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Sex-Dependent Effects of Induced Acute Inflammation on Glucose Homeostasis and RNA Editing Enzymes

Christian A. Rivas
Missouri State University, Rivas93@live.missouristate.edu

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SEX-DEPENDENT EFFECTS OF INDUCED ACUTE INFLAMMATION ON

GLUCOSE HOMEOSTASIS AND RNA EDITING ENZYMES

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Cell and Molecular Biology

By

Christian Alan Rivas

August 2023

SEX-DEPENDENT EFFECTS OF INDUCED ACUTE INFLAMMATION ON GLUCOSE HOMEOSTASIS AND RNA EDITING ENZYMES

Biomedical Science

Missouri State University, August 2023

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ABSTRACT

The first line of defense against bodily insults, like pathogen invasion, is the innate immune system. Innate immunity sets in motion countless cascades that result in inflammation. Inflammation simultaneously affects multiple biological processes like metabolism and gene expression. Males and females react differently to inflammation. To understand both molecular and physiological sex differences in inflammation, we examined how inflammation affects gene expression and glucose metabolism. Adenosine deaminase acting on RNA (ADAR1) is upregulated by inflammation and catalyzes RNA editing, a process where nucleotides encoded by the genome are modified. ADAR1 also controls the innate immune reaction by decreasing activity of a cytoplasmic RNA sensor, MDA-5. While MDA-5 expression is sex-dependent, differences in ADAR1 levels are unexplored. To look at how inflammation affects ADAR1 expression was measured from male and female mice injected with lipopolysaccharide (LPS), an agent known to cause inflammation. In order to look at the sex-dependent effects of inflammation on physiology, glucose tolerance tests were also done in LPS treated mice. Mice treated with LPS showed increased levels of ADAR1 and cytokines, as expected. There was also a sex-dependent effect on glucose tolerance, where inflammation affected blood glucose levels to a greater extent in male inflamed mice, compared to control males, or any female mice. The data suggests that inflammation induces insulin resistance in a sex-dependent manner and that female mice are protected from inflammation-dependent changes in glucose tolerance. We anticipate this information will help us understand sex-disparities in inflammatory-related disease progression and outcome.

Keywords: RNA editing, LPS, innate immunity, glucose tolerance testing, glucose metabolism, lipopolysaccharide, immunity, ADAR1, ADAR1p150, research

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Approved:

Randi J. Ulbricht, Ph.D., Thesis Committee Chair

Jianjie Wang, M.D., Ph.D., Committee member

Christopher Lupfer, Ph.D., Committee member

Julie Masterson, Ph.D., Dean of the Graduate College

In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

ACKNOWLEDGEMENTS

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INTRODUCTION

Innate immunity is made up of many biological components that work together to fight

pathogens and other threats to the body. The first line of defense a pathogen encounters is the

epithelium and mucosal tissue, which terminates most pathogenetic invasions[1]. Not only does

the skin serve as a physical barrier but also develops an indigenous microbiota that also plays a

role in the response of innate immunity, developing an inhospitable environment for pathogens,

as well as regulating other immunological processes[2]. If pathogens get past the epithelium and

mucosal tissue, innate immunity is the first and fastest response triggered. Innate immunity

triggers an inflammatory response that aids in eliminating the pathogen. Adaptive immunity will

eventually mount a specific response to an infection or pathogen. T lymphocytes and B

lymphocytes, which produce antibodies, aid in developing the adaptive immunity response. The

product of adaptive immunity is the development of memory T and B cells primed to prevent

reinfection of same pathogen.

Innate Immunity

Innate immunity begins by the activation of pattern recognition receptors (PRRs) such as

toll-like receptors (TLRs) and Rig-I-like receptors (RLRs)[3]. PRRs are receptors designed to

detect microorganisms through recognizing conserved structures among microbial species called

pathogen-associated molecular patterns (PAMPs)[4]. When PRRs bind to PAMPs, such as a

lipids, nucleic acids, or proteins from a pathogen, a signaling cascade is induced. A signaling

cascade is a mechanism that involves sequential activation of receptors and other biological

factors to aid in fighting pathogens. After activation of receptors, the recruitment of adapter

proteins including the Toll/IL-1 receptor (TIR) domains or Caspase Activation and Recruitment

domains (CARD) result in upregulation of transcription of specific genes involved in innate immunity, including cytokines and type 1 interferons[4]. Cytokines are small polypeptides and glycoproteins that mediate the inflammatory response by affecting how cells interact and communicate with one another[1,5]. Most immune cells, as well as non-immune cells, such as epithelial cells, respond to and express cytokines. Tumor necrosis factor – alpha (TNF-α) is a proinflammatory cytokine that is upregulated when inflammation is present. TNF-α binds to TNF receptors 1 and 2 (TNF-R1 and TNF-R2) on the cell surface. TNF-R1 binds more often to TNF-α due to it being ubiquitously expressed, while TNF-R2 is highly regulated. Once TNF-α binds to TNF-R1, TNF-R1 recruits a death domain containing adapter protein. The adapter protein then recruits TNF- receptor associated receptor 2 (TRAF2) and receptor interacting protein kinases RIPK) to form complex 1. The formation of complex 1 activates the transcription factors nuclear factor kappa light chain enhancer of activated B cells (NFk-b) and c- Jun N-terminus kinase (JNK) pathways that are known to activate transcription of type 1 interferons. Complex 1 simultaneously activates reactive oxygen species that promote apoptosis of the cell. Cytokines also activate chemokines that help recruit inflammatory cells to the infection site and cell adhesion molecules that aid in extravasation, all in order to protect the body from a perceived threat (Figure 1)[1].

Innate Immunity Receptors

Toll-like receptors (TLR) are PRRs and their main role in the innate immune system is to detect microbial infection and prompt a response from the host against it[6]. TLRs are made of up leucine rich repeats, transmembrane region, and cytosolic Toll-IL-1 domains[3]. The leucine rich repeats aid with the identification of PAMPS. PAMPs include proteins or lipids that are often present on the outside of bacteria, as well as the nucleic acid contents of a virus[7]. For

example, TLR-4 will bind to lipopolysaccharide (LPS), TLR-8 will bind to cytoplasmic or extracellular double stranded DNA (dsDNA), and TLR-7 will bind to single stranded RNA (ssRNA)[8]. Activation of the different TLRs will then be followed by recruitment of adapter proteins. The type of TIR domain-containing adapter proteins determines the rest of the cascade signaling pathway[9]. For example, if myeloid differentiation primary response 88 gene (MyD88) is the adapter protein, then the cascade will increase activation of NF-kB and activation of mitogen- activated protein (MAP) kinase. The adapter TIR-domain-containing adapter- inducing interferon- b (TRIF) induces a cascade that induces an alternative activation of NF-kB and interferon regulatory factor 3 (IRF3)[3]. Regardless of the adapter protein and resulting protein kinase cascade, the result of TLR activation is usually the production of cytokines or type 1 interferons.

Rig-1 like receptors (RLRs) are another family of receptors that participate in innate immunity. The RLR family includes retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology (LGP2). Unlike TLRs that are active in both cytoplasm and cell surface, RLRs are mainly active in the cytoplasm of the cell. They are comprised of two N-terminal CARMA/caspase – recruitment domain (CARDs), a central Asp-Glu-Ala-Asp (DEAD) box helicase/ATPase domain (DD), and a C-terminal regulatory domain (CTD)[4]. RLRs primarily interact with double stranded RNA (dsRNA) from both dsRNA viruses or replication intermediates of ssRNA viruses. For example, when a cell is infected with a dsRNA virus, RIG-1 monitors the 5' end and is activated by the foreign RNA that contains triphosphate group, uncapped diphosphate groups, or unmethylated RNA. MDA-5 also senses foreign RNA but MDA-5 has been noted to interact more exclusively with dsRNA. Activation of RIG-I or MDA-5 allows CARD domains to be accessible to interact

with mitochondrial antiviral-signaling protein (MAVS) that then results in activating NF-kB - induced transcription of cytokines and IRF3 induction of type 1 interferons[4]. Type 1 interferons are released to go to neighboring cells where they bind to interferon α and β receptors (Figure 1)[10]. Once a cytokine or ligand binds the receptor, phosphorylated janus kinases (JAK) then recruits signal transducer and activator of transcription proteins (STAT1/2). STAT1 is phosphorylated by JAK and then travels to nucleus to bind to specific genome sequences and activate expression of interferon stimulated genes (ISGs), including cytokines (Figure 2).

Lipopolysaccharide

Lipopolysaccharide (LPS) is a cell wall constituent of gram-negative bacteria and a potent PAMP. LPS is commonly used to stimulate the innate immune system and study various processes such as inflammation, signaling pathways, and disease. LPS is made up of a core oligosaccharide, an O-specific chain, and a lipid A component[11]. The lipid A is the main contributor to its ability promote inflammation. When LPS binds to an endothelial cell via the TLR4 receptor, it activates the endothelial cell to then cause innate immune activation by LPS-binding protein (LBP). LBP binds to LPS and then makes a ternary complex with the receptor molecule called CD14[12]. CD14 then aids in the transferring of LPS to TLR4 and myeloid differentiating protein-2 (MD2). TLR4 then oligomerizes and recruits adapters using the toll/interleukin receptor (TIR) domain to trigger the innate immune signaling cascade[12].

LPS also affects the glucose homeostasis. Induction of the cytokine IL-6 by LPS increases glucose-stimulated insulin secretion (GSIS)[13,14]. The increased amount of insulin in the blood allows more uptake of glucose from the blood into the tissues, reducing blood glucose levels. This is generally referred to as increased insulin sensitivity and glucose tolerance.

Glucose Metabolism and Innate Immunity

Glucose metabolism is the primary source of adenosine triphosphate (ATP) production, which a cell's major source of energy, allowing it to grow and proliferate. The pancreas, liver, and skeletal muscle work in unison to regulate glucose homeostasis by balancing the amount of glucose present in the blood (Figure 3). During fasting conditions, when glucose levels are low, pancreatic α cells release the hormone glucagon. Glucagon promotes production of glucose through glycogenolysis and gluconeogenesis. Glycogenolysis is the production of glucose through the breakdown of stored glycogen in the liver. Glucose can also be produced through gluconeogenesis from the processing of other substrates that include lactate, glycerol, and glucogenic amino acids. During fed conditions, when glucose from the diet is plentiful, the pancreatic β-cells secrete insulin. Insulin is an endocrine hormone that binds to receptors on the plasma membrane on targeted cells. Insulin has several different functions depending on the cell targeted. When insulin binds to receptors within skeletal muscle, it stimulates the uptake of glucose into cells from the blood. The glucose is used to produce ATP, or excess glucose will be used to produce glycogen. Glycogenesis occurs in the liver and is activated by insulin, which simultaneously blocks gluconeogenesis. Lastly, insulin bound to white adipocyte tissue (WAT) will prevent lipolysis but will increase lipogenesis and glucose transport[15].

Glucose in the blood is absorbed into the cell through a glucose transporter (GLUT). Once in the cell, the glucose is metabolized by either glycolysis or oxidative phosphorylation, resulting in the production of ATP[16]. More specifically, AMP-activated protein kinase (AMPK) is the main regulator of energy homeostasis through sensing the ratio of adenosine monophosphate (AMP) to ATP. When ATP is low, AMPK will be activated/phosphorylated to inhibit anabolic pathways that require ATP while simultaneously activating glycolysis and

expression of GLUTs, allowing more glucose to be transported into the cell by glucose transporters, and more ATP to be generated by glycolysis[17]. The physiological response to acute inflammation is one process know to increase the demand for ATP.

Innate immunity uses a great deal of ATP[17]. Limited glucose availability, and therefore limited ATP production, can result in blunting of the inflammatory response and decreasing the amount of proinflammatory cytokines produced[17]. Moreover, innate immunity also affects glucose metabolism. For example, activation by cytokines (specifically, IL-3) leads to the presence of the glucose transporter Glut1 on the surface of cells, allowing more uptake of glucose from the blood. However, Glut1 is degraded when IL-3 is no longer present[16]. IL-3 works by activating the PI-3K receptor, which converts phosphatidylinositol (PI) to PI 3,4,5 trisphosphate (PIP3), then phosphorylates Akt (Figure 4). Another pathway that leads to Akt activation and Glut1 translocation to the surface involves inactivation of GSK-3, causing the dephosphorylation of tuberous sclerosis complex subunit 2 (TSC2) which leads to an increase in mammalian target of rapamycin (mTOR)[18,19].

dephosphorylation of tuberous sclerosis complex subunit 2 (TSC2) which leads to an increase in mammalian target of rapamycin (mTOR)[18,19].

The ability of innate immune activation to increase uptake of glucose from the blood is supported by studies that show LPS injections cause hypoglycemia in mouse models[20,21]. Hypoglycemia is induced by the enhanced clearing of glucose, reduced breaking down glycogen from both liver and skeletal muscle, and disrupted hepatic gluconeogenesis. The LPS effect is specifically mediated by the TLR4, My88, and NFkB pathways.

Pro- and Anti-Inflammatory Cytokines

Interleukins (ILs), Interferons (IFNs), growth factors, and chemokines are all different types of cytokines. Fundamental cytokines in acute host response to infection are IL-1, IFN-α, and TNF-[1]. Both IL-1 and TNF- are small polypeptides that induce the inflammatory response as well as affect metabolism. Generally called proinflammatory cytokines, these cytokines alone prompt production of other cytokines or activate transcription from ISGs including IFN-, IFN-, IL-28, and ADAR1, all of which play a role in perpetuating or subduing inflammation[22,23]. Excessive inflammation can become harmful to the host cells, to the point of causing cell death, tissue damage and potentially progressing to death of the host. Therefore, both pro-inflammatory and anti-inflammatory cytokines are used to balance an ample response to pathogens while preventing injury to host cells. Anti-inflammatory cytokines decrease or completely stop inflammation. For example, the primary roles of IL-10 and TGF- are to activate the anti-inflammatory response by blocking the activation of cytokines and regulating cells of the adaptive immune response[24].

ADAR1 in Innate Immunity

One particular anti-inflammatory ISG is Adenosine deaminase acting on RNA 1 (ADAR1). ADAR1 has two isoforms, ADAR1p150 and ADAR1p110. Two constitutive promoters drive synthesis of the p110 isoform. The 150 kilodalton isoform (ADAR1p150) is made from an interferon inducible promoter. Transcription induction from this promoter includes an alternative translational start codon which ultimately leads to an extension of the N terminus of the protein. Although both ADAR1p150 and ADAR1p110 shuttle between the nucleus and cytoplasm, ADAR1p110 is located in the nucleus while ADAR1p150 primarily localizes to the cytoplasm. ADAR1p110 and ADAR1p150 are also expressed differently depending on the tissue, therefore the induction of each with inflammation may be different[25].

ADAR1p150 is an anti-inflammatory enzyme capable of blocking of RLR signaling, suppressing innate immunity[23]. ADAR1p150 reduces the efficiency of RLR signaling by blocking activation of melanoma differentiation-associated protein 5 (MDA-5) and mitochondrial antiviral-signaling protein (MAVS). MDA-5 is activated by dsRNA, which then activates MAVS. MAVS then proceeds to phosphorylate NF-kB. Once TLRs are activated, consequently induces the translocation of NF-kB to nucleus of cell. Interferon regulated factors 3 and 7 (IRF3/IRF7) and may induce activator protein – 1 (AP1), which is activated by a group of proteins called inhibitor of nuclear kappa kinase (IKKs). To prevent the MDA-5 cascade activation by endogenous cellular dsRNA, ADAR1p150 binds to dsRNA and does not allow binding by MDA-5. The reduction in MDA-5 activity dampens type 1 interferon production that prevents ISGs expression. Conversely, knocking out ADARp150 allows for MDA-5 to be activated more readily by endogenous dsRNA[23]. Loss of ADAR1p150 produces up to 300-fold more gene expression of some interferon stimulated genes[26]. ISGs affected by loss of

ADAR1p150 include STAT1, STAT2, interferon regulatory factor (IRF1), IRF7, IRF9; the GTPases Mx1 and Mx2; protein kinase R (PKR); the 2′,5′-oligoadenylate synthases OAS1, OAS2, and OAS3; the ubiquitin-like modifiers ISG15 and ISG20; and the interferon-induced proteins Ifit1–Ifit3[27]. Therefore, ADAR1p150 plays an important role in attenuating the immune response.

RNA Editing

ADAR1 is a member of the ADAR family of RNA editing enzymes that deaminate select adenosines in regions of double stranded RNA. When adenosine is deaminated, it is converted to inosine (Figure 5). While adenosine normally hydrogen bonds with uridine, inosine base pairs similarly to guanosine, preferring to hydrogen bond to cytosine[28]. If editing occurs in the coding region of a pre-mRNA, the protein encoded from the transcript may be affected. For example, FLNA codes for filamin-. The genomically encoded transcript contains a CAG codon that encodes glutamine (Q). Adenosine to inosine RNA editing by ADAR2 changes the codon to CIG that codes for arginine (R)[29]. The change in amino acid sequence in FLNA likely changes the protein-protein interactions with this protein, altering its ability to regulate cytoskeletal structure. Eliminating or decreasing RNA editing of FLNA alters the function of the protein and therefore affects the stability of the actin cytoskeleton[29]. This alteration of actin skeleton increases the amount of smooth muscle contraction. With increased blood vessel contraction, increased blood pressure follows. Prolonged blood pressure increases the damage on blood vessels and through cardiac remodeling causes stiffness. The complete or high editing of FLNA aids in preventing cardiac remodeling that may cause heart disease.

There are 3 different ADAR genes in mammals: ADAR1, ADAR2 and ADAR3. All the ADARs have the double stranded RNA binding domain (dsRBD), that allows binding to double

stranded RNA targets. ADAR1 and ADAR2 contain an active deaminase domain that catalyzes hydrolytic deamination of select adenosines within the dsRNA structure. ADAR1 has Z-DNA-binding domains that allow both p110 and p150 isoforms to bind to left-handed Z-RNA. The p150 isoform of ADAR1 has two of these Z-DNA-binding domains, Zα and Zβ, while the p110 isoform only has Zβ[23]. ADAR1 and ADAR2 can have overlapping as well as distinct targets. For example, both ADARs are able to target and deaminate the serotonin receptor $5HT_{2C}$ pre-mRNA at the A and C editing sites. However, the B site on this RNA is edited specifically by ADAR1, while the D site is edited specifically edited by ADAR2[30]. ADAR1 and ADAR2 both contain nuclear localization signal (NLS) domain. This domain helps keep ADARs in the nucleus to aid in endogenous RNA editing that happens co-transcriptionally, thus usually requiring introns. ADAR1p150 differs from the rest by also have a nuclear export signal (NES) that allows it to edit in the cytoplasm and participate in innate immunity. ADAR1 and ADAR2 are known to be expressed in most tissues while ADAR3 resides only in mammalian brain[19]. The main role of ADAR3 seems to be control of ADAR1 and ADAR2 activity. Because ADAR3 can bind dsRNA but cannot catalyze RNA editing, it can compete for binding dsRNA and inhibit ADAR1 and ADAR2 from binding and editing this RNA.

ADAR1p150 RNA editing activity is critical to its role in regulating innate immunity through regulation of MDA-5. ADAR1p1500catalyzed RNA editing of cytoplasmic dsRNA is thought to disrupt the double stranded structure and thus the ability of MDA-5 to recognize it and activate innate immune reaction. Mutations in the ADAR1 gene that interfere with its ability to process or edit double stranded RNA can lead to inappropriate activation of the immune response, a characteristic of autoimmune disease. For example, Aicardi-Goutieres syndrome is an autoimmune condition in which there are elevated levels of interferon stimulated genes and is

associated with mutations to ADAR1[31]. Deletion of ADAR1 in mice leads to embryonic lethality at embryonic day 11.5[32]. Moreover, while eliminating expression of just ADAR1p150, but keeping ADAR1p110, is lethal, this phenotype is repressed by the deletion of MDA-5 or MAVS[32,33]. This indicates that ADAR1p150 repression of MDA-5 and MAVS activation through dsRNA sensing is integral to animal development. Moreover, ADAR1p150-mediated control of innate immunity can prevent auto-immune disease, likely by controlling that amount of immune activation through MDA-5/MAVS.

Sex-differences in Innate Immunity and Glucose Tolerance

There are known sex-differences in both immunity and in glucose homeostasis. Many of the genes for innate immunity reside in the X chromosome. For example, the PRR toll-like receptor 7 (TLR7) gene is located on the X chromosome. Usually, due to X chromosome inactivation (XCI), duplicate X-genes are inactivated to control expression of gene[34]. If the TLR7 gene does not get inactivated in one X chromosome, it will be expressed at a higher rate in females compared to males[35]. This escape from XCI happens 15-23% of all X-linked human genes[36]. This increase in TLR7 expression predisposes females to a more robust response when activated by PAMPs, such as ssRNA. When activated, the TLR7 increases the expression of IFN-α, a pro-inflammatory interferon. The effect of increased TLR7 ultimately increases the response as well as increases the chances of acquiring auto-immune disease[36]. Males have higher expression of TLR4 on macrophages that makes them induce pro-inflammatory responses greater than females response when introduced to LPS[37]. Although TLR7 responds primarily to ssDNA and TLR4 responds primarily to LPS, does not mean there are not cross over. Male mice have shown to have an increase amount of TLR4 and other interferons in the heart that have been infected with ssRNA virus coxsackieviruses , compared to females. Females had

lower amounts of TLR4 and higher amount of anti-inflammatory cytokines throughout the experiment[38]. Uninfected and infected female mice also have increased levels of MDA-5 and RIG1 proteins in the heart, compared to males[39].

Sex hormones also have an impact on how the organism reacts to inflammation. Overall, females are much more prone to autoimmune diseases while males are prone to tissue damage due to inflammation[40]. Estrogen receptors are found on monocyte derived cells and prevent/ controls the inflammation response by producing both anti and pro-inflammatory cytokines. For example, 17beta-estradiol (E2) is one of four most prominent endogenous estrogens. E2 is also the most prominently expressed as well as capable estrogens[41]. Expression and potency of E2 in addition to the quantity, circulation, and type of estrogen receptors influence the immune system. Deceased levels of E2 results in a T_H1 response that causes expression of interferon gamma driven pro-inflammatory response. In contrast, increases in expression of E2 induces a T_H2 response driven by the expression of IL-13, IL-5, IL-4, as well as the anti-inflammatory IL-10[41]. For males, androgens such as testosterone are considered immunosuppressive as well anti-inflammatory[41]. This is due to testosterone decreasing natural killer cell activity as well as NFk-b attenuation.

Sex hormones play an important role in glucose metabolism. Without the induction of inflammation, females are more sensitive to insulin than males[42]. This is despite the fact that females having less skeletal muscle, more adipose tissue mass, and more free fatty acids, all of which are factors known to increase insulin resistance[42]. It has also been shown that females induce more insulin secretion per the amount of insulin action[42]. Sex differences have also been seen in pre-diabetic syndromes such as impaired glucose tolerance and impaired fasting glucose. Females have shown to have more impaired glucose tolerance while males usually have

impaired fasting glucose compared to females[42]. The reason for this discrepancy is not understood but data suggests that this difference is due to sex hormones. To support the hypothesis, some studies have shown that during reproductive age women are less likely to develop type II diabetes than males. However, the reverse happens when comparing post-menopausal females compared to males[42]. To try and understand what effect sex has on glucose clearance while inflamed, Underwood and Thomas induced chronic inflammation by giving mice a high fat diet. Glucose tolerance testing showed that males had higher blood glucose levels throughout the glucose tolerance test, compared to their female counterparts, suggesting that males absorb glucose less readily than females. To our knowledge, there have been no published data comparing the effects of acute inflammation on glucose homeostasis between the sexes.

Research Goal and Aims

The goal of this project is to investigate potential sex-dependent effects of acute inflammation. We are particularly interested in differential regulation of glucose homeostasis and levels of anti-inflammatory ADAR1p150 in male and female mice.

Aim 1: Sex-Dependent Glucose Tolerance During Induce Acute Inflammation. Innate immunity and glucose metabolism have a strong association, and it is known that there are sex-dependent differences in both glucose metabolism and innate immune activation. However, research has not determined if there are sex dependent differences on inflammatory driven glucose metabolism in an acute inflammation model. My first research aim is to investigate sex-dependent effects of acute inflammation on glucose metabolism in mice. Female and male mice will be injected with 2 mg/kg body weight LPS to stimulate an innate immune reaction. After 24 hours, mice are injected with dextrose and a glucose tolerance test (GTT)is completed which

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involves determining the blood glucose is measured over a time course. We will take a blood glucose reading with the glucometer every 10-15 minutes for 1 ½ hours after dextrose injection. While we expect blood glucose to rise over the first 20-30 min of the GTT, it will fall as glucose from the blood is absorbed up by the tissues. The more tolerant the mouse is to glucose, the more readily the mouse will take up the glucose from the blood, resulting in lower blood glucose and/or reducing blood sugar at a faster rate.

Previous studies showed that female mice were more efficient at clearing blood glucose compared to male mice under normal conditions[43,44]. Therefore, we expect female mice injected with saline (control) to have lower blood glucose throughout GTT, compared to male mice. LPS will induce acute inflammation, therefore activating production of various cytokines, and also increasing glucose uptake by allowing more Glut1 receptors on the surface of cells. Therefore, blood glucose is expected to decrease during GTT of LPS treated mice, showing a greater glucose tolerance during inflammation. Since females usually instigate a Th-1 cell response, this allows a more controlled inflammation response with more anti-inflammatory cytokines. Therefore, we may expect female mice to be more resistant to LPS-dependent changes in glucose tolerance. Although we expect glucose tolerance to increase with LPS treatment in both males and females, the change is expected to be more exaggerated in males, compared to the female counterparts[13]. In order to confirm that the selected dose of LPS induces inflammation in the mice, we will collect blood and measure the levels of certain cytokines in the blood. The levels of the anti-inflammatory cytokine IL-6 is expected to be increased in mice with induced inflammation, compared to controls, therefore the level of IL-6 will be determined with an enzyme linked immune assay (ELISA). TNF-α is also expected to increase in expression in inflamed mice. It's expression will be measured with quantitative reverse transcription –

polymerase chain reaction (qRT-PCR).

Aim 2: Sex-Dependent Differences in ADAR Levels During Induced Acute Inflammation. ADAR1p150 has an important role in regulating innate immune reactions, by preventing activation of MDA-5 and RIG1. The levels of MDA-5 and RIG1 are different between sexes, and so is the immune reaction itself. However, all of the studies investigating ADAR1p150 have been completed either exclusively in male mice or have not compared males and females when both sexes were used. No study has critically analyzed potential sex-differences in ADAR1p150 induction. Therefore, we will induce acute inflammation and then measure the levels of ADAR1p150 mRNA with qRT-PCR. We expect LPS to trigger a robust inflammatory response and increase the levels of ADAR1p150 and other cytokines in males and females. ADAR levels and ADAR1p150 induction are known to be tissue dependent, therefore, we will complete this analysis in a few organs. In other ongoing projects, the level of RNA editing in these mice will be determined. In order to conclude that any changes in RNA editing are due exclusively to changes in ADAR1p150, we will also determine the levels of ADAR2 in this thesis, which we expect to remain consistent between saline and LPS treated mice.

To accomplish aim 2, adult mice will be injected intraperitoneally with LPS or saline[45,46]. Four hours later, we will dissect the following organs: heart, brain, pituitary gland, eye, kidney, abdominal muscle. Organs will be snap frozen in liquid nitrogen to preserve RNA and protein. Gene expression of ADAR1p150, ADAR2, and the cytokine TNF- will be quantified using quantitative reverse transcription PCR (qRT-PCR). We expect TNF- and ADAR1p150 expression to be induced by inflammation, and therefor increase the levels of the mRNA in the LPS treated mice, compared to saline treated mice. While we do not expect LPS to change ADAR2 levels, we will confirm that its expression is unchanged. Moreover, we will

repeat these experiments in both male and female mice.

Study Implications

This study will complement other ongoing studies in the Ulbricht lab that are trying to understand sex-dependent changes in RNA editing that might occur during acute inflammation. While other projects are analyzing levels of RNA editing, this study is necessary to confirm systemic inflammation, and ADAR1p150 induction. Additionally, these studies will contribute to the understanding on what role sex plays in inflammation and diseases or conditions caused by inflammation. Specifically, this work will help determine how glucose homeostasis is affected during an infection or times of stress. This study may help explain why females are more prone to auto-immune diseases and have a more sustained activation of the innate immune response, while males are more prone to negative effects from inflammation specifically when it comes to cardiac tissue damage[40,47].
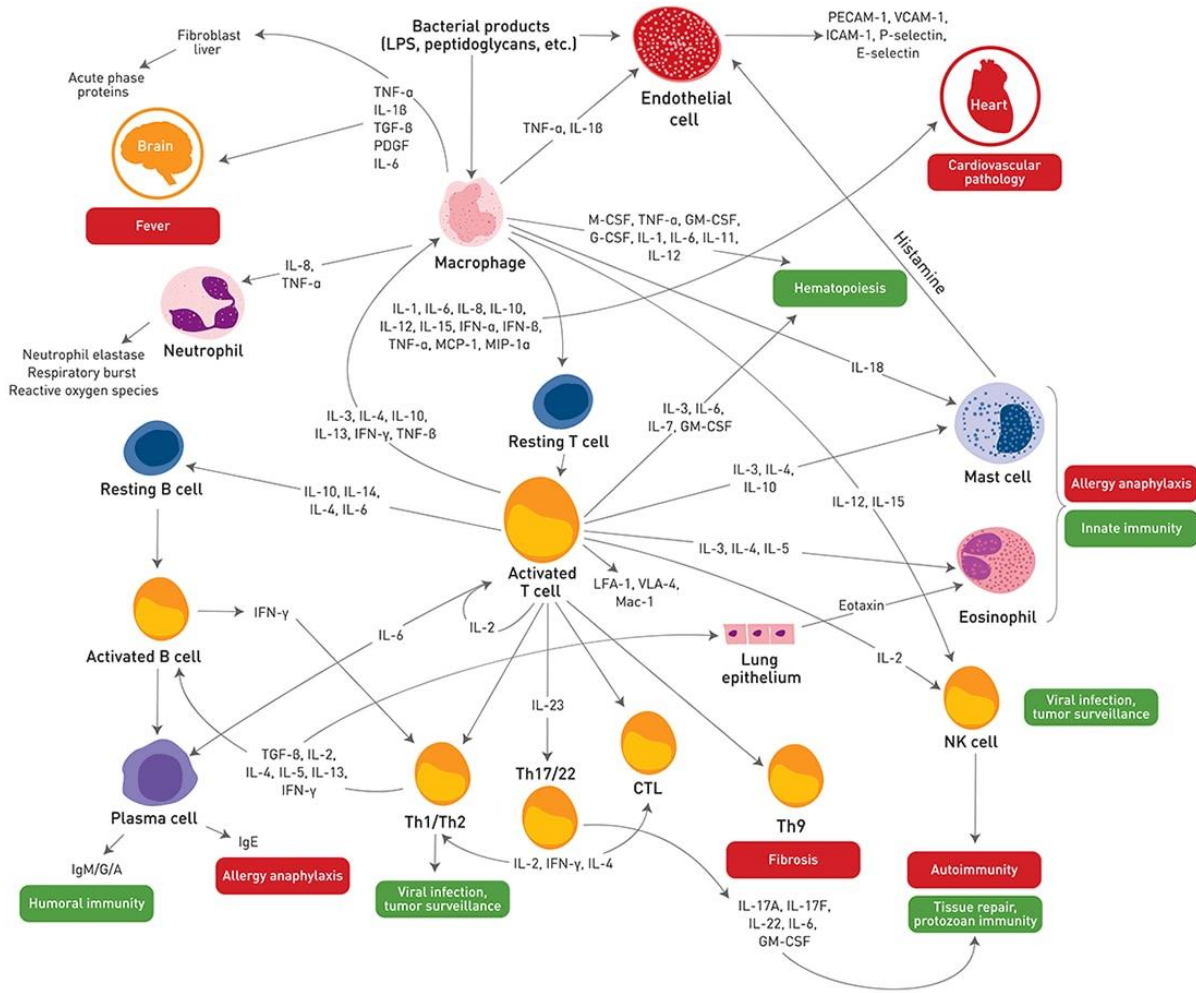
Figure 1. Overview of the function of cytokines in immunity. Figure is from Thermofisher. Green represents favorable reactions for the organism while red indicates unfavorable side effects from inflammation. Orange ovals represent activated immunological cells or signaling proteins.
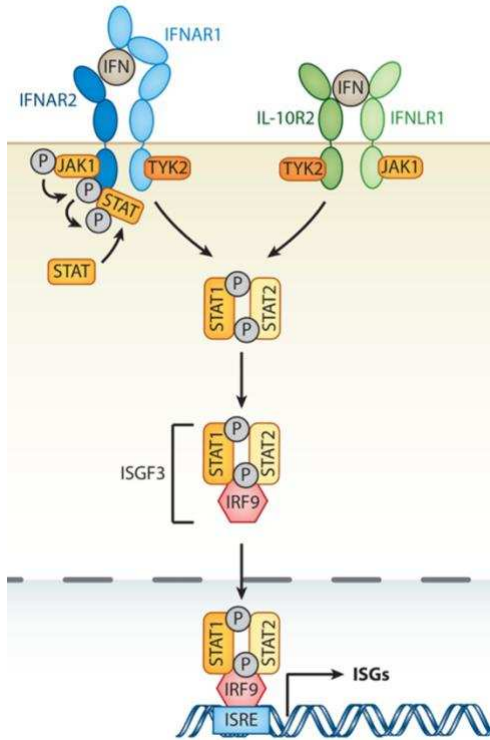
Figure 2. JAK1/STAT signaling through IFN. Interferon receptor (IFNAR; dark blue and light blue on plasma membrane) activation by interferon (IFN; gray). JAK1 is phosphorylated inside the cell, that induces STAT phosphorylation. Phosphorylated STAT1 then travels from cytoplasm into the nucleus where it activates transcriptions of ISGs. Modified from Schneider et al 2014[22].
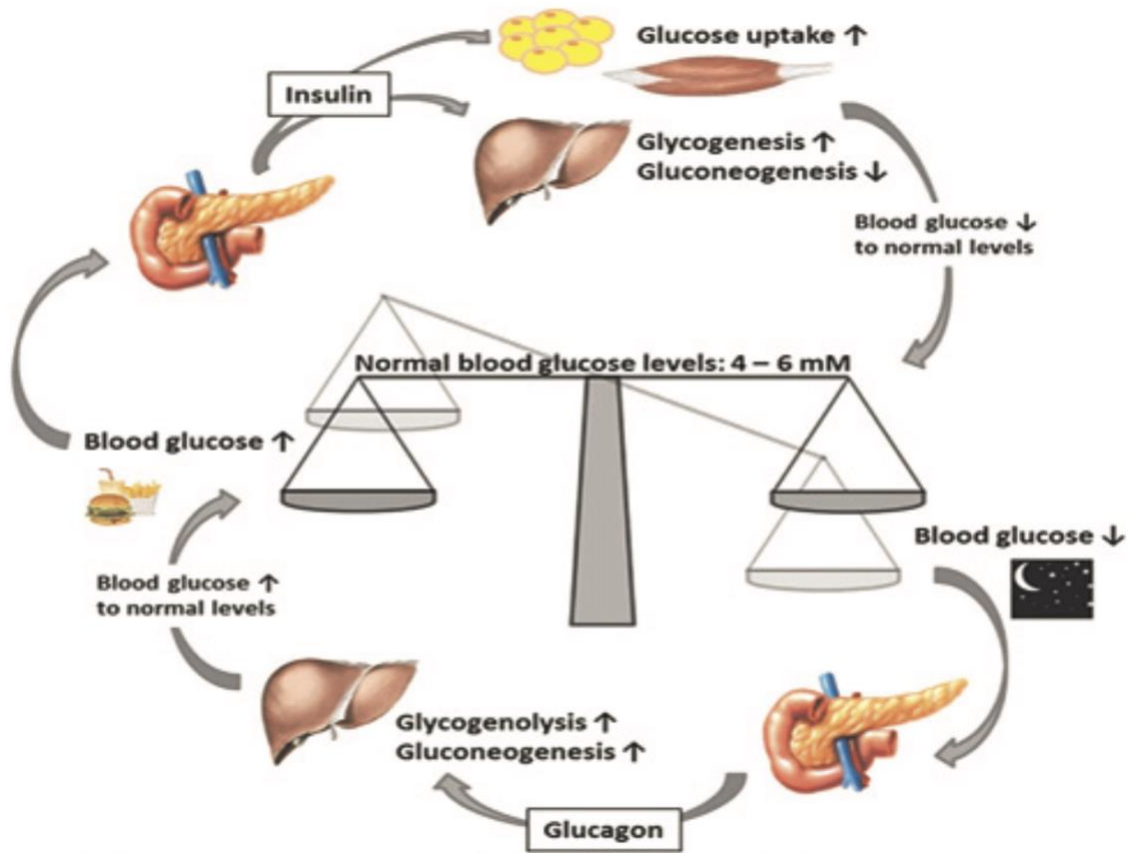
Figure 3. Balance of normal blood glucose[48]. Maintenance of glucose homeostasis. When there is an increase in exogenous glucose the pancreas releases β-cells that secrete insulin that allow the cells to uptake the glucose and reduce the amount of exogenous glucose. When glucose levels are low the pancreas α cells secrete glucagon that increase the amount of gluconeogenesis, allowing the amount of exogenous glucose levels to come back to normal.
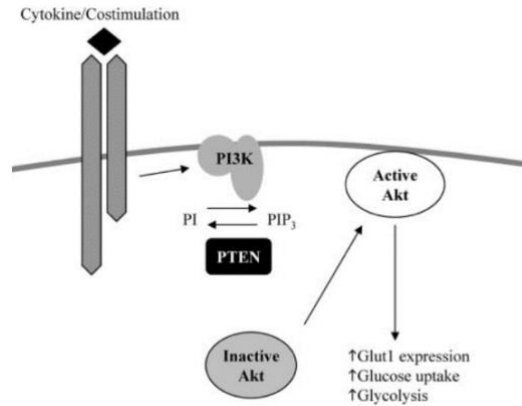
Figure 4. PI3K and Akt Pathway[19]. Cytokine (black diamond) attaches to the receptor (long grey bars), activating PI-3K that then converts PI to PIP3. The activation of PIP3 activates the Akt to allow for more glucose uptake by allowing expression of Glut1 and increasing glycolysis. Figure from MacIver et al 2008.
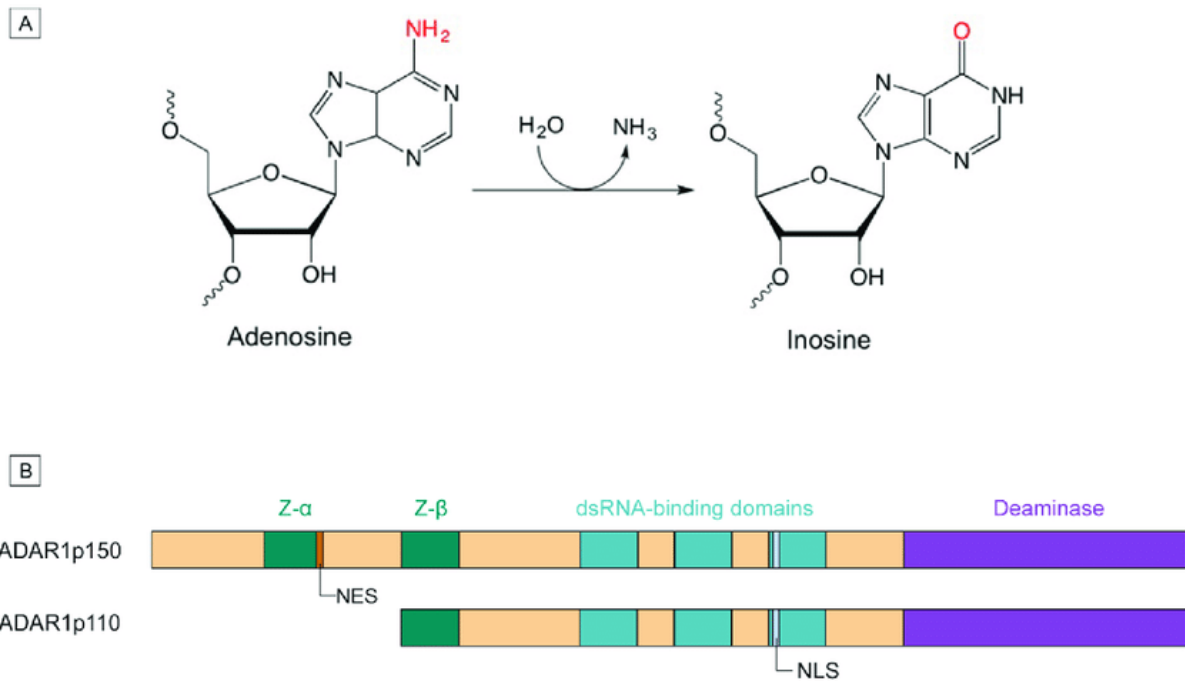
Figure 5. Adenosine deamination by ADARS[49].(A) Both catalytic isoforms of ADAR1 deaminate adenosine to inosine. (B) ADAR1p150 and ADAR1p110 isoforms both contain dsRNA binding domains (teal) as well as a deaminase domain (purple). ADAR1p150 also contains an extra Z-DNA binding domain (Z-α, green) as well as a nuclear export signal (NES, red).

METHODS

All work presented in this thesis was completed prior to 2022 and approved by the Institutional Animal Care and Use Committee (Appendix A) and Institutional Biosafety Committee (Appendix B). Required biosafety and animal care and use training was also completed (Appendix C).

Mice

Mus musculus C57Bl6 mice between the ages of 8 and 12 weeks were used for these experiments. All mice studies were approved by the Missouri State University Institutional Animal Care and Use Committee (IACUC), protocols 18.006 and 19.004. Mice were maintained on a diet consisting of Laboratory Rodent Diet 5001 (Purina, catalog # 0001319, St. Louis, MO). Mice were born and bred in Temple Hall in Missouri State University. At approximately 20 days, ear tags were placed on mice for identification. At 8 weeks of age mice were transferred to the Professional Building vivarium of Missouri State University. The light cycle in the Professional Building vivarium was inverse light cycle consisting of dark from 6am to 6pm and light from 6pm to 6am. The vivarium held a 72˚ C temperature and roughly a 55 % humidity.

Glucose Tolerance Testing (GTT)

Twenty-four hours before the start of GTT, the mice were weighed and injected with LPS or saline. LPS was injected at 2 mg/kg, or control mice were injected with an equal volume of 0.9% saline. Five hours before start of GTT mice were weighed and put in fresh cages without food but with water to begin the fast. The cages of fasting mice were placed a different room in vivarium with the light on to allow the mice to acclimate to the room for future experimentation. Fifteen minutes prior to start of GTT test, mice were weighed then placed in tall styrofoam

tower. A rechargeable handwarmer topped with a piece of absorbent benchpaper was placed in a styrofoam box. Mice undergoing GTT were placed on the handwarmer for the duration of the experiment. Lidocaine was applied to the tip of tail for approximately 10 minutes to numb the tail. Tail was wiped with 70% ethanol 1-2 minutes before starting GTT. The terminal 2-5cm of tail are snipped off with clean shears. The first drop of blood was wiped off and discarded. The second drop was applied to a glucometer and glucose test strips. After measuring fasting glucose levels, animals were injected intraperitonially with 2 g/kg dextrose. Intraperitoneal (IP) Injections were made on the mouse's left side left to the middle nipple. All injections were done as swiftly as possible to avoid further stress on mice. Blood glucose was then measured at 10, 20, 30, 45, 60, 75, and 90 minutes after dextrose injection through the end of previously cut tail.

Cardiac Puncture

After GTT, mice were anesthetized with isoflurane and cardiac punctures are performed to extract the blood from the mice. Cardiac punctures had the mouse face up and we palpated the xyphoid process. A 25 gauge needle was inserted at $25\degree - 35\degree$ angle, to the left side of the xyphoid process, until the needle reached the left ventricle. The syringe was used to remove blood from the ventricle. After acquiring 500 µL to 800 µL of blood, the mice are then euthanized by cervical dislocation. Eppendorf tubes as well as syringes were flushed with 2% EDTA to prevent blood coagulation. Blood was centrifuged at 10,000 rpm at 4 ˚C for 10 minutes to allow separation of plasma and erythrocytes. Plasma was extracted and stored at -80 ˚C.

Acute Inflammation and Dissection

LPS from *E. Coli* 0111:B4 (Sigma, St. Louis, MO) was used as the endotoxin for this experiment[45]. The LPS was resuspended in sterile saline. Sixty percent of lethal dose[45] (15 mg/kg) was injected into the peritoneum of mice. The lethal dose and dissection time was

determined from Jing-Hua et al 2003to induce the highest amount ADAR1p150 and

inflammation[46]. Injections were done by grabbing scruff on the back of neck with index finger

and thumb while tucking tail in with pinky. A 25 gauge needle on a 1 cc syringe was inserted

between the midline and leg muscle. Injections were performed around noon and mice were

dissected 4 hours after injection. Control mice were injected with an equal volume of saline

only.

Four hours after saline or LPS injections, the mice were anesthetized with isofluorane and

sacrificed by cervical dislocation. The brain, eye and pituitary gland were dissected from the

head, while the pancreas, heart, kidney and abdominal muscle were dissected from the body.

Organs were snap frozen in liquid nitrogen, and stored at -80 ˚C until homogenization.

qRT-PCR

Homogenization and RNA isolation was performed by C. Nichols for a parallel thesis

project. RNA samples were made into cDNA by using High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems) using random primers. One microgram of RNA is mixed

with 1 X buffer, 0.5 X dNTPs, 1 X random hexamers, and 1 mL of either reverse transcriptase or

water (for negative control) were placed in a 20 mL reaction. The reaction was performed in a

thermocycler using protocol HCRT (25 °C for 10min, 37 °C for 1 hr, 85 °C for 5 min).

The cDNA was then subjected to qPCR to determine the quantity of TNF-α,

ADAR1p150 and ADAR2 cDNA present in each sample. Using iTaq Universal SYBR green

mix (Bio-Rad), sense and antisense primers (Table 1), and 1:10 dilution of cDNA was added to

white PCR tubes (Bio-Rad). The reaction was cycled in a CFX connect real-time detection

system (Bio-Rad) under the following conditions: 95 °C for 2 min, 57 °C for 30 s, repeated 40

times, followed by melt curve analysis of 65-95 °C in 0.5 °C increments. Each reaction was

performed in duplicate.

Primer Efficiency

Primers were mixed in master mix containing: 5 µl of cDNA, 2 µl of sense and anti-sense primers, 25 µl of Dream Taq Master Mix(Thermo), and 16 µl of H_2O per primer set and cycled with Tm of 60 °C. PCR product was then electrophoresed on a 2% agarose gel. The product was purified from the gel slice with the Promega SV Wizard Gel and PCR Purification kit. The produce was ligated to pGEM from the T Easy Vector Systems (Promega). Ligation was done using: 3 µl of PCR product, 1 µl of T-easy vector, 5 µl of 2 x rapid ligation buffer, 1 µl T4 DNA ligase. Ligation was left in 4˚C overnight then 5 µl of the ligation was transformed into 100µl of DH5α competent cells. The cells were incubated on ice for 30 min, then heats hocked at 42 °C for 35 sec. The cells recovered in 900µl of NZY+ medium at 37˚C for 1.5 hours. Lastly 350µl of ligation was spread on Lysogeny Broth (LB) ampicillin plates. Plates were then incubated overnight at 37˚C.

Colonies from the transformation were isolated and grown in liquid LB with ampicillin at 37 C overnight. DNA was isolated using the Promega SV Wizard miniprep kit. The average C_T was determined from duplicate qPCR of a dilution series (from 1/10 to 1/1,000,000) of each cloned product. A scatter plot and the trendline was made from the C_T average and the log of the dilution was made. The slope of the final trendline was used to determine the efficiency using the equation $E = -1 + 10^{(-1/slope)}$. To determine the relative gene expresion, the Ep value ($Ep = E + 1$) was used to determine the fold change $= (Ep)^{\Delta CT}$, where the ΔC_T was calculated by taking the average of each target C_T and subtracting that from control average C_T from the same target. The fold change for each target was then divded by the fold change for the control, GAPDH, from the same sample[50].

ELISA

Mouse IL-6 Uncoated ELISA kit (Invitrogen) was used to quantify the amount of IL-6 present in blood plasma. Reagents were diluted twice the recommended amount, including: capture antibody (1:500), detection antibody (1:500), and streptavidin – horse radish peroxidase (HRP) (1:200). Corning Costar 9018 ELISA plates were coated with 100 µL/well with capture antibody and incubated overnight at 4˚C, nutating at 60 rpm. Wells were then washed three times by adding 250 µL of 1 X PBS, 0.05 % Tween-20 (wash buffer) and incubating for 1 minute, then dumped out and the plate blotted dry. Wells were blocked with 200 µL/well of ELISA/ELISPOT diluent (1 X) and incubated for 1 hour at room temperature at 60 rpm. After incubation, the plates were washed two times. The standard included in kit was reconstituted with water. Undiluted standard was added to the first well (200 µL), a dilution was made by adding 100 µL of ELISA/ELISPOT dilution buffer to 100 µL of the standard. Serial dilutions were continued by adding 100 µl of the diluted sample to 100 µL ELISA/ELISPOT dilution buffer until there were 8 different dilutions. Plasma samples were added (100µL) to each well and a well with 100µL of ELISA/ELISPOT diluent was also included as a negative control. All samples were run in duplicates. The plate was then incubated for 2 hours at room temperature on the orbital shaker at 60 rpm. After incubation, the plate was then washed five times to then 100 µL of detection antibody was added to all the wells being tested. The plate was then incubated at room temperature at shaking at 60 rpm for 1 hour. After incubation the plate was washed five times before adding 100 µL of streptavidin-HRP to each wells. The plate was once again incubated at room temperature, shaking at 60 rpm for 30 minutes. The plate was then washed seven times and then 100µL of TMB solution was added to each used well. The plate was incubated at room temperature for 15 minutes. Stop solution (100 µL H_3PO_4) was then added to the wells. The plate

was read under plate reader at 450nm.

Statistical Analysis

GTT Analysis. GTT data is presented as both raw blood glucose and normalized. The normalized values were developed by comparing the blood glucose value from each mouse to their respective starting fasting blood glucose level. For each data set, the individual data points were assembled and first checked for outliers. Outliers were assessed univariately by examining the standardized z-scores with a cutoff score of ± 3. Outliers were removed from the primary analyzed data. Descriptive statistics were then done by splitting saline and LPS as well as males and females. Each split was checked for normality using Shapiro-Wilk method. Both splits failed the normality test. Greenhouse-Geisser was used as sphericity correction. Repeated measures ANOVA was run on each split. Post hoc tests comparisons were run using the Holm correction. All errors bars are based on the standard error of the mean.

Area under the curve was subsequently performed on absolute blood glucose via GraphPad to obtain average total area as well as standard error. Ordinary one way ANOVA was then done with Tukey's multiple comparison tests.

Fasting glucose data was compared by first testing for normality. Deviation from normality was observed through Shapiro-Wilk test. ANOVA analysis was then done with Tukey correction for post hoc tests.

Gene Expression.ELISA duplicates were averaged. A standard curve was produced using the standard provided in the ELISA kit. Averages were placed in equation to solve for concentration of IL-6. Outlier test (robust regression and outlier removal) was then done to remove any outliers (1 sample was removed). Normality tests including D'Agostino, Anderson Darling, Shapiro-Wilk, Kolmogorov-Smirnov were then run to identify no deviation from

normality. Paired T-test was then performed to identify significant difference between LPS and saline mice.   qRT-PCR was run on both male mice heart and brain to find the amount of gene expression. After extracted RNA was converted into cDNA the Pfaffle method was used to identify gene expression[50]. The ratios were then analyzed via multiple unpaired T tests.

Table 1. Summary of Primers

Lab Name	Gene	Sense or Antisense	Sequence
oRU 85	ADAR2	Sense	TCACCGTTGATACACTTCGTC
oRU 86	ADAR2	Antisense	AGTGCTCTCTGGAGTAGTGAT
oRU 97	IL6	Sense	CAA GAA AGA CAA AGC CAG AGT C
oRU 98	IL6	Antisense	GAA ATT GGG GTA GGA AGG AC
oRU 89	ADAR1 p150	Sense	GCA CTA TGT CTC AAG GGT TC
oRU 90	ADAR1 p150	Antisense	GGT ATC TCC ACT TGC TAT GC
oRU 109	GAPDH	Sense	CCCAGCTTAGGTTCATCAGG
oRU 113	GAPDH	Antisense	GCCGTTGAATTGCCGTGAG
oRU 93	TNF- α	Sense	ATCCTGGCCATCAAGGGCAATC
oRU 94	TNF- α	Antisense	TCCCTGGGGCCTCATTAGCATATCA

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RESULTS

The research goal was to identify if sex plays a role in expression of RNA editing enzymes as well as glucose metabolism when induced with acute inflammation. Inflammation was induced by injecting male and female mice with LPS, then glucose tolerance (Figure 6) and gene expression (Figure 7) was determined from these mice.

Inducing Acute Inflammation

In order to determine that the LPS injections successfully induced inflammation, the amount of IL-6 was determined from blood. Whole blood was extracted via cardiac puncture and the plasma was subjected to ELISA. There was 48.3 pg/ml IL-6 in LPS treated mice, versus 36.0 pg/ml concentration in saline treated mice (Figure 8). A significant ($p = 0.0009$) increase in IL-6 concentration was detected in LPS injected male mice compared to saline injected male mice. From the data, we concluded that the dose (2 mg/kg) and timing (24 hrs) of LPS injection appropriately induced inflammation.

Significance in Sex in Glucose Metabolism

In order to determine if there are sex-dependent effects of acute inflammation on glucose metabolism, glucose tolerance tests were performed on male and female mice treated with LPS or Saline. For the glucose tolerance tests, the blood glucose was measure from a tail snip before (time = 0 min) and after glucose injection. As is characteristic for glucose tolerance test, the blood glucose in these mice rose quickly from 0 min until around 20 min after injection, making a very steep slope. After reaching the peak, blood glucose starts decreasing at a much slower rate, making a much flatter slope (Figure 9). In general, blood glucose levels from female mice is lower than the male mice, throughout GTT. A repeated measures ANOVA test showed overall

there was a significant (p < 0.001) difference between the sexes. Post hoc testing indicates that the 10, 20, and 30 minute time points have significant differences between males and females (p < 0.001, Figure 9A). The area under the curve was determined from the GTT, but there were no significant differences between males and females (Figure 9B).

GTT and Inflammation in both Male and Female Mice

The GTT data for was further analyzed to determine the effects of inflammation on glucose tolerance. In both males and females, the amount of blood glucose was lower in LPS treated animals compared to saline treated animals (p < 0.001). In male mice treated with LPS (Figure 9C), blood glucose levels were significantly lower than saline treated in almost all time points including 0 min (p < 0.05), 10 min (p < 0.05), 30 min (p < 0.01), 45 min (p < 0.001), 60 min (p < 0.001), 75 min (p < 0.001), 90 min (p < 0.01). In female mice treated with LPS, the decrease (p < 0.001) in blood glucose starts at 20 min post glucose injection (Figure 10D) and continued in time point 30 min (p < 0.01) and 45 min (p < 0.05). Interestingly, the area under the curve (Figure 9B) was not different between the LPS and saline treated animals. The lack of difference in the AUC but significant difference in blood glucose suggests that LPS causes hypoglycemia, but that the GTT curves are parallel to one another.

Fasting Glucose Levels

From the glucose tolerance test curves (Figure 9A), we noticed that the fasting glucose were lower in LPS treated animal, compared to saline. Closer analysis of fasting glucose levels was done by determining the relative fasting glucose levels (Figure 10). All groups' fasting blood glucose levels were averaged and all individuals were normalized to the smallest average (Male LPS). While the fasting glucose levels were similar between males and females, fasting glucose levels were significantly lower in LPS treated animals, compared to Saline, in both male

($p < 0.001$) and female ($p = 0.006$) mice (Figure 10).

Normalized Blood Glucose

All LPS treated mice had reduced fasting blood glucose levels, but those levels were also generally lower over the course of the entire GTT. That the area under the curve is not different between sexes or between treatments, suggesting that the rate of glucose accumulation and uptake is similar between groups. We suggest that the glucose tolerance is better reflected by presenting the GTT data by percent of baseline. Therefore, the blood glucose levels over the course of an individual animal GTT were normalized to their own fasting blood glucose (time = 0 min). Normalizing allows all groups to start at the same point. There is a more significant increase in male LPS treated male mice at the 10, 20 and 30 min time points ($p < 0.001$), indicating that these mice have reduced glucose tolerance compared to all other groups (Figure 11A). There were no differences between treated and control female mice, after normalizing the blood glucose levels, indicating that the glucose tolerance of females is not affected by LPS. The males treated with saline also had similar GTT to the female mice, suggesting that sex-differences in GTT are isolated to those with acute inflammation.

Quantification of ADAR1p150 and TNF-α

In order to identify if there are significant differences in the expression of ADAR1p150 and ADAR2 between sexes when induced with acute inflammation, 4 hours after LPS injection, mice were euthanized by isoflurane followed by cervical dislocation. Brain and heart were dissected promptly and stored in liquid nitrogen to avoid RNA degradation. Organ homogenizing was then followed by RNA extraction and reverse transcriptase to gather cDNA. qPCR of cDNA was then used to measure the amount of expression of TNF-α, ADAR1p150, and ADAR2 (Figure 12). Samples were run in duplicate and analyzed using the Pfaffel method[50]. For mice

heart gene expression, the increased ratio of TNF-α was 6.6 fold expression LPS injection in

contrast to 1.7 fold expression for saline injected mice (Figure 11). ADAR1 also had higher

expression by exhibiting 25.6 fold expression LPS injection in contrast to 2.1 fold expression in

saline injected mice (Figure 11). Expression of ADAR2 did not differ much between the LPS

and Saline injected mice by having 1.7 and 1.2 fold expression respectively (Figure 11). There

was no significant difference between the LPS and saline for any of the targets analyzed here
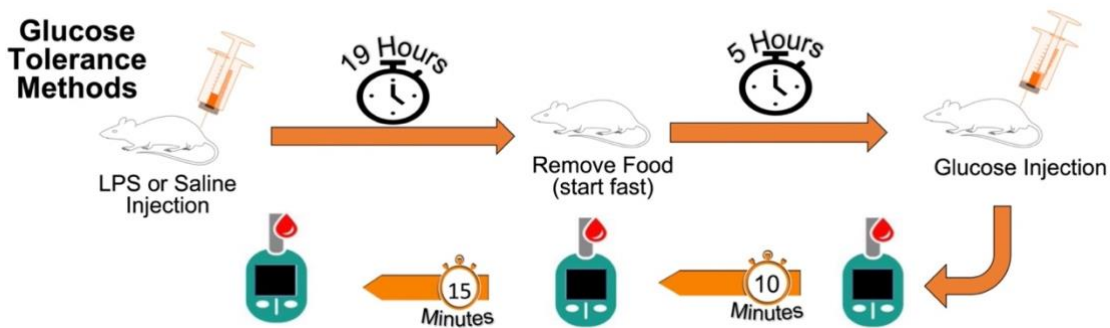
(Figures 11 and 12).

Figure 6. GTT method schematic.LPS or saline was injected intraperitoneally in mice. After 19 hours, food was removed from mice and commenced the 5 hour fast. Glucose injection was done via IP injection 24 hours after LPS or saline injection and blood glucose was measured every 10 or 15 minutes until 1 hour and 30 minutes had transpired.
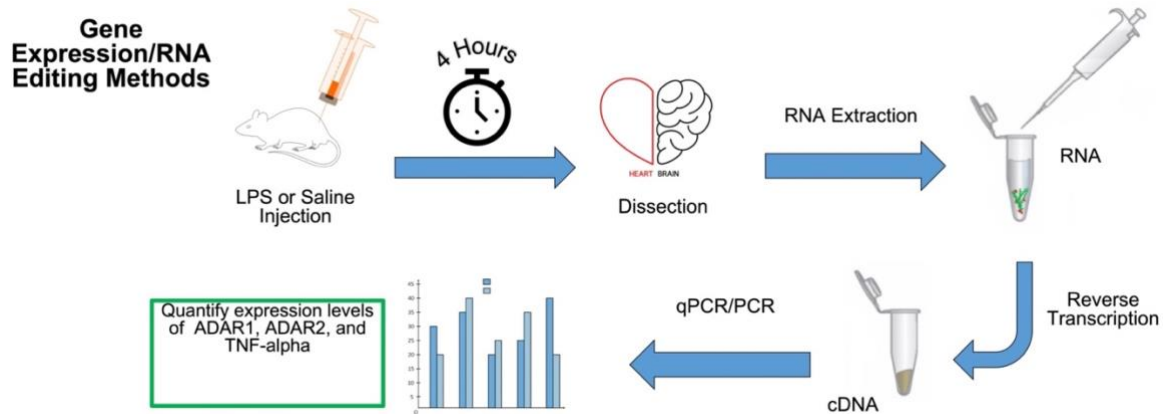
Figure 7. Analysis of gene expression methods. Mice were injected intraperitoneally with either saline or LPS. After 4 hours mice were dissected. RNA extracted from dissected tissues is then reverse transcribed to cDNA and subjected to PCR amplification in order to quantify the amount of expression of ADAR1, ADAR2, and TNF-α.
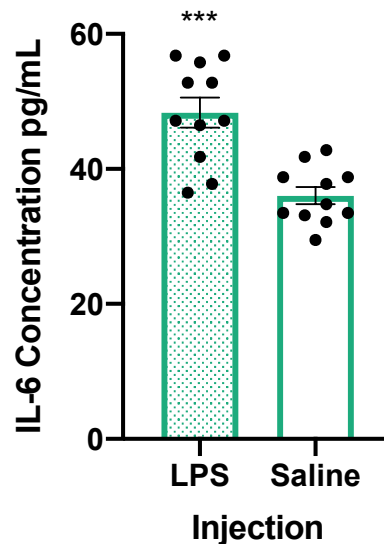
Figure 8. LPS induces acute inflammation. Plasma IL-6 levels were measured from male mice by ELISA. All samples were run with duplicates. Filled column represents the LPS injected male mice. The unfilled column is the saline injected male mice. A standard curve was developed and the equation of the line on this curve was used to determine the IL-6 concentration from the average of duplicates. Outliers were assessed univariately by examining the standardized z-scores. Paired t-test, $p = 0.0009$, N = 12, n = 24, error bars = SEM.
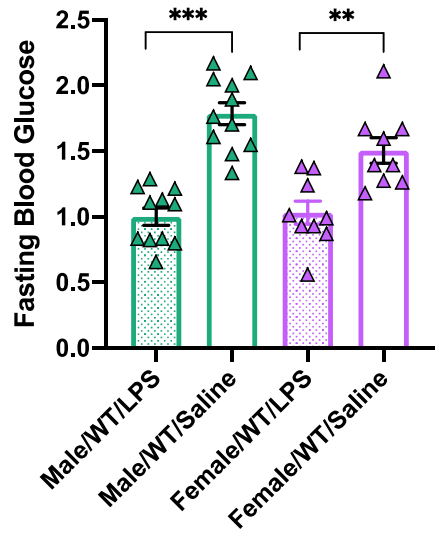
Figure 9. Relative Fasting Glucose. Blood glucose measured after 5 hours without food, and 24 hours after either LPS or saline injection. Green bars represent male mice while purple bars represent female mice. Filled bars represent LPS injected mice while non-filled bars represented saline injected mice. Each individual triangle indicates blood glucose from an individual mouse. All fasting levels of individual conditions were normalized to average LPS in each group. Male, N = 11, n = 22; Female N = 9, n = 18, one way ANOVA, *** p < 0.001.

A

Relative to Fasting GTT Males vs Female

B

Relative to Fasting GTT Female

C

Relative to Fasting GTT Males
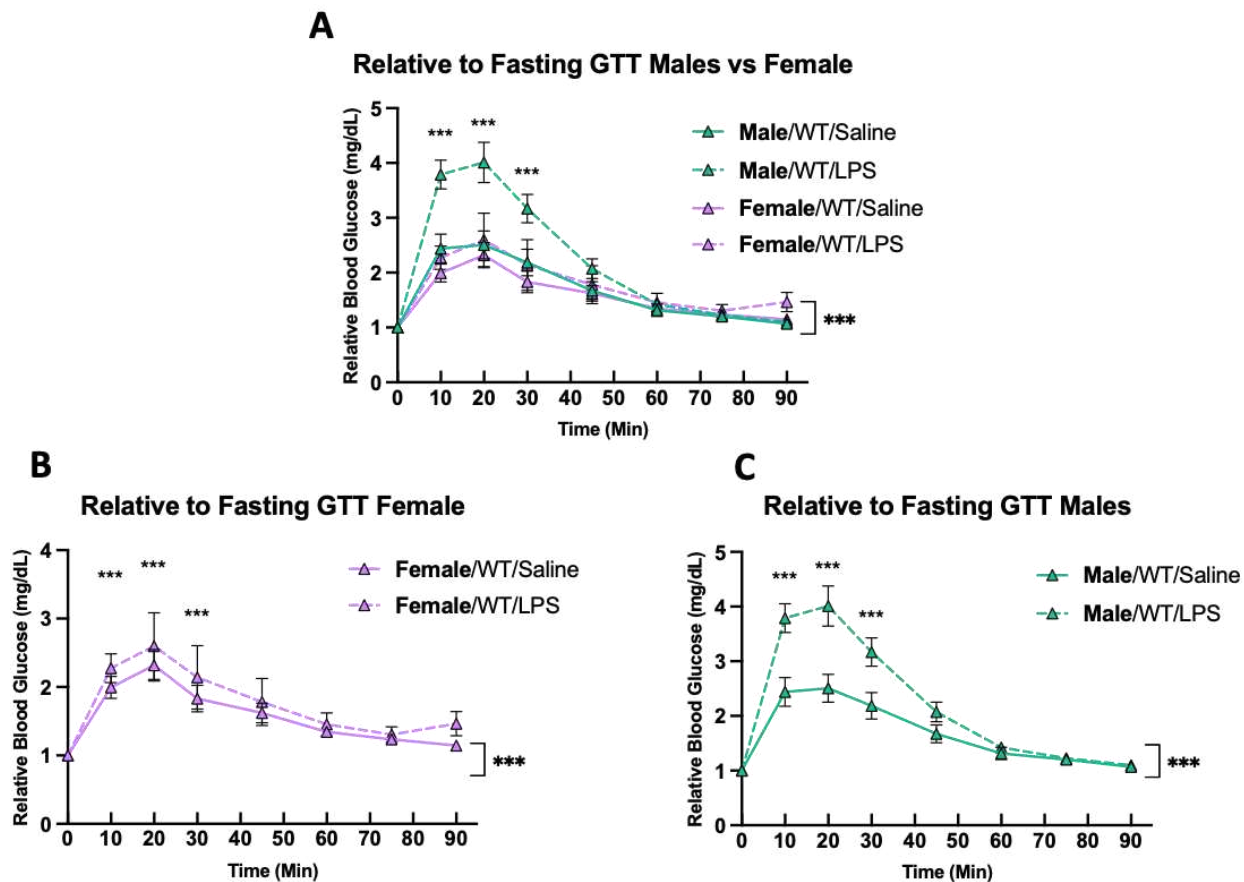
Figure 10. Normalized GTT. Mice were injected with LPS (dashed lines) or saline (solid lines) 24 hours before glucose injection (time 0). Blood glucose was determined over 90 min time course. Green lines represent the male mice (N = 11, n =2 2). Purple lines represent female mice (N = 9, n =1 8). All data is presented in A, while female and male data is separated in B and C, respectively. Outlier test was done to eliminate any outliers. Data was normsalized to starting fasting blood glucose for each individual (time 0). Check for normality was done on both sex and injection. Two-way ANOVA with repeated measures was performed, followed by post hoc Tukey test for multiple comparisons, ***$p < 0.001$, **$p < 0.01$, *$p<0.05$.

Figure 11. Gene expression form LPS and saline treated mouse heart. Four hours after IP injection with saline (blue) or LPS (red), mice were euthanized. RNA was extracted from dissected heart and expression levels of ADAR1, ADAR2, and TNF-α were measured via qRT-PCR. Independent T-test indicates no significant difference. TNF-α N = 4, ADAR1 N = 7, ADAR2 *N = 7.*

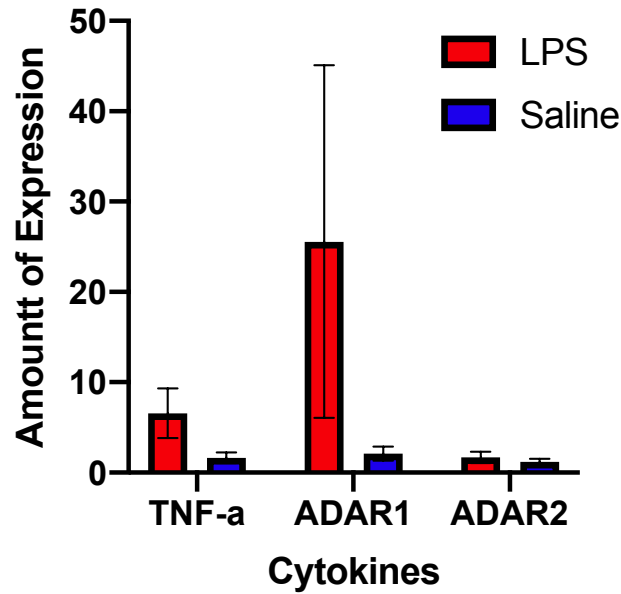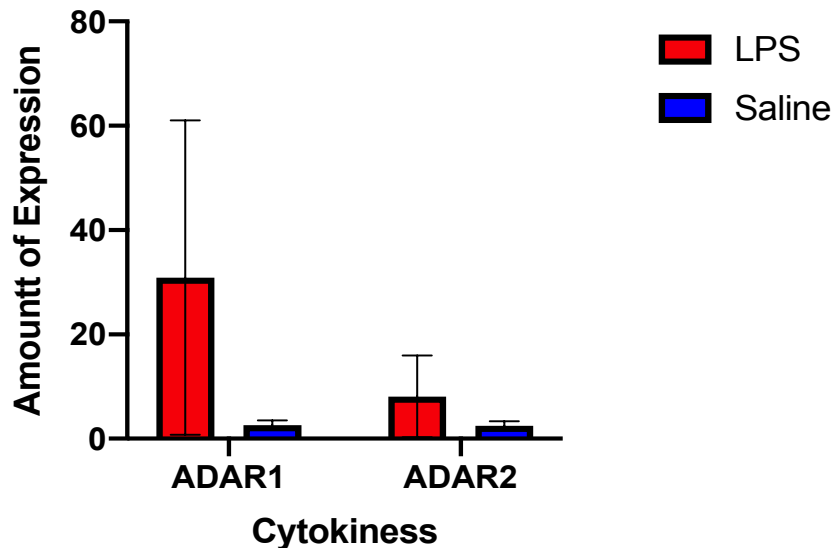Figure 12. Gene expression form LPS and saline treated mouse brain. Four hours after IP injection injected with saline (blue) or LPS (red), mice were euthanized. RNA was extracted from dissected brain and expression levels of ADAR1 and ADAR2 were measured via qRT-PCR. ADAR1 N = 7, ADAR2 N = 7

DISCUSSION

The goal of this thesis was to determine the physiological effects that sex and induced acute inflammation had on glucose tolerance and RNA editing enzymes. Importantly, we show significant differences in glucose metabolism between the sexes. The sex-dependent differences occurred in both saline and LPS treated mice, indicating that biological sex has a significant effect on glucose levels in the presence and absence of inflammation.

Confirming Innate Immune Activation

Inflammation was induced by injecting male and female mice with LPS. LPS has been well documented to cause inflammation. The dosage selected (2 mg/kg) was used in previous literature to induce an acute inflammatory response[13]. We verified that this dose of LPS affected innate immune system 24 hours after injection by measuring IL-6 levels at this time. IL-6 is an pro-inflammatory cytokine who expression is induced later and remains active longer[51]. The significant increase in induction of IL-6 in our experiments indicates that the innate immune pathway was activated by LPS and was still perpetuating immune reaction throughout the organism.

It was our intention to measure and compare innate immune reaction between male and female mice. However, IL-6 quantification was performed in only male mice. The blood extraction required for IL-6 ELISAs is performed after the completion of GTT. The GTT experiments were performed by teams of graduate and undergraduate students. While I performed the GTT and blood extractions, the GTT and blood extraction from female mice was performed by other students. I extracted blood via cardiac puncture, while other teams used tail snipping. Cardiac punctures require brief sedation of the mice, but quickly and efficiently

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extracts the maximum amount of blood from the animal with minimal stress. Tail snipping is crude and not recommended to extract the volume of blood required for ELISA analysis as it also includes both venous and arterial blood[52]. As the researcher is essentially milking the blood from tail, both blood and tissue fluid is extracted. Furthermore, the stress and repeated injury and inflammation potentially introduces confounding variables. Therefore, we cannot compare cytokine levels from blood obtained from two different methods. Future studies will perform cardiac punctures from mice immediately after fasting to determine cytokine levels from both male and female mice.

Before IL-6, we actually attempted to measure TNF-α levels. However, there was no detectible levels of TNF-α in the plasma (data not shown). More research revealed that TNF-α induction is at its highest around the second hour when involved in acute cytokine response, therefore it was likely that TNF-α had already peaked and lowered by the time we drew blood for plasma[53]. On the other hand, IL-6 induction had much higher expression 24 hours after an acute cytokine response and thus was chosen as the cytokine to quantify[53].

The dose and timing of LPS treatment was different in the ADAR1p150 studies compared to the GTT studies. For ADAR1p150, a higher dose of LPS (15 mg/kg compared to 2 mg/kg) was used due to previous studies showing induced peak levels of ADAR1 p150 four hours after injection of LPS[46]. Because we isolated tissues and plasma only 4 hours after LPS injection, we used TNF-α as the cytokine to verify innate immune activation. We also measured expression using qRT-PCR, rather than ELISA. There were not significant differences in the expression of ADAR1p150, TNF-α, and ADAR2. However, the variation between samples was significant. Increasing the number of samples is required to reduce the error and get a more accurate description of gene expression levels of all targets. We expected no significant

difference in the expression of ADAR2 since it is not induced by inflammation, but expected induction of TNF-α and ADAR1p150. Moreover, these studies only measured expression from the brain, while the goal was to analyze multiple tissues. Multiple tissues such as abdominal muscle, kidney, heart, brain, pituitary gland, eye, and pancreas were isolated from treated and control mice. Future students will focus on qRT-PCR from these additional tissues. We expect systemic induction of inflammation but it is possible that some tissues have a more robust response, compared to others. There were some issues that arose due to qPCR results not being consistent and expanding the standard error. Alternatively, instead of using semiquantitative analysis, a western blot may also be run to hopefully achieve more consistent results.

Other concurrent projects in the Ulbricht lab used these same mice to measure the endogenous RNA editing, with the goal of determining if induction of ADAR1p150 affected nuclear RNA editing of endogenous targets. These experiments showed that there were no changes in editing of the targets FLNA, FLNB or CAPS1 (C. Nichols Thesis, 5/2022) in the heart or brain. However, preliminary data showed that there were significant alterations in FLNA and FLNB editing in abdominal muscle, suggesting that injection of LPS indeed induced alterations to ADAR1 levels that then altered RNA editing levels. Quantification of ADAR1p150 from this thesis is meant to confirm the induction of ADAR1p150 in these mice, however, the extremely high variability in ADAR1 levels indicate that additional samples may be needed for proper estimates of ADAR1 level. It is necessary to determine if ADAR1p150 is induced in the heart brain and skeletal muscle of these mice. It would also be interesting to know if ADAR1p150 induction is greater in skeletal muscle, resulting in more dramatic, detectible alterations to RNA editing.

This study meant to induce temporary and acute inflammation. To induce ADAR1p150,

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our time frame between injection and tissue isolation was very short (4hrs) but the time frame for GTT studies was 24 hrs. By altering this time period, we could get a better idea on how innate immune activation affects physiology. Nguyen et al (2013) performed GTT 6 hrs after LPS injection and saw very similar LPS-induced effects of GTT, indicating that innate immune effects on glucose homeostasis are maintained for at least a day. Extension of the time period would give a better idea of the lasting effects of a single innate immune event. Alternatively, repeated LPS injections over several weeks or high fat diet over several months could be used to induce more chronic inflammation. These conditions could imitate natural situations such as seasonal allergies in which the immune response is activated daily. The conditions may also mirror those situations in which the host is infected with Epstein Barr virus and it triggers an autoimmune disorder such as lupus. Chronic inflammation may also be attempted in the future to study the influence of sex on chronic inflammatory conditions such as obesity or autoimmune disease. Previous literature has shown that males are more prone to chronic conditions such as type 2 diabetes, atherosclerosis, and greater incidences of infections compared to females whom are more prone to autoimmunity diseases as well as having adverse reactions to vaccines[54]. Further studies on chronic conditions would allow confirmation of previous literature as well as investigating the role RNA editing enzymes plays in those instances as well as how that would affect blood glucose metabolism.

Effects on Glucose Homeostasis

GTT of LPS and saline treated mice concurred with previous literature stating that inflammation causes hypoglycemia, or lower blood glucose[13]. The amount of blood glucose in male mice is very similar to previous reports with males starting about 150 mg/dL but decreasing to around 100 mg/dl after LPS treatment[21]. The LPS treated mice had lower blood glucose

throughout the whole GTT experiment, similar to previous studies[13]. The sex of the mice was not noted in this study, but we also see that sex has a significant effect on glucose levels. Male mice have significantly higher blood glucose, compared to female mice, particularly from the 0-30 min time frame of GTT (Figure 8A). This indicates that male mice have higher blood glucose than female mice, regardless of the inflammatory state of the mouse.

While the blood glucose levels were different between groups through most of the GTT, we noticed that the curves seemed parallel between groups. This suggested that the rate of glucose clearance may be similar between groups, even though the overall amounts of glucose were different. To further investigate on the rate of glucose clearance, we normalized the data from all the time points to the fasting glucose level. This normalized data allows each experiment to start at the same value. After normalizing the data, it was clear that the rate of glucose clearance from the blood was significantly reduced in male mice, compared to female mice. Also, LPS resulted in significantly lower rates of clearance of glucose than saline in males, but LPS did not affect the rates of glucose accumulation or clearance in females. This result suggests that glucose homeostasis is altered during inflammation in male animals, but that females are resistant to this[55].

Acute inflammation likely affects both glucose uptake and glucose production. The LPS treatment reduced blood glucose at fasting in both males and females. LPS has been shown to also increase glucose uptake[56,57] but also reduce it in some cells such as adipose cells[58]. LPS raises insulin levels, promoting cells to uptake glucose more efficiently. However, if cells fail to respond to insulin, then the cell's response will be blunted and blood glucose levels will not respond as robustly, no matter the insulin levels. Mice that are genetically engineered to be insulin resistant have GTT results that look very similar to our data from male mice indicating

that LPS may have caused a temporary insulin resistance in male mice[55]. To investigate if male mice are more prone to LPS induced insulin resistance, fasting insulin levels could be determined in male and female mice. No significant differences in fasting blood glucose levels were observed between male and female mice (Figure 9). Significant differences in amount of fasting insulin would indicate cell sensitivity to insulin due to sex of mouse. If no significant cell sensitivity to insulin is observed the same test could then be run on LPS and Saline mice. There was significant difference in fasting blood glucose when comparing LPS to saline. No significant differences in amount of fasting insulin could indicate change in sensitivity of cells to insulin. Significant difference of blood insulin levels observed due to injection would suggest change in production of insulin due to injection. Insulin tolerance testing may also be done on LPS and Saline mice. If LPS induces more blood glucose in male mice when challenged with insulin compared to female mice, this would indicate that LPS is affecting the sensitivity of cells to insulin in males. Insulin levels increase as insulin resistance increases[59]. If the previous test are done, LPS injected mice would most likely have additional fasting insulin compared to saline due to having higher fasting blood glucose. The higher fasting blood glucose is most likely due to cells being resistant to insulin. The resistance to insulin is what causes the host to produce more insulin to achieve desired blood glucose.

Our data demonstrates a lower amount of fasting blood glucose for both sexes with acute inflammation. Female mice how lower levels of TLR4, the major receptor for LPS, as well as fewer pro-inflammatory cytokines[38]. If female mice would have blood extracted via cardiac puncture, it would provide the ability to compare the cytokine expression of specifically IL-6 to male mice and see if there is a difference in IL-6 induction. Of course, induction of IL-6 is only one of dozens of cytokines, thus a potential lack of sex-dependent differences in this cytokine

does not rule out sex-dependent differences in other cytokines, or overall immune response.

This work can lead to further understanding of what acute inflammation does to glucose metabolism and how it is different for each sex. The different sex dependent effects of inflammation on glucose metabolism could also allow for more specialized drugs that could help males be able to increase their tolerance to maybe mimic the protection that females seem to have. This drugs could later be developed for humans suffering from diabetes or are suffering from inflammation to achieve a more precise glucose response without as many possible side effects. This work could also help guide more studies done on RNA editing enzymes such as ADAR1p150 to be done not only on more tissues but to also see if there are indeed significant changes in the expression of the enzyme due to sex.

REFERENCES

1. Tosi, M.F. Innate Immune Responses to Infection. J Allergy Clin Immun 2005, 116, 241–249, doi:10.1016/j.jaci.2005.05.036.

2. Cogen, A.L.; Nizet, V.; Gallo, R.L. Skin Microbiota: A Source of Disease or Defence? Brit J Dermatol 2008, 158, 442–455, doi:10.1111/j.1365-2133.2008.08437.x.

3. Kawai, T.; Akira, S. Toll-like Receptor and RIG-1-like Receptor Signaling. Ann Ny Acad Sci 2008, 1143, 1–20, doi:10.1196/annals.1443.020.

4. Takeuchi, O.; Akira, S. Pattern Recognition Receptors and Inflammation. Cell 2010, 140, 805–820, doi:10.1016/j.cell.2010.01.022.

5. Zhang, J.-M.; An, J. Cytokines, Inflammation, and Pain. Int Anesthesiol Clin 2007, 45, 27–37, doi:10.1097/aia.0b013e318034194e.

6. Heeg, K. The Innate Immune System. Neuroimmune Biology 2007, 7, 87–99, doi:10.1016/s1567-7443(07)00205-0.

7. Bianchi, M.E. DAMPs, PAMPs and Alarmins: All We Need to Know about Danger. J Leukocyte Biol 2007, 81, 1–5, doi:10.1189/jlb.0306164.

8. Abdul-Cader, M.S.; Senapathi, U.D.S.; Nagy, E.; Sharif, S.; Abdul-Careem, M.F. Antiviral Response Elicited against Avian Influenza Virus Infection Following Activation of Toll-like Receptor (TLR)7 Signaling Pathway Is Attributable to Interleukin (IL)-1β Production. Bmc Res Notes 2018, 11, 859, doi:10.1186/s13104-018-3975-4.

9. Verstak, B.; Stack, J.; Ve, T.; Mangan, M.; Hjerrild, K.; Jeon, J.; Stahl, R.; Latz, E.; Gay, N.; Kobe, B.; et al. The TLR Signaling Adaptor TRAM Interacts with TRAF6 to Mediate Activation of the Inflammatory Response by TLR4. J Leukocyte Biol 2014, 96, 427–436, doi:10.1189/jlb.2a0913-487r.

10. Rehwinkel, J.; Gack, M.U. RIG-I-like Receptors: Their Regulation and Roles in RNA Sensing. Nat Rev Immunol 2020, 20, 537–551, doi:10.1038/s41577-020-0288-3.

11. Dauphinee, S.M.; Karsan, A. Lipopolysaccharide Signaling in Endothelial Cells. Lab Invest 2006, 86, 9–22, doi:10.1038/labinvest.3700366.

12. Lu, Y.-C.; Yeh, W.-C.; Ohashi, P.S. LPS/TLR4 Signal Transduction Pathway. Cytokine 2008, 42, 145–151, doi:10.1016/j.cyto.2008.01.006.

13. Nguyen, A.T.; Mandard, S.; Dray, C.; Deckert, V.; Valet, P.; Besnard, P.; Drucker, D.J.; Lagrost, L.; Grober, J. Lipopolysaccharides-Mediated Increase in Glucose-Stimulated

Insulin Secretion: Involvement of the GLP-1 Pathway. Diabetes 2013, 63, 471–482, doi:10.2337/db13-0903.

14. Müller, T.D.; Finan, B.; Bloom, S.R.; D'Alessio, D.; Drucker, D.J.; Flatt, P.R.; Fritsche, A.; Gribble, F.; Grill, H.J.; Habener, J.F.; et al. Glucagon-like Peptide 1 (GLP-1). Mol Metab 2019, 30, 72–130, doi:10.1016/j.molmet.2019.09.010.

15. Petersen, M.C.; Shulman, G.I. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev 2018, 98, 2133–2223, doi:10.1152/physrev.00063.2017.

16. Palmer, C.S.; Ostrowski, M.; Balderson, B.; Christian, N.; Crowe, S.M. Glucose Metabolism Regulates T Cell Activation, Differentiation, and Functions. Front Immunol 2015, 6, 1, doi:10.3389/fimmu.2015.00001.

17. Turner, M.L.; Cronin, J.G.; Noleto, P.G.; Sheldon, I.M. Glucose Availability and AMP-Activated Protein Kinase Link Energy Metabolism and Innate Immunity in the Bovine Endometrium. Plos One 2016, 11, e0151416, doi:10.1371/journal.pone.0151416.

18. Buller, C.L.; Loberg, R.D.; Fan, M.-H.; Zhu, Q.; Park, J.L.; Vesely, E.; Inoki, K.; Guan, K.-L.; Brosius, F.C. A GSK-3/TSC2/MTOR Pathway Regulates Glucose Uptake and GLUT1 Glucose Transporter Expression. Am J Physiol-cell Ph 2008, 295, C836–C843, doi:10.1152/ajpcell.00554.2007.

19. MacIver, N.J.; Jacobs, S.R.; Wieman, H.L.; Wofford, J.A.; Coloff, J.L.; Rathmell, J.C. Glucose Metabolism in Lymphocytes Is a Regulated Process with Significant Effects on Immune Cell Function and Survival. J Leukocyte Biol 2008, 84, 949–957, doi:10.1189/jlb.0108024.

20. Oguri, S.; Motegi, K.; Iwakura, Y.; Endo, Y. Primary Role of Interleukin-1α and Interleukin-1β in Lipopolysaccharide-Induced Hypoglycemia in Mice. Clin Vaccine Immunol 2002, 9, 1307–1312, doi:10.1128/cdli.9.6.1307-1312.2002.

21. Raetzsch, C.F.; Brooks, N.L.; Alderman, J.M.; Moore, K.S.; Hosick, P.A.; Klebanov, S.; Akira, S.; Bear, J.E.; Baldwin, A.S.; Mackman, N.; et al. Lipopolysaccharide Inhibition of Glucose Production through the Toll-like Receptor-4, Myeloid Differentiation Factor 88, and Nuclear Factor Kb Pathway. Hepatology 2009, 50, 592–600, doi:10.1002/hep.22999.

22. Schneider, W.M.; Chevillotte, M.D.; Rice, C.M. Interferon-Stimulated Genes: A Complex Web of Host Defenses. Annu Rev Immunol 2014, 32, 513–545, doi:10.1146/annurev-immunol-032713-120231.

23. Lamers, M.M.; Hoogen, B.G. van den; Haagmans, B.L. ADAR1: "Editor-in-Chief" of Cytoplasmic Innate Immunity. Front Immunol 2019, 10, 1763, doi:10.3389/fimmu.2019.01763.

24. Siede, J.; Fröhlich, A.; Datsi, A.; Hegazy, A.N.; Varga, D.V.; Holecska, V.; Saito, H.; Nakae, S.; Löhning, M. IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGFβ Release. Plos One 2016, 11, e0161507, doi:10.1371/journal.pone.0161507.

25. Hood, J.L.; Morabito, M.V.; Martinez, C.R.; Gilbert, J.A.; Ferrick, E.A.; Ayers, G.D.; Chappell, J.D.; Dermody, T.S.; Emeson, R.B. Reovirus-Mediated Induction of ADAR1 (P150) Minimally Alters RNA Editing Patterns in Discrete Brain Regions. Molecular and Cellular Neuroscience 2014, 61, doi:10.1016/j.mcn.2014.06.001.

26. Wang, Q.; Li, X.; Qi, R.; Billiar, T. RNA Editing, ADAR1, and the Innate Immune Response. Genes-basel 2017, 8, 41, doi:10.3390/genes8010041.

27. Hartner, J.C.; Walkley, C.R.; Lu, J.; Orkin, S.H. ADAR1 Is Essential for the Maintenance of Hematopoiesis and Suppression of Interferon Signaling. Nat Immunol 2009, 10, 109–115, doi:10.1038/ni.1680.

28. Nishikura, K. Functions and Regulation of RNA Editing by ADAR Deaminases. Biochemistry-us 2010, 79, 321–349, doi:10.1146/annurev-biochem-060208-105251.

29. Jain, M.; Mann, T.D.; Stulić, M.; Rao, S.P.; Kirsch, A.; Pullirsch, D.; Strobl, X.; Rath, C.; Reissig, L.; Moreth, K.; et al. RNA Editing of Filamin A Pre-mRNA Regulates Vascular Contraction and Diastolic Blood Pressure. Embo J 2018, 37, doi:10.15252/embj.201694813.

30. Lehmann, K.A.; Bass, B.L. Double-Stranded RNA Adenosine Deaminases ADAR1 and ADAR2 Have Overlapping Specificities †. Biochemistry-us 2000, 39, 12875–12884, doi:10.1021/bi001383g.

31. Rice, G.I.; Kasher, P.R.; Forte, G.M.A.; Mannion, N.M.; Greenwood, S.M.; Szynkiewicz, M.; Dickerson, J.E.; Bhaskar, S.S.; Zampini, M.; Briggs, T.A.; et al. Mutations in ADAR1 Cause Aicardi-Goutières Syndrome Associated with a Type I Interferon Signature. Nat Genet 2012, 44, 1243–1248, doi:10.1038/ng.2414.

32. Kapoor, U.; Licht, K.; Amman, F.; Jakobi, T.; Martin, D.; Dieterich, C.; Jantsch, M. ADAR-Deficiency Perturbs the Global Splicing Landscape in Mouse Tissues. Genome Res 2020, 30, gr.256933.119, doi:10.1101/gr.256933.119.

33. Sun, T.; Yu, Y.; Wu, X.; Acevedo, A.; Luo, J.-D.; Wang, J.; Schneider, W.M.; Hurwitz, B.; Rosenberg, B.R.; Chung, H.; et al. Decoupling Expression and Editing Preferences of ADAR1 P150 and P110 Isoforms. Proc National Acad Sci 2021, 118, e2021757118, doi:10.1073/pnas.2021757118.

34. Shen, Y.; Matsuno, Y.; Fouse, S.D.; Rao, N.; Root, S.; Xu, R.; Pellegrini, M.; Riggs, A.D.; Fan, G. X-Inactivation in Female Human Embryonic Stem Cells Is in a Nonrandom

Pattern and Prone to Epigenetic Alterations. Proc National Acad Sci 2008, 105, 4709–4714, doi:10.1073/pnas.0712018105.

35. Pisitkun, P.; Deane, J.A.; Difilippantonio, M.J.; Tarasenko, T.; Satterthwaite, A.B.; Bolland, S. Autoreactive B Cell Responses to RNA-Related Antigens Due to TLR7 Gene Duplication. Science 2006, 312, 1669–1672, doi:10.1126/science.1124978.

36. Souyris, M.; Mejía, J.E.; Chaumeil, J.; Guéry, J.-C. Female Predisposition to TLR7-Driven Autoimmunity: Gene Dosage and the Escape from X Chromosome Inactivation. Semin Immunopathol 2019, 41, 153–164, doi:10.1007/s00281-018-0712-y.

37. Klein, S.L.; Flanagan, K.L. Sex Differences in Immune Responses. Nat Rev Immunol 2016, 16, 626–638, doi:10.1038/nri.2016.90.

38. Frisancho-Kiss, S.; Davis, S.E.; Nyland, J.F.; Frisancho, J.A.; Cihakova, D.; Barrett, M.A.; Rose, N.R.; Fairweather, D. Cutting Edge: Cross-Regulation by TLR4 and T Cell Ig Mucin-3 Determines Sex Differences in Inflammatory Heart Disease. J Immunol 2007, 178, 6710–6714, doi:10.4049/jimmunol.178.11.6710.

39. Whitacre, C.C. Sex Differences in Autoimmune Disease. Nat Immunol 2001, 2, 777–780, doi:10.1038/ni0901-777.

40. Derry, H.M.; Padin, A.C.; Kuo, J.L.; Hughes, S.; Kiecolt-Glaser, J.K. Sex Differences in Depression: Does Inflammation Play a Role? Curr Psychiat Rep 2015, 17, 78, doi:10.1007/s11920-015-0618-5.

41. Slavich, G.M.; Sacher, J. Stress, Sex Hormones, Inflammation, and Major Depressive Disorder: Extending Social Signal Transduction Theory of Depression to Account for Sex Differences in Mood Disorders. Psychopharmacology 2019, 236, 3063–3079, doi:10.1007/s00213-019-05326-9.

42. Mauvais-Jarvis, F. Gender Differences in Glucose Homeostasis and Diabetes. Physiol Behav 2018, 187, 20–23, doi:10.1016/j.physbeh.2017.08.016.

43. Marino, H.A. Modulation of Glucose Homeostasis by Nucleotide P2Y2 Receptor and Biological Sex, 2021.

44. Dockman, R.L.; Carpenter, J.M.; Diaz, A.N.; Benbow, R.A.; Filipov, N.M. Sex Differences in Behavior, Response to LPS, and Glucose Homeostasis in Middle-Aged Mice. Behav Brain Res 2022, 418, 113628, doi:10.1016/j.bbr.2021.113628.

45. Kabir, K.; Gelinas, J.-P.; Chen, M.; Chen, D.; Zhang, D.; Luo, X.; Yang, J.-H.; Carter, D.; Rabinovici, R. Characterization of a Murine Model of Endotoxin-Induced Acute Lung Injury. Shock 2002, 17, 300–303, doi:10.1097/00024382-200204000-00010.

46. Yang, J.; Luo, X.; Nie, Y.; Su, Y.; Zhao, Q.; Kabir, K.; Zhang, D.; Rabinovici, R. Widespread Inosine-containing MRNA in Lymphocytes Regulated by ADAR1 in Response to Inflammation. Immunology 2003, 109, 15–23, doi:10.1046/j.1365-2567.2003.01598.x.

47. Quintero, O.L.; Amador-Patarroyo, M.J.; Montoya-Ortiz, G.; Rojas-Villarraga, A.; Anaya, J.-M. Autoimmune Disease and Gender: Plausible Mechanisms for the Female Predominance of Autoimmunity. J Autoimmun 2012, 38, J109–J119, doi:10.1016/j.jaut.2011.10.003.

48. Ahmed, A.; Khalique, N. Molecular Basis of Blood Glucose Regulation. 2019, doi:10.5772/intechopen.89978.

49. Song, C.; Sakurai, M.; Shiromoto, Y.; Nishikura, K. Functions of the RNA Editing Enzyme ADAR1 and Their Relevance to Human Diseases. Genes-basel 2016, 7, 129, doi:10.3390/genes7120129.

50. Pfaffl, M.W. A New Mathematical Model for Relative Quantification in Real-Time RT–PCR. Nucleic Acids Res. 2001, 29, e45–e45, doi:10.1093/nar/29.9.e45.

51. Liravi, B.; Piedrafita, D.; Nguyen, G.; Bischof, R.J. Dynamics of IL-4 and IL-13 Expression in the Airways of Sheep Following Allergen Challenge. Bmc Pulm Med 2015, 15, 101, doi:10.1186/s12890-015-0097-9.

52. Diehl, K.H.; Hull, R.; Morton, D.; Pfister, R.; Rabemampianina, Y.; Smith, D.; Vidal, J.M.; Vorstenbosch, C. van de; Methods, E.F. of P.I.A. and E.C. for the V. of A. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. J Appl Toxicol 2001, 21, 15–23, doi:10.1002/jat.727.

53. Matsui, A.; Stephens, D.; Kantarci, A.; Rittling, S.R. Early Cytokine Response to Infection with Pathogenic vs Non-Pathogenic Organisms in a Mouse Model of Endodontic Infection. Plos One 2015, 10, e0132752, doi:10.1371/journal.pone.0132752.

54. Bupp, M.R.G. Sex, the Aging Immune System, and Chronic Disease. Cell. Immunol. 2015, 294, 102–110, doi:10.1016/j.cellimm.2015.02.002.

55. Dridi, L.; Seyrantepe, V.; Fougerat, A.; Pan, X.; Bonneil, É.; Thibault, P.; Moreau, A.; Mitchell, G.A.; Heveker, N.; Cairo, C.W.; et al. Positive Regulation of Insulin Signaling by Neuraminidase 1. Diabetes 2013, 62, 2338–2346, doi:10.2337/db12-1825.

56. Lang, C.H. Neural Regulation of the Enhanced Uptake of Glucose in Skeletal Muscle after Endotoxin. Am J Physiology-regulatory Integr Comp Physiology 1995, 269, R437–R444, doi:10.1152/ajpregu.1995.269.2.r437.

57. Mészáros, K.; Lang, C.H.; Bagby, G.J.; Spitzer, J.J. Contribution of Different Organs to Increased Glucose Consumption after Endotoxin Administration. J Biol Chem 1987, 262, 10965–10970, doi:10.1016/s0021-9258(18)60912-3.

58. Johnson, A.R.; Milner, J.J.; Makowski, L. The Inflammation Highway: Metabolism Accelerates Inflammatory Traffic in Obesity. Immunol Rev 2012, 249, 218–238, doi:10.1111/j.1600-065x.2012.01151.x.

59. Bhat, S.L.; Abbasi, F.A.; Blasey, C.; Reaven, G.M.; Kim, S.H. Beyond Fasting Plasma Glucose: The Association between Coronary Heart Disease Risk and Postprandial Glucose, Postprandial Insulin and Insulin Resistance in Healthy, Nondiabetic Adults. Metabolism 2013, 62, 1223–1226, doi:10.1016/j.metabol.2013.04.012.

APPENDICES

Appendix A

June 14, 2023

RE: IACUC protocol 18.006, 19.004, & 2021-02

Christian Rivas,

IACUC protocol # 18.006 entitled "Glucose Tolerance Test in the P2Y2 Knockout mouse" was approved by the committee on October,12, 2017 through October 11, 2020 and reapproved by the committee as protocol # 2021-02 on February 5, 2021 expiring February 4, 2025. IACUC protocol # 19.004 entitled "Mouse breeding protocol" was approved by the committee on January 14, 2019 – January 13, 2022.

The protocols reflect that you were approved to work with Dr. Jianjie Wang and Dr. Randi Ulbricht on these projects.

Thank you and if you need anything in the future regarding these protocols, please contact me either via email (johnnapedersen@missouristate.edu) or at 417-836-3737.

Sincerely,

Johnna Pedersen

Johnna Pedersen
IACUC Administrator/Member
Interim Director of Research Administration

Appendix B

Missouri State
UNIVERSITY

June 14, 2023

RE: IBC protocol 2016-10

Christian Rivas,

IBC protocol # 2016-10 entitled "RNA editing" was approved by the committee on September 13, 2016 - September 12, 2019 and reapproved by the committee on September 22, 2019 – April 28, 2022.

The protocol reflect that you were approved to work with Dr. Randi Ulbricht on this project.

Thank you and if you need anything in the future regarding these protocols, please contact me either via email (johnnapedersen@missouristate.edu) or at 417-836-3737.

Sincerely,

Johnna Pedersen

Johnna Pedersen
IBC Administrator
Interim Director of Research Administration

OFFICE OF RESEARCH ADMINISTRATION
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Appendix C

CITI PROGRAM

Completion Date 04-Aug-2019
Expiration Date N/A
Record ID 32664144

This is to certify that:

Christian Rivas

Has completed the following CITI Program course:

Reducing Pain and Distress in Laboratory Mice and Rats
(Curriculum Group)
Reducing Pain and Distress in Laboratory Mice and Rats
(Course Learner Group)
1 - Basic Course
(Stage)

Not valid for renewal of certification through CME.

Under requirements set by:

Missouri State University

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w3f1f1842-d1ab-43eb-b054-3be6a91d2822-32664144

Completion Date 04-Aug-2019
Expiration Date 03-Aug-2021
Record ID 32664150

This is to certify that:

Christian Rivas

Has completed the following CITI Program course:

Not valid for renewal of certification through CME.

Biosafety and Biosecurity (BSS)
(Curriculum Group)
Basic Biosafety Course
(Course Learner Group)
1 - Basic Course
(Stage)

Under requirements set by:

Missouri State University

Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w3fd9a665-c714-4efa-b012-6ce1a18da174-32664150