics

	Lean Mass (g)	Obese Mass (g)
Control (n=6)	28.4±0.8 *	48.4±0.7 **
Control+Feb (n=6)	27.9±1.1 *	51.4±1 **
UCMS (n=6)	26.7±1.8 *	41.5±0.8
UCMS+Feb (n=6)	28.8±0.8 *	45.5±3.1 **
*p<0.0001 vs Obese Control; **p<0.05 vs Obese UCMS		

Coat Scores

Coat scores were collected to assess acquisition of depressive phenotype. UCMS significantly increased coat score values in both lean and obese groups (**Table 4-2**).

Table 4-2: Coat Scores

	Lean Coat Score	Obese Coat Score
Control (n=6)	0.1±0.1	0±0
Control+Feb (n=6)	0.3±0.2	0±0
UCMS (n=6)	7.3±0.3 **	7.2±0.2 *
UCMS+Feb (n=6)	7.3±0.4 **	7.5±0.2 *
*p<0.0001 vs Obese Control, **p<0.05 vs Lean Control Scoring scale: 0=normal grooming behavior. Max value=8 and indicates worst grooming and self-care behavior.		

Figure 4-1 shows the changes observed in total white blood cells (CD45⁺) for lean and obese mice, with and without febuxostat treatment. Obese controls had significantly less CD45⁺ cells than their lean counterparts ($p < 0.05$). In contrast, the febuxostat treatment did not result in any significant differences in the prevalence of CD45⁺ cells, in either lean or obese mice.

After identifying the white blood cells, specific cell populations were parsed out. In Figure 4-2, T-cells and T-cell subsets were examined. Compared to lean mice, obese mice had significantly lower CD3⁺ cells ($p < 0.05$), indicating an overall decrease in total T-cell populations (Fig. 4-2A). In the obese control group, there was a significant increase in positive staining for both CD4⁺ cells ($p < 0.01$; Fig. 4-2B) and CD8⁺ ($p < 0.05$, Fig. 4-2C) compared to lean controls. The ratio of CD4⁺ to CD8⁺ cells was also calculated, with higher numbers being associated with an anti-inflammatory phenotype and lower numbers being associated with a pro-inflammatory phenotype. Between the lean and obese groups, no statistically significant differences were observed in the CD4⁺ to CD8⁺ ratio (Fig. 4-2D). The addition of febuxostat did not result in any statistically significant differences in CD3⁺, CD4⁺, or CD8⁺ cells, nor in the CD4⁺ to CD8⁺ ratio.

In addition to T-cells and their subsequent populations, B-cells (CD45R⁺) were identified (Figure 4-3). The percentage of B-cells was shown to be significantly lower in the obese controls' brains, compared to lean controls ($p < 0.05$). Febuxostat treatment did not result in any significant differences in CD45R⁺ cells, in either lean or obese mice.

Figure 4-4 examines macrophage populations, specifically focusing on the pro-inflammatory M1 phenotype (CD11c⁺CD206⁻) versus the anti-inflammatory M2 phenotype (CD206⁺CD11c⁻). No statistically significant differences were observed in the M1

populations in the lean and obese control groups (Fig. 4-4A), though the obese controls showed a significant decrease ($p<0.05$) in M2 populations (Fig. 4-4B). The M1 to M2 ratio was also calculated, with higher values corresponding to a pro-inflammatory environment, and lower values being associated with an anti-inflammatory environment. There were no significant differences amongst the lean and obese controls (Fig. 4-4C). Febuxostat treatment did not result in any significant differences in M1 or M2 macrophages, or the M1 to M2 ratio.

Effects of Chronic Febuxostat Treatment on Brain Immune Cell Populations and the Role of Obesity

Figure 4-1: Percentage of white blood cells (CD45⁺) in lean and obese animals with febuxostat (Non-UCMS+Feb) treatment. * $p<0.05$ vs lean control (Non-UCMS). $n=4-5$ per group.

Figure 4-2: Percentage of A) T-cells (CD3⁺), B) Helper-T cells (CD4⁺), C) Cytotoxic-T cells (CD8⁺), and D) the CD4⁺ to CD8⁺ ratio (an indicator of inflammation) in lean and obese animals with febuxostat (Non-UCMS+Feb) treatment. $p < 0.05$ vs lean control (Non-UCMS). $n = 4-5$ per group.

Figure 4-3: Percentage of B-cells (CD45R⁺) in lean and obese animals with febuxostat treatment (Non-UCMS+Feb). $p < 0.05$ vs lean control (Non-UCMS). $n = 4-5$ per group.

Figure 4-4: Percentage of A) M1 macrophages, B) M2 macrophages, C) the M1/M2 ratio (an indicator of inflammation) in lean and obese animals with febuxostat treatment (Non-UCMS+Feb). $p < 0.05$ vs lean controls (Non-UCMS). $n = 4-5$ per group.

Effects of UCMS on Brain Immune Cell Populations in Lean and Obese Mice

Figure 4-5 represents the CD45⁺ populations in controls versus UCMS implementation. No significant differences were observed between the lean controls and the lean UCMS mice. In comparison to the obese controls, the obese UCMS cohort showed a significant increase in total white blood cells ($p < 0.01$).

T-cell populations were also identified, as seen in Figure 4-6. In terms of total T-cells (CD3⁺), there were no significant differences between the lean and obese controls compared to the UCMS groups (Fig. 4-6A). As such, CD4⁺ cells, CD8⁺ cells, and the CD4⁺

to CD8⁺ ratio was similar between lean and obese controls vs. UCMS groups (Fig. 4-6B-4-6D).

Figure 4-7 shows the populations of CD45R⁺ cells (B-cells). UCMS implementation did not significantly alter the B-cell populations in the lean cohort. While not statistically significant, the obese UCMS group exhibited a downward trend, in comparison to the non-stressed obese group.

M1 and M2 macrophages are represented in Figure 4-8. Figure 4-8A shows the M1 phenotype, in which a positive trend was observed between lean controls and the lean UCMS group. However, no differences were noted between obese controls and obese UCMS group. In regard to M2 macrophages, there was no significant difference observed between the lean controls and lean UCMS groups (Fig 4-8B) and no differences were noted in the M1 to M2 ratio (Fig. 4-8C). However, the obese UCMS cohort experienced a significant decrease ($p < 0.05$) in comparison to obese controls (Fig. 4-8B). However, the UCMS protocol did not result in differences in the M1 to M2 ratio (Fig. 4-8C).

Figure 4-5: Percentage of white blood cells (CD45⁺) in lean and obese animals with UCMS. *p<0.05 vs obese control (Non-UCMS). n=4-5 per group.

Figure 4-6: Percentage of A) T-cells (CD3⁺), B) Helper-T cells (CD4⁺), C) Cytotoxic-T cells (CD8⁺), and D) the CD4⁺ to CD8⁺ ratio (an indicator of inflammation) in lean and obese animals with UCMS. n=4-5 per group

Figure 4-7: Percentage of B-cells (CD45R⁺) in lean and obese animals with UCMS. n=4-5 per group.

Figure 4-8: Percentage of A) M1 macrophages, B) M2 macrophages, C) the M1/M2 ratio (an indicator of inflammation) in lean and obese animals with UCMS. $p < 0.05$ vs obese controls (Non-UCMS). n=4-5 per group.

Effects of Febuxostat Treatment on Brain Immune Cell Populations in Lean and Obese Mice with UCMS

Expanding on the UCMS paradigm, Figure 4-9 shows the total population of CD45⁺ cells, examining febuxostat treatment. In regard to total white blood cells, the chronic delivery of febuxostat did not result in any statistically significant differences, between the groups.

Figure 4-10 illustrates alterations in the respective T-cells and T-cell subsets with the chronic treatment of febuxostat in lean and obese mice exposed to UCMS. Overall, there were no statistically significant differences in total T-cells (CD3⁺), Helper-T cells (CD4⁺), Cytotoxic-T cells (CD8⁺), or the CD4⁺ to CD8⁺ ratio (Figures 4-10A-4-10D) between groups.

B-cells are seen in Figure 4-11. Again, chronic febuxostat treatment did not have any significant effects on the CD45R⁺ populations between groups.

Figure 4-12 shows macrophage subsets. Chronic febuxostat treatment did not lead to any significant alterations in the M1 phenotype (Fig. 4-12A), the M2 phenotype (Fig. 4-12B), or the M1 to M2 ratio (Fig. 4-12C) between groups.

Figure 4-9: Percentage of white blood cells (CD45⁺) in lean and obese animals with UCMS and febuxostat treatment (UCMS+Feb). n=4-5 per group.

Figure 4-10: Percentage of A) T-cells (CD3⁺), B) Helper-T cells (CD4⁺), C) Cytotoxic-T cells (CD8⁺), and D) the CD4⁺ to CD8⁺ ratio (an indicator of inflammation) in lean and obese animals with UCMS and febuxostat treatment (UCMS+Feb). n=4-5 per group.

Figure 4-11: Percentage of B-cells (CD45R⁺) in lean and obese animals with UCMS and febuxostat treatment (UCMS+Feb). n=4-5 per group.

Figure 4-12: Percentage of A) M1 macrophages, B) M2 macrophages, C) the M1/M2 ratio (an indicator of inflammation) in lean and obese animals with UCMS and febuxostat treatment (UCMS+Feb). n=4-5 per group.

Chapter 5

Discussion

5.1 Alterations to Immune Cell Populations

The primary purpose of this study was to assess alterations in neuroimmune cell populations, as a result of obesity, chronic stress, and a combination of the two. Furthermore, the study aimed to gauge if febuxostat, an inhibitor of xanthine oxidase, would attenuate any negative alterations. Consistent with our hypothesis, we observed an alteration to immune cell populations in response to chronic stress delivery, in some cases. However, contrary to our hypothesis, we did not find a consistent pro-inflammatory response with obesity, and observed minimal involvement from febuxostat.

Previous work has shown that obesity leads to a constant state of systemic inflammation, indicating an absolute increase in white blood cells.¹⁰⁶ Adipose tissue serves as a reservoir for activated immune cells, with macrophages being one of the most populous cell types. In a pro-inflammatory environment, the M1 phenotype of macrophages has a higher prevalence than the generally anti-inflammatory M2. In terms of cytokine secretion, M1 macrophages are notorious for releasing messengers including TNF- α and IL-6, causing inflammation and recruitment of additional immune cells.^{42,43} To a lesser degree, M2 macrophages contribute to inflammation due to their (albeit, lower) expression of MHC II. This extracellular immune complex allows for the presentation of a pathogenic antigen to a CD4⁺ T-cell and leads to its activation.⁵⁶ In the study at hand, diet-induced obesity did not produce the expected results, based on the understanding of the physiological consequences of obesity. Beginning with total white blood cells, the obese phenotype did not result in the significant increase that was expected. In the context of microglia, obesity did not lead to the expected increase in the M1 phenotype, though it did result in a significant decrease in the anti-inflammatory M2 phenotype.

However, this did not translate to the M1:M2 ratio. As microglia present antigens, activating CD4⁺ T-cells, additional immune cells become activated; active CD4⁺ T-cells secrete IFN- γ , acting in a positive feedback loop, increasing macrophage recruitment.⁵⁶ Additionally, CD4⁺ T-cells induce the activation and proliferation of both CD8⁺ T-cells and B-cells, further contributing to the systemic inflammation.^{42,52} As such, it was expected that there would be an overall increase in T-cells, T-cell subsets, and B-cells and a decrease in the CD4⁺ to CD8⁺ ratio. The ratio of CD4⁺ to CD8⁺ cells is used as a general reference point for immune system activation, with normal values considered to be 1.5-2.5; an intense immune response would result in CD8⁺ T-cell proliferation, causing an inversion of the ratio.^{105,106,108} The data did not entirely align with what would be expected; there was an overall decrease in T-cells, though there were greater percentages staining CD4⁺ and (independently) CD8⁺. There were no significant differences in the T-cell ratio, and unexpectedly, B-cell quantity decreased. Overall, the acquisition of the obese phenotype did not result in much of a significant difference, compared to controls. This conclusion is also true for lean and obese animals that were given febuxostat in their drinking water; the xanthine oxidase inhibitor did not seem to significantly affect the obese cohort.

The implementation of the unpredictable chronic mild stress protocol sought to deliver a chronic stress component. Bouts of stress activate the HPA axis, which leads to the downstream secretion of glucocorticoids. While the mechanism is a negative feedback loop that is programmed to turn itself off, chronic stress overloads the system and can lead to tangential, adverse immune effects. For example, the system has the potential of overexpressing cortisol, causing immunosuppression. Conversely, another

fate is hypocortisolism, preventing immunosuppression. While cortisol levels were not assessed, two pieces of evidence that provide support for the theory of hypocortisolism: a significant decrease in total white blood cells in the obese UCMS population, compared to the lean UCMS group, and a significant decrease in M2 macrophages. Though they were statistically significant, these data are not sufficient to fully explain what is happening in the system. Cortisol values were not measured for this study and there were no other significant cell population alterations. Similar studies that have used the UCMS protocol have reported data both on cellular alterations, as well as changes in cytokines. For example, Chiang et al. found pro-inflammatory cytokines pre- vs. post-stress protocol, show a positive relationship between stress and inflammation.¹⁰⁹ Additional reviews detailed a correlation between stress, immunosuppression, and the neuroimmune profile, such as activated microglia recruit peripheral T-cells to the central nervous system, creating an exacerbated pro-inflammatory environment, resulting in harm to the host.^{110,111}

Due to the lack of evidence that a pro-inflammatory environment was established with obesity in our study, our ability to interpret the effectiveness of chronic febuxostat delivery as a therapy to combat inflammation is limited.

Under normal circumstances, xanthine oxidase catalyzes the reaction of hypoxanthine to uric acid – the primary problem with this is the downstream production of ROS. An increase in ROS leads to a reduction in available NO⁻, and greater potential to sustain tissue injury.¹²⁶ This additionally exacerbates the pro-inflammatory response, happening concurrently in the stressed system. This chronic occurrence leads to a decline in vascular function, in the form of dilatory impairment.^{78,126} Although, the use of

febuxostat has been challenged – while it has been shown to be successful in inhibiting XO, febuxostat has demonstrated an inability to cross the blood brain barrier, mitigating the compound's ability to be especially successful at reducing oxidative stress in the central nervous system.¹²⁵ However, instances of intense stress can compromise the integrity of the blood brain barrier;. Previous studies have determined the reduced function of the blood brain barrier can allow for larger molecules, including pro-inflammatory cytokines, to cross.^{112,113,114}

5.2. Confounding Factors

Body mass and assessment of coat scores were conducted to provide both qualitative and quantitative assessment of the exposure model/conditions. Based on the significant difference in body mass and coat score between the experimental groups, it is safe to say that the exposure was successfully implemented. However, conducting the flow cytometry protocol was met with several experimental challenges.

Flow cytometry itself was done in an unconventional way, to accommodate concurrent project aspects. Flow cytometry-centric projects usually aim to keep the number of surgery days to a minimum – having the number of trials in the study be spread over the course of 6 weeks made for a huge challenge by itself, as data for flow cytometry is acquired by 1) homogenizing and staining the cells, 2) incubating for a 12-hour minimum to allow for plasma membrane fixation/up to 2 weeks, 3) running the samples on the LSRFortessa, 4) creating a gating strategy to discern cell populations, and 5) developing plots to determine the percentage of each cell type.

Flow cytometry consists of mostly basic steps, but is lengthy, utilizes multiple incubation periods, and requires full attention. In addition to this, certain steps, such as adding the percoll underlay, required a great deal of practice to properly execute. Inconsistencies occurred due to the limited use of equipment due to conflicting time schedules. This conflict was most exaggerated with running the samples, as some were run as early as possible, while others had to wait the full two weeks. This impacts the size of the cells, and can cause the LSRFortessa to inaccurately classify certain cells. In this study, given the time invested in the exposure protocol, we sought to differentiate using 9 fluorescently-labeled antibodies to gain the most information possible. However, having this quantity increased the difficulty of data acquisition and subsequent analysis. Flow cytometry also involves a degree of bias, as there are somewhat less-defined setpoints on the exact spot to put gates on single-stain controls, with a similar problem occurring during gate analysis. Due to all of this, the original schema of 6 animals per group dwindled to 4-5 per group, with inconsistencies being prevalent throughout them. If surgery days were minimized to focus on this immune aspect, the consistency of the end products would likely be more similar, as well. In addition to flow cytometry, utilizing complementary methodologies would create a more comprehensive picture. Intracellular staining could be used to expand on the flow cytometry method, allowing for data collection of cytokines. In addition, integrating a methodology such as an ELISA or immunohistochemistry could be done. Either of these protocols could be used to verify the presence of pro- and anti-inflammatory cytokines, confirming cellular actions at the molecular level.

5.3. Limitations

The examination of the pro-inflammatory milieu requires a complex approach. Utilizing flow cytometry as a means of identifying cell populations was an appropriate first step to uncovering the cellular adaptations. With that being said, successful completion of the protocol is dependent on the skilled execution of many crucial and meticulous steps – due to the high potential of error, one of the limitations was having a relatively low sample size, that became even lower in some groups due to errors. In addition, the protocol implementation was not ideal, as it is more common to process all of the animals for flow cytometry in one day, in contrary to this 6-week approach.

We only evaluated flow cytometry for this study. While this aids in identifying cell populations and can help to establish comparisons between controls and stressors, the types of cells themselves do not totally define what the systemic physiology looks like, as we have not looked at the concentration of pro- and anti-inflammatory cytokines, or determined whether or not the mice showed any other signs of systemic dysfunction. The aim was to highlight a shift to a pro-inflammatory cellular profile, and later distinguish evaluate cytokines.

5.4. Future Research

In this study, the only methodological approach used was flow cytometry as a means of discerning general cell populations. This methodology can be expanded on – because the phenotype of macrophages is so crucial to their function, isolating enough brain immune cells could help to parse out M1 and M2 macrophages in the brain, instead of just having a wholistic picture.

This same concern also applies to the adaptive immune system assessment of T-cells. The current approach for determining pro- vs anti-inflammatory involves determining the CD4⁺ to CD8⁺ ratio. While it is a noted approach, using this ratio may not necessarily be the most ideal due to gauge the inflammatory status as both have previously been shown to increase with stress. However, a subset of CD4⁺ T-cells, known as regulatory T-cells, show a more direct correlation with the inflammatory status of the environment.

In addition to the surface staining protocol, it would also be informative to employ intracellular staining. This is a subset of flow cytometry that involves permeabilizing the plasma membrane to allow the infiltration of antibodies. This provides a more in-depth look at the actions of the cells, as cytokines are able to be identified and quantified. As an additional alternative, the use of protocols such as immunohistochemistry can be utilized to discover the cytokines at hand.

Because febuxostat acts as an antagonist to a pathway that ultimately results in the production of superoxides, it would be wise to measure any alterations in that activity. Superoxides have the potential to damage the vasculature, and are upregulated with stress – monitoring alterations in their production could give insight into the functionality of chronic febuxostat delivery.

Another component to add is the inclusion of female mice. Sex-associated immunological differences are primarily resultant from the respective quantities of sex hormones. Estrogen has been shown to be influential in immune cell mediation, including pathways that affect activation, differentiation, and apoptosis.¹¹⁵ In estrogen receptor

knockout models, the development of the thymus was stunted, which would be especially impactful on the development and function of T-cells.¹¹⁶

Immune cells have been shown to express estrogen receptor mRNA, indicating a link to the sex hormone as an effector molecule on a variety of cell types.¹²⁰ This is further supported by Escribese et al., whilst examining Dendritic Cells, a pivotal link between the Innate and Adaptive Immune Systems.¹¹⁹ While normally a part of the process to activate CD4 + T-cells, Dendritic Cells that were isolated from a system that was primed with estrogen were shown to have a reduced functional capacity, indicating a suppressive role by the hormone.

The priming of the immune system may be necessary to combat pathogenic threats, but overstimulation may lead to autoimmune consequences. Mor et al. demonstrated the complexity of such consequences, showing that receptor isoforms are critical.¹¹⁷ A potential outcome, mediated by estrogen, is the delaying of monocyte apoptosis, leading to the presentation of symptoms of an autoimmune disease.¹¹⁷ Similar to monocytes, B- cells have also been shown to exhibit a more robust phenotype, supporting estrogen's potential to be both anti-apoptotic and pro-inflammatory.¹¹⁸

Chapter 6

Conclusion

6.1. Conclusion

In conclusion, the study is suggestive that the implementation of stressors has an impact on the immune system, with significant differences in white blood cells and M2 macrophages seen. Although cell populations were assessed, the baseline expectation of obesity causing a pro-inflammatory environment was not met. While psychological stress lead to a significant decrease in M2 macrophages, overall, the data did not support the validate that chronic stress and obesity caused a drastic alteration of the systemic homeostasis. Additionally, the chronic delivery of febuxostat did not result in significant change, though this interpretation is hampered due to the lack of evidence showing an increase in the pro-inflammatory state induced by chronic stress or obesity.

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