The Role of Aconitate Decarboxylase 1 in Inflammation and Disease

by

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DEDICATION

To my family (four legged included), friends and Kelsey. Your endless love and support are the structure I stand on today. It is an enormous understatement to say I would not be where I am without what you have given me each day of my life. Thank you.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Acod1	Aconitate decarboxylase
PRR's	pattern recognition receptors
PAMPs	pathogen associated molecular patterns
DAMPs	damage associated molecular patterns
IFN	interferon
CAM	classically activated macrophage
AAM	alternatively activated macrophage
TLRs	toll-like receptors
ROS	reactive oxygen species
LPS	lipopolysaccharide
LBP	LPS binding protein
CD14	cluster of differentiation 14
TLR4	toll-like receptor 4
MD2	myeloid differentiation factor 2
NF-κB	nuclear factor kappa B
IRF3	interferon regulatory factor 3
MyD88	myeloid differentiation primary response gene 88
TRIF	TIR domain-containing adaptor -inducing interferon-beta
TIR	toll/IL-1R
TCA	tricarboxylic acid
ATP	adenosine triphosphate
OXPHOS	oxidative phosphorylation
PPP	pentose phosphate pathway
OCR	oxygen consumption rate
ECAR	extracellular acidification rate

Irg1	immunoresponsive gene 1
SDH	succinate dehydrogenase
DMI	dimethyl itaconate
4-OI	4-octyl itaconate
BMDM	bone marrow derived macrophage
WT	wild type
Acod1KO	Acod1 knockout
Nrf2	nuclear factor-erythroid factor 2
KEAP1	kelch-like ECH associated protein 1
ATF3	activating transcription factor 3
GSH	glutathione
ICL	isocitrate lyase
SARS-CoV2	Severe Acute Respiratory Syndrome Coronavirus 2
MS	multiple sclerosis
DMF	dimethyl fumarate
I/R	ischemia/reperfusion
MCA	middle cerebral artery
BBB	blood brain barrier
LDH	lactate dehydrogenase
GPX4	glutathione peroxidase 4
DIO	diet induced obesity
DSS	dextran sulfate sodium

ABSTRACT

Immune cell metabolism, or immunometabolism, has recently become of interest for its role in inflammation and disease. A growing field of research has identified that metabolic rewiring and immune cell activation are intimately connected, however the mechanisms driving these connections have remained poorly understood. The tricarboxylic acid cycle and its intermediates have become recognized as major players in disease and inflammation. The immunometabolite itaconate has been identified as a potent immunomodulator produced in high quantities in activated macrophages. Itaconate is produced by the enzyme aconitate decarboxylase 1 (Acod1), which is highly upregulated in proinflammatory macrophages. Although itaconate and Acod1 have been found to be upregulated in macrophages under stimulated conditions, the potential role of itaconate production in other non-immune cells remains poorly understood. Itaconate and its exogenous derivative forms have been found to be potent mediators of inflammation, and specifically have been found to decrease proinflammatory cytokine production in cultured macrophages.

In this dissertation, we sought to identify the role of itaconate in three separate murine models of disease: cerebral ischemia/reperfusion injury, diet-induced obesity, and ulcerative colitis. We hypothesized that deletion of Acod1 would lead to greater disease severity in these models and that macrophages would be the primary cell type responsible. To understand the role of endogenously produced itaconate, mice lacking Acod1 ($Acod1^{-/-}$) were used. We demonstrate that global Acod1 deletion leads to significantly worsened disease severity in all three models studied. Specifically, Acod1 deletion leads to increased lesion volume size compared to wild type (WT) mice in a model of ischemia/reperfusion stroke. The observed increased lesion volume did not appear to be caused by increased inflammation, indicating a separate potential mechanism driving these changes. In a model of diet-induced obesity, mice lacking Acod1 showed similar weight gain compared to WT mice, however, $Acod1^{-/-}$ mice had elevated blood glucose levels after 12 weeks of high fat diet. $Acod1^{-/-}$ mice also had elevated inflammatory gene expression. Furthermore, naïve $Acod1^{-/-}$ mice had significant increases in fat deposition when on chow diet at 3 and 6 months of age. Lastly, $Acod1^{-/-}$ mice exposed to an acute ulcerative colitis model induced by dextran sulfate

sodium (DSS) treatment showed increased disease severity with more severe and sustained body weight loss and increased inflammatory gene expression.

Importantly, cell specific knockout of Acod1 in myeloid cells (MyAcod1$^{-/-}$) with LysM-Cre did not phenocopy disease severity in any of the three in vivo models. This suggests that myeloid cells, specifically macrophages, are not the primary cell type responsible for the observed phenotypes seen in the global Acod1$^{-/-}$ studies. These data define a novel role for Acod1 in transient ischemia/reperfusion occlusion stroke, diet-induced obesity, and ulcerative colitis. Furthermore, these differences do not appear to be regulated by Acod1 and itaconate in macrophage/myeloid cells. These findings suggest that Acod1 and itaconate are likely working through other cell types.

To further explore potential mechanisms driving the observed Acod1-/- phenotypes, we sought to identify if Acod1 and endogenous itaconate were capable of modulating ferroptosis induced cell death. Using bone marrow derived macrophages as a model cell type capable of expressing Acod1, we found that macrophages lacking Acod1 had significantly increased susceptibility to RSL3 induced ferroptosis death compared to WT cells. Further analysis found that Acod1-/- macrophages also showed decreased glutathione levels compared to their WT counterparts. Lastly, we found that supplementing Acod1-/- cells with exogenous itaconate restored protection from RSL3 induced cell death and increased glutathione level to what is observed in WT macrophages. These findings would suggest that Acod1 and endogenous itaconate play a role in ferroptosis protection through sustaining intracellular glutathione levels, and could be a relevant mechanism regarding the protective role of Acod1 from ischemia/reperfusion injury.

CHAPTER 1

Introduction

1.1 Innate immune cell metabolism and inflammatory responses

The human body is under constant exposure to external threats from viruses, bacteria, fungi, and other opportunistic pathogens. Although this constant exposure is a regular threat, our bodies have systems in place to fight off these pathogens. The immune system is a complex and highly regulated safeguard that detects and eliminates potential threats in an organism. There are two main parts of the immune system that serve specific functions and roles. The first being the innate immune system and is a general, nonspecific response that includes leukocytes such as macrophages, mast cells, neutrophils, dendritic cells among others[1-4]. The second is the adaptive immune system, which primarily relies on immunological memory to mount a response and in general, is the slower of the two responses. This chapter will focus on the innate immune response, relevant cell signaling pathways involved in the innate inflammatory response, and how these responses are regulated.

1.2 Innate immune system function and inflammatory responses

The innate immune system is the most evolutionarily conserved immune response and is responsible for generating a quick, non-specific response that is primarily activated by pattern recognition receptors (PRR's). Innate immunity can be viewed in four major categories: anatomic, physiologic, endocytic/phagocytic, and inflammatory. (1) Skin and mucous membranes are common examples of the anatomic barriers of the innate immune system by acting as an effective barrier against microorganisms[2, 4, 5]. (2) Common physiologic barriers include pH, oxygen tension, temperature, and various soluble factors. These physiologic barriers act to deter pathogens by limiting their ability to survive by simply not being compatible with the hosts environment. Examples of this can include the internal body temperature not being suitable for specific

microorganisms to grow, or the low pH in the stomach from gastric acid limiting the growth of microorganisms[2, 4]. (3) The endocytic/phagocytic barriers are derived from cells ability to internalize (endocytosis) and break down foreign molecules found in the cell's environment. Furthermore, several specialized cells have the ability to capture, internalize, and kill entire pathogens. The most common phagocytic cells, or professional phagocytes, include monocytes, neutrophils, dendritic cells, and macrophages. (4) The inflammatory barrier of the innate immune system is caused by invasion of microorganisms and pathogens. The invasion of these foreign pathogens leads to a complex and robust sequence of highly regulated responses known as the immune response. The most classic signs of inflammation are often referred to as the "four cardinal signs of inflammation" and include redness, swelling, heat and pain at the sight of injury or infection. Along with these hallmark signs, the inflammatory response involves the trafficking of immune cells, and large amounts of cytokine production[2, 4]. These cardinal signs of inflammation are key indications of three major steps that take place during inflammation: vasodilation, increases in capillary permeability, and an influx of circulating phagocytic cells.

Innate immunity is considered the first line of defense when pathogens and injury occur. The major cells of the innate immune response include natural killer cells, neutrophils, dendritic cells, mast cells, basophils, and macrophages[2]. For this chapter, macrophages, their role in inflammation, and the changes they undertake during activation will be discussed in detail. Macrophages are professional phagocytes that play a major role in inflammation by detecting, capturing, and destroying microbial threats[6]. This function is performed by continuous scanning of the tissue environment where resident tissue macrophages reside. Macrophages also play a major part in regulation of wound healing and resolution of inflammation, where they play an active role in homeostasis and integrity[6, 7]. In this capacity, they can act by clearing debris from an injury site and promote tissue repair. It has recently been appreciated that macrophages and other immune cell types undergo distinct shifts in metabolic states upon activation[8, 9]. Activation of macrophages is primarily through chemical signal recognition through binding on PRRs. These PRRs are found on macrophage and respond to pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). Upon detection of PAMPs, the cells of the innate immune system are activated resulting in an inflammatory cascade. This activation also initiates phagocytosis, notably in macrophages, as well as cytokine and interferon (IFN) secretion. The secretion of these inflammatory signaling molecules leads to inflammation and induction of

antimicrobial/antiviral responses[2, 4, 9]. During infection or tissue injury, resident immune cells are activated through recognition of PAMPs or DAMPs, eliciting the release of proinflammatory cytokines and chemokines that recruit additional circulating immune cells[10]. This leads to a major influx of immune cells starting with neutrophils, monocytes, dendritic cells, and lymphocytes[6, 10]. The recruitment of macrophages specifically, can originate from the spleen which has been shown to be a significant contributor of immune cells in early stages of injury[10-13]. The more classic site of macrophage and monocyte recruitment is from bone marrow. Macrophages and monocytes are derived from hematopoietic precursor cells that are recruited to the circulation following detection of pathogens or tissue injury (Figure 1.1) [1, 10, 14].

1.3 Macrophage polarization (M1 vs. M2), TLR4 signaling pathway (general)

Macrophages are important innate immune cells and play a critical role in both normal physiology as well as during the pathophysiology of disease. Originally, macrophages were classified into two major groups, one being the classically activated macrophage (CAM) and the other alternatively activated macrophage (AAM) and were defined as M1 or M2 respectively (Figure 1.2). This classification was dependent on induction by specific Th1 and Th2 cytokines in vitro. However, in vivo macrophages display a wide range of diverse phenotypes during injury and infection and cover a wide spectrum spanning M1 and M2 markers, emphasizing that this

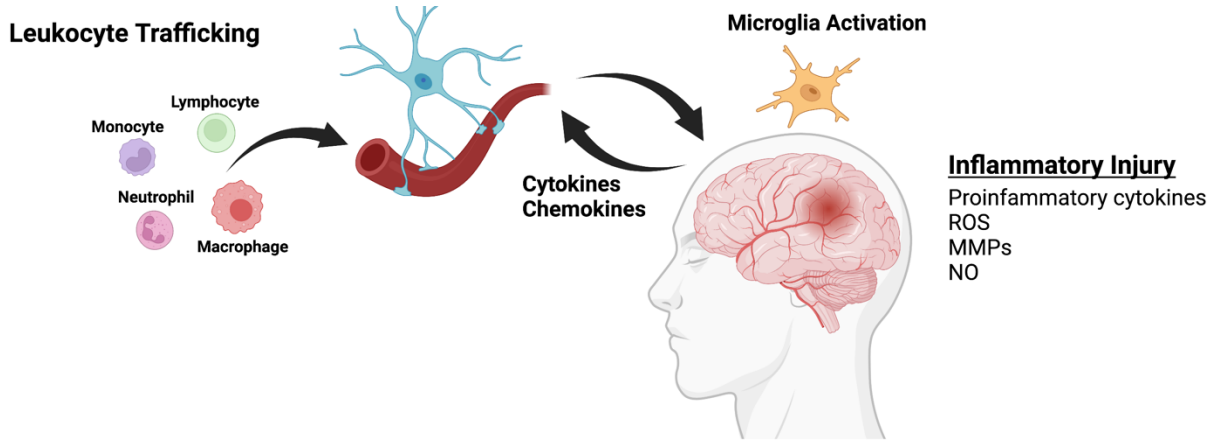

Figure 1.1. Immune cell trafficking during cerebral ischemia-reperfusion injury. Following injury, tissue resident immune cells activate and release proinflammatory cytokines and chemokines in conjunction with other affected tissue resident cell populations. This leads to recruitment and activation of circulating immune cells from the bone marrow niche and spleen leading to an influx of immune cells and inflammation.

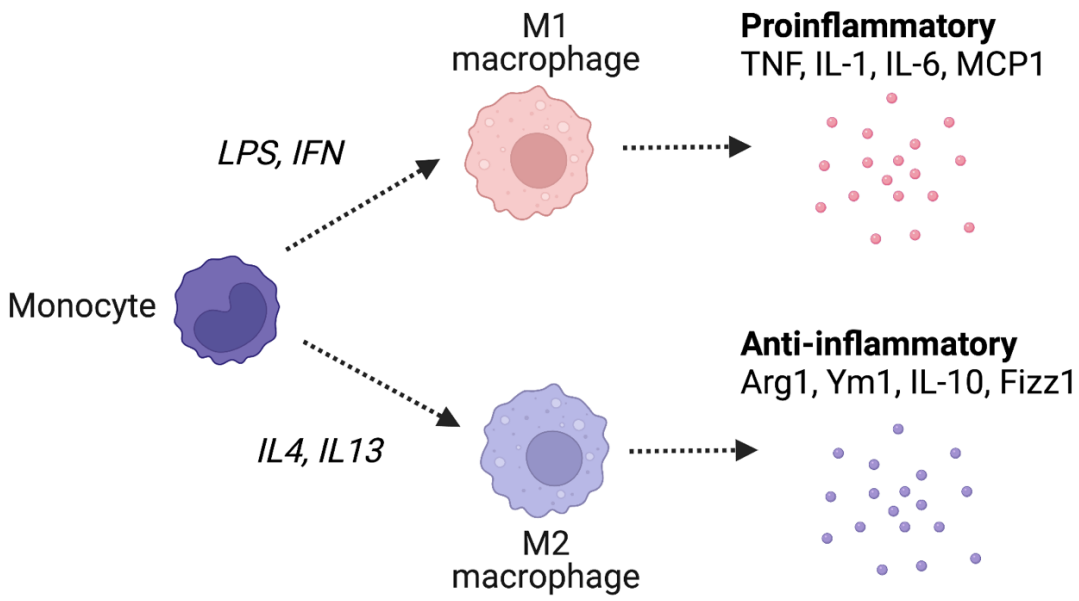

Figure 1.2. In vitro induction of M1 and M2 macrophage polarization. Classical activation, or M1 activation, is commonly caused through stimulation with LPS, IFN, or a combination of the two in vitro. This leads to the release of proinflammatory cytokines and chemokines including IL-1β, TNFα, IL-6 and MCP1. Alternative activation, or M2 activation is achieved with IL-4 or IL-13 stimulation and leads to the release of anti-inflammatory cytokines and chemokines including Arg1, YM1, IL-10 and Fizz1.

simplistic dichotomous classification is not sufficient to characterize the numerous macrophage phenotypes observed in vivo[14-18].

In general, CAMs are considered pro-inflammatory and can be induced by stimulation with IFN-γ and lipopolysaccharide (LPS) during type 1 immune responses[15, 16]. Macrophages are a major cell type involved in the innate immune system response to microbial invasion and sensing damaged tissue in injury[18, 19]. The sensing of these signals is primarily accomplished through PRRs, which include toll-like receptors (TLRs)[20]. During type 1 immune responses, CAMs contribute to inflammation by releasing pro-inflammatory cytokines including IL-1β, TNFα and IL-6 and by producing reactive oxygen species (ROS)[21]. The increase in ROS is primarily thought to occur through up-regulation of iNOS and NADPH oxidase[4, 22-24]. In contrast, AAMs are considered to be important in wound healing and have anti-inflammatory properties. AAMs have their own unique set of markers associated with them as well, and can include Arg1, IL-10, Ym1, and CD206[18]. It is believed that AAMs play a protective role in a number of cardiovascular diseases, although the exact mechanisms associated with this protection are not

clear. Specifically, studies of myocardial infarction and stroke have identified a transition to M2 macrophages as being critical for wound healing and resolution of inflammation[25-27]. In general, AAMs are considered to be involved in inflammation resolution and wound healing[28].

1.4 TLR4 signaling and immune cell metabolic shifts

Macrophage activation occurs through a variety of stimuli such as DAMPs and PAMPs[4] and is critical in response to bacterial, viral, and fungal infection. Toll-like receptors (TLR's) are one the major groups of proteins responsible for recognition of PAMPs and DAMPs leading to activation of inflammatory cascades and a pro-inflammatory response[19, 29]. One of the most well studied pro-inflammatory stimuli is lipopolysaccharide (LPS). LPS is commonly found on the outer membrane of gram-negative bacteria and acts as a potent activator of macrophages[29, 30]. Excessive exposure to high levels of LPS leads to uncontrolled production of pro-inflammatory cytokines and in extreme cases can lead to septic shock, highlighting the extent of LPS's pro-inflammatory activation potential[31]. During bacterial infection, LPS recognition is first initiated by binding to LPS binding protein (LBP)[30]. LBP works to bind LPS from bacterial cell membranes or vesicles released by bacteria found in serum of the infected host. Once LPS is bound to LBP it is then transferred to cluster of differentiation 14 (CD14) on immune cell membranes. CD14 then plays a crucial role by presenting monomeric LPS molecules to toll-like receptor 4 (TLR4)/ lymphocyte antigen 96 (MD2) complex[31, 32]. Activation of this TLR4/MD2 complex is achieved through dimerization and allows for initiation of multiple pro-inflammatory signaling components such as nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 (IRF3)[30, 33]. This series of events leads to expression and eventual release of pro-inflammatory cytokines and interferons.

TLR4 is unique in the toll-like receptor family by its ability to synergistically activate both myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor - inducing interferon-beta (TRIF) dependent pathways of activation[32]. The MyD88 dependent pathway is initiated directly from TLR4 activation, allowing MyD88 to bind its cytosolic Toll/IL-1R (TIR) domain[32]. The TRIF dependent pathway works separately and relies on endocytosis of TLR4 for initiation[33, 34]. Both pathways lead to pro-inflammatory activation of macrophages and other innate immune cells highlighting redundancy in immune cell activation pathways (Figure 1.3). However, recent studies have shown that NF-κB is primarily regulated through the

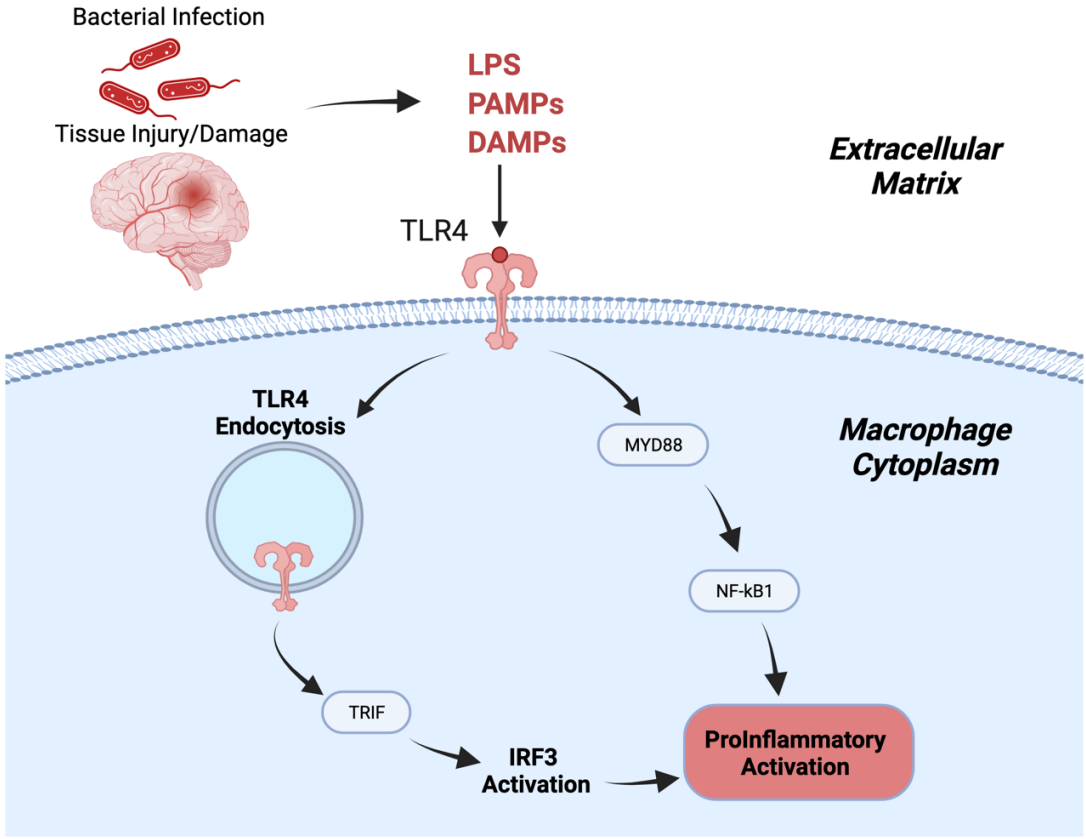

Figure 1.3. TLR4 signaling pathways. Activation of TLR4 can occur through a variety of stimuli, including LPS, PAMPs and DAMPs. Dimerized TLR4 leads to activation of proinflammatory activation through two pathways. The first, involving endocytosis of the TLR4 which leads to TRIF activation. TRIF activation subsequently leads to IRF3 activation. The second path is commonly known as the MYD88 dependent pathway which is activated directly through TLR4 activation. Once activated, MYD88 then activates NF-kB1. Together, these two pathways lead to proinflammatory activation of macrophages and other immune cells that express TLR4.

MyD88 dependent pathway, whereas TNFα promoter activation is primarily through TRIF dependent mechanisms[33, 35].

1.5 Macrophage activation and tricarboxylic acid cycle changes

Along with these distinct polarization profiles seen in macrophages, it has become increasingly appreciated that they also display unique metabolic profiles as well. Macrophages and other innate immune cells have been found to have drastic shifts in the tricarboxylic acid (TCA) cycle after pro-inflammatory stimulation. Alterations in cellular metabolism have been observed back to the late 1920's when Otto Warburg first observed tumor cells preferentially using glycolysis over oxidative metabolism even in normoxic conditions. This process that has since been coined

"aerobic glycolysis" and is commonly called the Warburg effect[36, 37]. This observation was later identified in innate immune cells in the 1950's when it was found that neutrophils would similarly prefer the use of aerobic glycolysis when generating adenosine triphosphate (ATP)[38]. In 1970, it was observed that LPS activated macrophages showed decreases in oxygen consumption and had an increased reliance on glycolysis[39]. Dendritic cells have similar metabolic changes as activated macrophages, highlighting that LPS activation can lead to drastic metabolic changes in innate immune cells[34, 40]. Although it has been widely known for decades that activated macrophages and innate immune cells undergo metabolic shifts, the mechanisms governing these changes have only recently begun to be elucidated.

1.6 TCA cycle and oxidative phosphorylation

The TCA cycle, also commonly referred to as the Krebs cycle or citric acid cycle, is viewed as the major metabolic pathway in cells[41]. This cycle is composed of eight key enzymes, seven of which are located inside the mitochondrial matrix, and the eighth (succinate dehydrogenase) located in the inner membrane of the mitochondria[41, 42]. Under normal conditions, macrophages primarily generate ATP by converting glucose into pyruvate, which is then transformed into acetyl CoA[20, 41, 42]. The TCA cycle is initiated by fusing acetyl CoA and oxaloacetate to generate citric acid. The TCA cycle is called a "cycle" because oxaloacetate is regenerated at the end of the cycle. Over the course of the cycle, the four-carbon oxaloacetate molecule is transformed into several molecules by either addition or removal of carbon atoms[42]. ATP generation is possible from this cycle because it has several energetically favorable steps that produce NADH. At its most simplistic level, the TCA cycle uses pyruvate to generate acetyl CoA, which is then used to generate NADH at multiple steps in the cycle pathway[43]. The mitochondrial electron transport chain then uses NADH generated by the TCA cycle, allowing oxidative phosphorylation (OXPHOS) to generate ATP for energy usage by the cell (Figure 1.4) [43]. The method of ATP production from macrophages in normal conditions primarily relies on OXPHOS, which requires cellular oxygen consumption[9, 44].

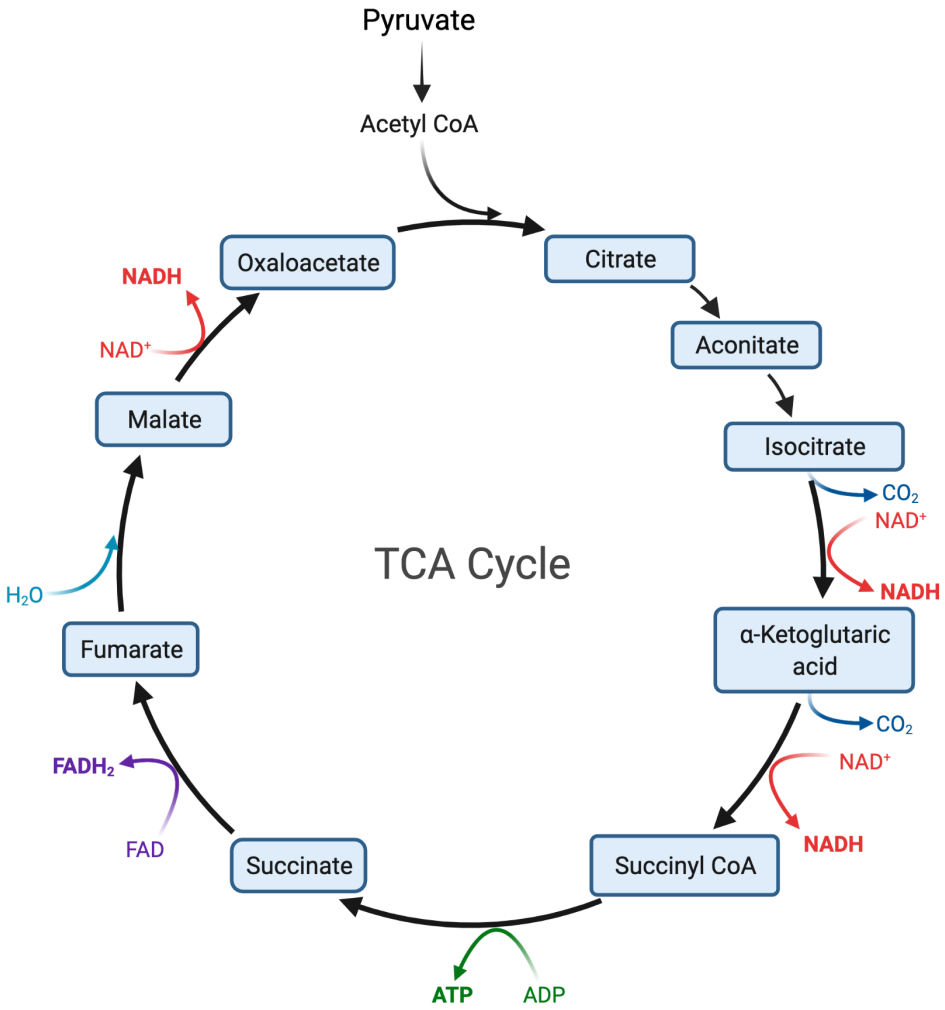

Figure 1.4. Tricarboxylic acid (TCA) cycle. The TCA cycle (or Krebs cycle) is initiated when pyruvate is used to generate acetyl-CoA. This leads to a series of reactions that are energetically favorable that generate three NADH, one FADH2, and one ATP molecule.

1.7 Metabolic reprogramming during macrophage activation

Upon activation by a stimulus like LPS, macrophages make a dramatic shift away from OXPHOS and rely primarily on glycolysis for ATP production[45]. This shift to glycolysis is similar to the Warburg effect and leads to pyruvate being used for lactate production rather than being used by the TCA cycle[45, 46]. Furthermore, ATP production by glycolysis is primarily accomplished through the pentose phosphate pathway (PPP). Increased activity of the PPP in activated immune cells also leads to increased levels of available purines and pyrimidines for biosynthesis and provides NADH which is used by NADH oxidase enzyme to generate ROS[20]. Together, this increase in biosynthesis and ROS is thought to aid activated immune cells by

allowing them to rapidly generate a response to invading pathogens[20, 45, 47]. This upregulation in glycolysis involves a change from pyruvate being used by the TCA cycle to it being converted into lactate and other glycolytic intermediates that are used to supply the high-energy demands of stimulated macrophages. Along with this shift to glycolysis, there is also a measurable decrease in the oxygen consumption rate (OCR) and increase in extracellular acidification rate (ECAR), emphasizing the change of energy production[48, 49]. This decrease in OCR happens even in the presence of ample oxygen present for the cell to use[45].

Along with distinct metabolic reprograming, macrophages also show a dramatic shift in metabolic intermediates that are formed when activated. Under normal conditions, macrophage TCA cycle remains functional and acts as the primary metabolic pathway. Once activated, macrophage OXPHOS is downregulated and undergoes several alterations to the TCA cycle's metabolic intermediates. This results in the accumulation of TCA cycle intermediates succinate and fumarate, as well as the induction of the immunometabolite itaconate (also referred to as itaconic acid) (Figure 1.5) [48]. These compounds have all been found to be immunomodulatory and have recently become of interest for their therapeutic potential. Itaconate specifically has been identified as a highly immunomodulatory compound in mammalian cells and will be the primary focus.

1.8 Itaconate production and inflammation in macrophages

Immune cell metabolites have recently become appreciated for their ability to modulate immune responses through mechanisms outside of their conventional role in bioenergetics[40, 50-55]. Itaconate represents one of the most recently identified immunometabolites, and its role in immunomodulation has become of great interest in the past decade[9, 44, 46, 48, 54, 55]. However, itaconate has had a long history dating back over a hundred years outside of biomedical research. The initial identification of itaconate was outside of its production in mammalian cells, rather it was first isolated as a product from citric acid distillation back in 1836[56]. Shortly after this initial discovery, the synthesis of itaconate was reported in 1841 through the decarboxylation of cis-aconitate[57]. Production of itaconate in vivo was later identified in 1931 in a fungal *Aspergillus*

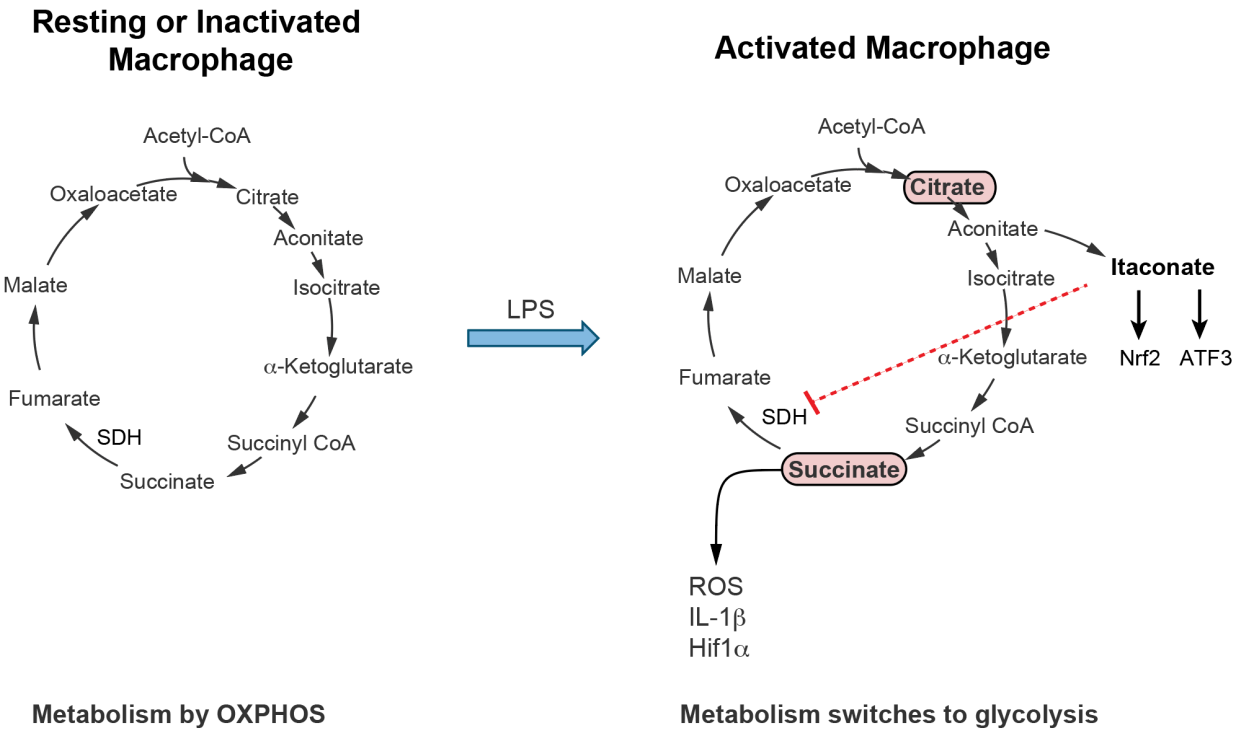

Figure 1.5. Metabolic reprogramming during macrophage activation. In a resting or inactive state, macrophages primarily rely on oxidative phosphorylation (OXPHOS) driven by the TCA cycle to generate ATP. Upon activation, there are two breaks in the TCA cycle: the first being caused by aconitate being used to generate itaconate, and the second caused by itaconate-induced inhibition of SDH. This leads to a decrease in reliance on OXPHOS, and a dramatic increase in glycolysis to generate ATP under this proinflammatory activation state.

species and was later named *Aspergillus itaconcus* because of its ability to produce itaconate. It was shortly after this finding that itaconate quickly became of focus in an industrial role, as it was an excellent building block in production of polymers because of the reactive methylene groups in its structure[58]. This finding led to itaconate production efficiency from *Aspergillus* to be a primary focus from an industrial standpoint and to this day is still an active field of study[59].

It was not until 1995 that itaconate unknowingly found its place outside of industrial relevance when Lee et al. cloned immunoresponsive gene 1 (*Irg1*). *Irg1* was found to be a highly upregulated gene in LPS stimulated macrophages and was the first step leading to the identification of itaconate's role in mammalian innate immunity[60]. After the identification of *Irg1,* multiple groups identified mammalian itaconate production from in vivo and in vitro studies[61-63]. These findings led to the eventual unification of *Irg1* and itaconate in 2013, when Michelucci et al found that IRG1 was responsible for the production of itaconate in mammalian cells[54]. Upon

identifying itaconate production by *Irg1*, the gene and protein identification (originally *Irg1* and IRG1) has since been renamed aconitate decarboxylase 1 (*Acod1*), sometimes also called cis-aconitate decarboxylase (CAD). In the past decade, itaconate and Acod1 have gained significant interest in the field of immunometabolism.

1.9 Endogenous itaconate production in macrophages and derivative forms

Macrophage itaconate production occurs during activation by pro-inflammatory stimuli and involves two major breaks in the TCA cycle. *Acod1* expression is highly upregulated following stimulation from toll-like receptor agonists, especially TLR4 activation with LPS[9]. This leads to increased protein levels of cis-aconitate decarboxylase which decarboxylates cis-aconitate to generate itaconate, causing the first break in the cycle[64]. The second break in the TCA cycle occurs at succinate dehydrogenase (SDH). Itaconate is an inhibitor of SDH, which then leads to accumulation of succinate[48]. Intracellular levels of itaconate have been reported to range from 40 μM all the way up to 8 mM in activated macrophages[40, 54, 63]. In fact, expression of *Acod1* in murine models has mainly focused on its expression solely in macrophages, mainly because *Acod1* expression and itaconate production are increased to such a high degree in LPS stimulated macrophages relative to other cell types at steady state[65]. As mentioned previously, itaconic acid is produced in vast quantities from a commercial standpoint, but itaconic acid/itaconate itself has not been employed in most biomedical studies. In the past decade, several derivative forms of itaconate have been developed with the goal of increasing their cellular uptake to better understand the mechanisms of itaconate. The two most common itaconate derivatives are dimethyl itaconate (DMI) and 4-octyl itaconate (4-OI). Although these derivatives have led to increased interest in itaconate, there remain serious questions regarding their mechanisms of action compared to endogenous itaconate. It has only been in the past couple of years that groups have begun to understand the potential differences between these distinct compounds.

1.10 Mechanisms of itaconate action

Over the past decade, several publications have identified potential mechanistic pathways of itaconate action. This section will summarize the major pathways that have been identified and will highlight whether itaconate or a derivative form was used to elucidate the mechanism.

Itaconate as an SDH inhibitor:

The enzyme SDH, also known as complex II, is located in the mitochondrial membrane of eukaryotes. SDH is responsible for the oxidation reaction that converts succinate to fumarate. In 2016, two separate groups identified itaconate as an inhibitor of SDH, highlighting the first potential immunoregulatory role of *Acod1* and itaconate[48, 66]. These studies found that SDH inhibition by itaconate blocked mitochondrial ROS production by inhibiting the oxidation of succinate to fumarate. It is believed that itaconate's structural similarities to succinate allows it to act as an inhibitor of SDH[67]. Cordes et al. found that bone marrow derived macrophages (BMDMs) and RAW264.7 cells showed increased levels of itaconate and succinate following LPS stimulation, and itaconate inhibits SDH activity leading to elevated succinate levels. Furthermore, this group found that BMDMs collected from mice lacking *Acod1* (Acod1KO) had significantly lower levels of succinate compared to wild type (WT) counterparts, likely caused by loss of itaconate in the Acod1KO primary cells[66]. Separately, Lampropoulou et al. found that treatment with the derivative form dimethyl itaconate leads to increased levels of succinate and inhibition of proinflammatory cytokines, including IL-6, IL-1β and IL-12[48]. Succinate accumulation has been previously shown to play a role in IL-1β accumulation and promote mitochondrial ROS production. Inhibition of SDH has been found to be protective against ischemia/reperfusion injury in stroke and cardiac models[68-70], highlighting the therapeutic potential of itaconate as a modulator of ischemic damage. Itaconate was found to inhibit reverse electron transfer caused by ischemia/reperfusion injury and protected against oxidative damage (Figure 1.6) [66].

KEAP/NRF2 modulation by itaconate:

Nuclear factor-erythroid factor 2 (Nrf2) is a master transcription factor responsible for driving cellular antioxidative responses following oxidative stress and has been found to inhibit proinflammatory responses in macrophages[71]. Nrf2 regulation is managed post-translationally through Kelch-like ECH associated protein 1 (KEAP1). Under homeostasis, KEAP1 acts as a regulator of Nrf2 through proteasome-dependent degradation, hindering Nrf2 to act as a master

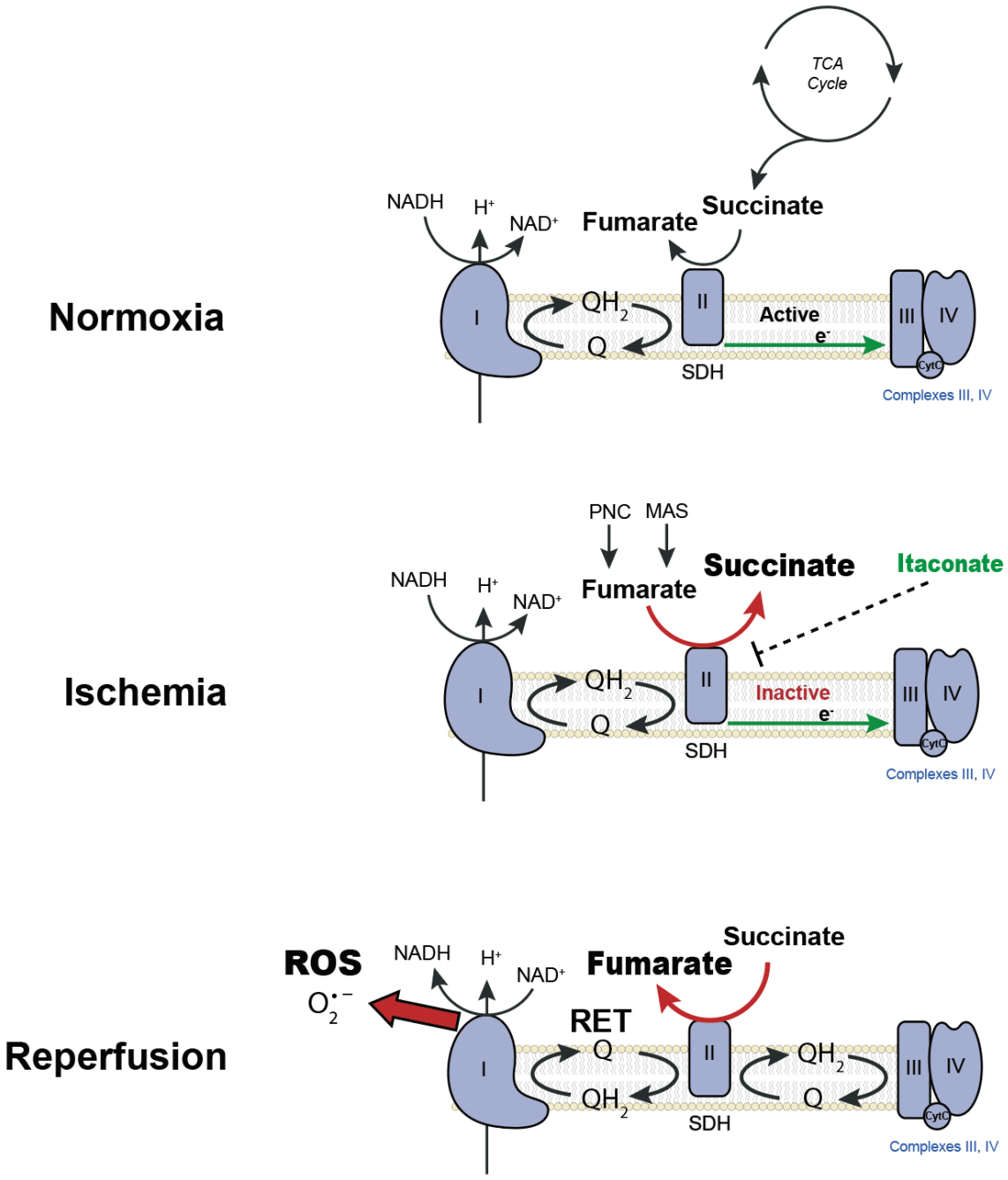

Figure 1.6. Itaconate inhibition of SDH. Under normoxia conditions, succinate is oxidized to fumarate by SDH (or respiratory complex II). Under ischemic conditions this natural flow is reversed causing fumarate to be converted back into succinate and leading to accumulation of succinate. In ischemic conditions fumarate is believed to be supplied by the malate/aspartate shuttle (MAS) and purine nucleotide cycle (PNC). Upon reperfusion the accumulated succinate is quickly oxidized back into fumarate and causing ROS production through reverse electron transport (RET).

transcription factor. During oxidative stress, KEAP1 is released from Nrf2, a mechanism that is facilitated through sensor cysteines locating on KEAP1. The release of KEAP1 allows for accumulation and translocation of Nrf2 to the nucleus where it then acts as the major transcription

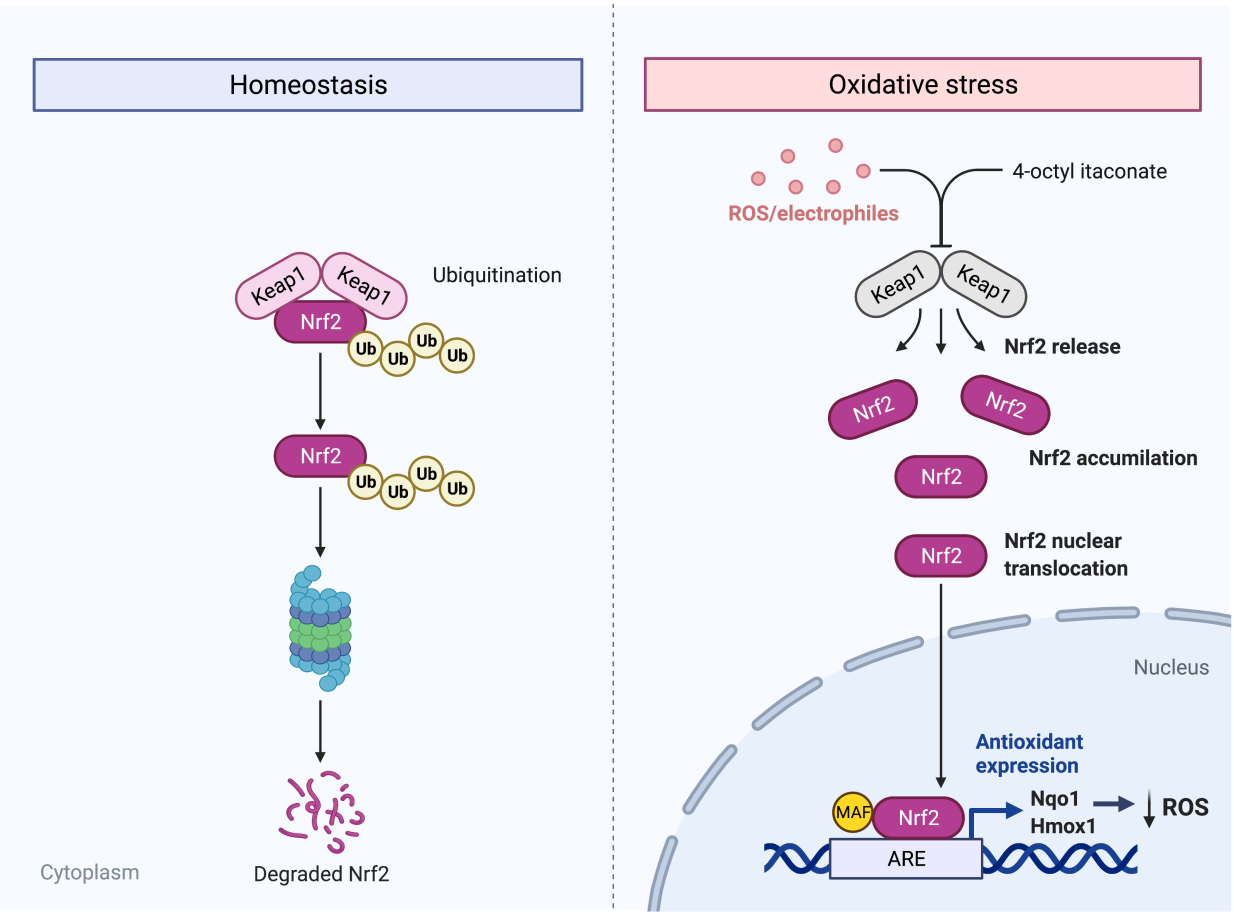

Figure 1.7. Itaconate-mediated induction of Nrf2. Under normoxia conditions KEAP1 is bound to Nrf2, keeping it in the cytoplasm. Nrf2 induction occurs through release of KEAP1 from Nrf2 caused by ROS and electrophilic stress. Itaconate similarly acts by binding cysteine residues on KEAP1, causing release of Nrf2. This leads to nuclear translocation of Nrf2, where it then can activate antioxidant response elements leading to decreased ROS and proinflammatory levels.

factor regulating antioxidant responses and leads to expression of genes including *Hmox1* and *Nqo1* (Figure 1.7) [71]. Past studies have identified that mice lacking Nrf2 (Nrf2KO) have increased susceptibility to oxidative injury caused by disease [72-76]. Separately, Nrf2 activation and subsequent expression of antioxidant response elements have been found to be potent inhibitors of proinflammatory markers [77-81].

Itaconate contains an electrophilic α,β-unsaturated carboxylic acid that allows it to alkylate cysteine residues through Michael addition. In 2018 Mills et al. identified that the itaconate derivative 4-OI enhanced Nrf2 stabilization and increased Nrf2 protein levels during LPS treatment in BMDMs[82]. Further analysis showed that 4-OI was acting through alkylation of multiple cysteine residues on KEAP1 including the primary regulator sensory Cys151 and thereby releasing Nrf2 from its negative regulation by KEAP1 (Figure 1.7). Treatment with 4-OI led to

increased levels of Nrf2 target gene Nqo1 and a dose dependent increase in HMOX1 protein levels. 4-OI also dose dependently decreased the proinflammatory IL-1β. These effects were reduced in Nrf2KO BMDMs, where they found cells lacking Nrf2 showed significantly lower levels of HMOX1 compared to WT controls, and the dose dependent inhibition of 4-OI on IL-1β was no longer observed. Taken together the authors concluded that itaconate, in the derivative form 4-OI, required Nrf2 and was responsible for the observed antioxidant and anti-inflammatory responses.

Itaconate modulation of ATF3/IkBζ axis:

Shortly after identifying Nrf2 activation as a potential mechanism of action by itaconate, Bambouskova et al. identified a separate Nrf2 independent pathway[81]. Activating transcription factor 3 (ATF3) and IkBζ are inflammatory axis regulators that interact with NF-κB and act independently of the Nrf2 pathway. This study found that itaconate and its derivatives were activators of ATF3 by inducing electrophilic stress, which then acts as an inhibitor of IkBζ. Treatment of BMDMs with DMI led to electrophilic stress where DMI reacted strongly with glutathione (GSH). Further analysis found that the electrophilic stress caused by DMI treatment would selectively upregulate secondary transcriptional responses by inhibition of IkBζ. The cause of IkBζ inhibition was found to be through ATF3, which led to suppression of a subset of proinflammatory cytokines, including IL-6 and IL-12. Acod1KO BMDMs that were tolerized with LPS showed decreased levels of ATF3 and increased IkBζ compared to WT controls. These findings supported their conclusion that itaconate was acting through ATF3 activation and subsequent IkBζ inhibition to modulate proinflammatory responses in macrophages (Figure 1.8) [81]. This finding marked a second potential pathway of itaconate that would be considered Nrf2 independent using the itaconate derivate DMI.

Antibacterial/antiviral properties of itaconate:

In addition to itaconate's role in immune cell regulation, it also has been found to have direct antibacterial and antiviral properties. One of the first antibacterial mechanisms identified for

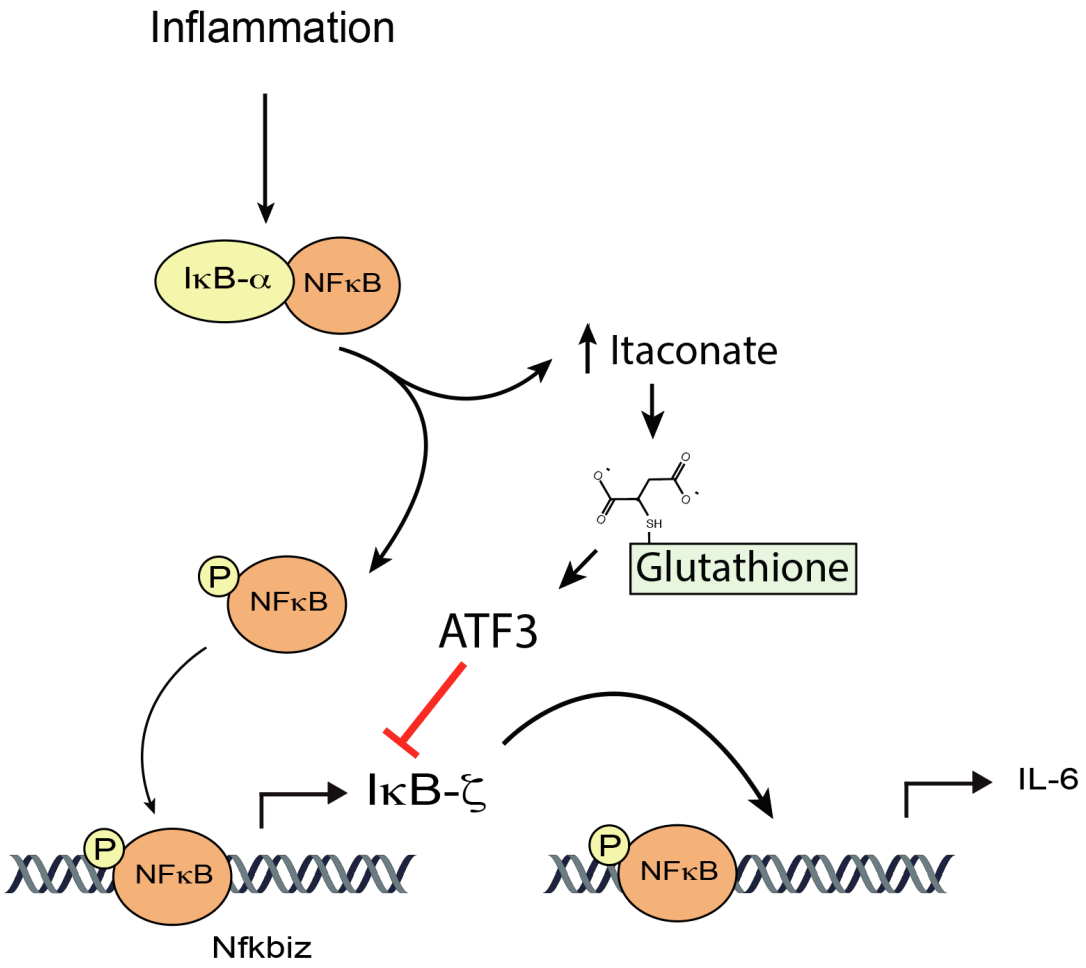

Figure 1.8. Itaconate-mediated induction of IκBζ–ATF3 Axis. Itaconate directly activates AFT3 through electrophilic stress. ATF3 then directly inhibits IkBζ and decreases the proinflammatory response.

itaconate was through inhibition of bacterial isocitrate lyase (ICL). ICL is a key enzyme that is required in the bacterial glyoxylate shunt. It has been shown by multiple groups that itaconate can effectively inhibit ICL, and through this mechanism limit bacterial growth during infection. In addition, itaconate can have indirect, ICL-independent, antibacterial effects through modulating immune cell responses. A recent finding has identified that itaconyl-CoA, a byproduct of itaconate metabolism, was capable of inhibiting *M. tuberculosis* growth through regulation of inflammatory responses[83]. Itaconate was found to decrease *M. tuberculosis* immunopathology through inhibition of macrophage chemokine production and through decreasing neutrophil recruitment. Using Acod1 deficient mice, they found that lack of itaconate rapidly led to significant lethality during *M. tuberculosis* infection. This increased mortality in Acod1KO mice was associated with

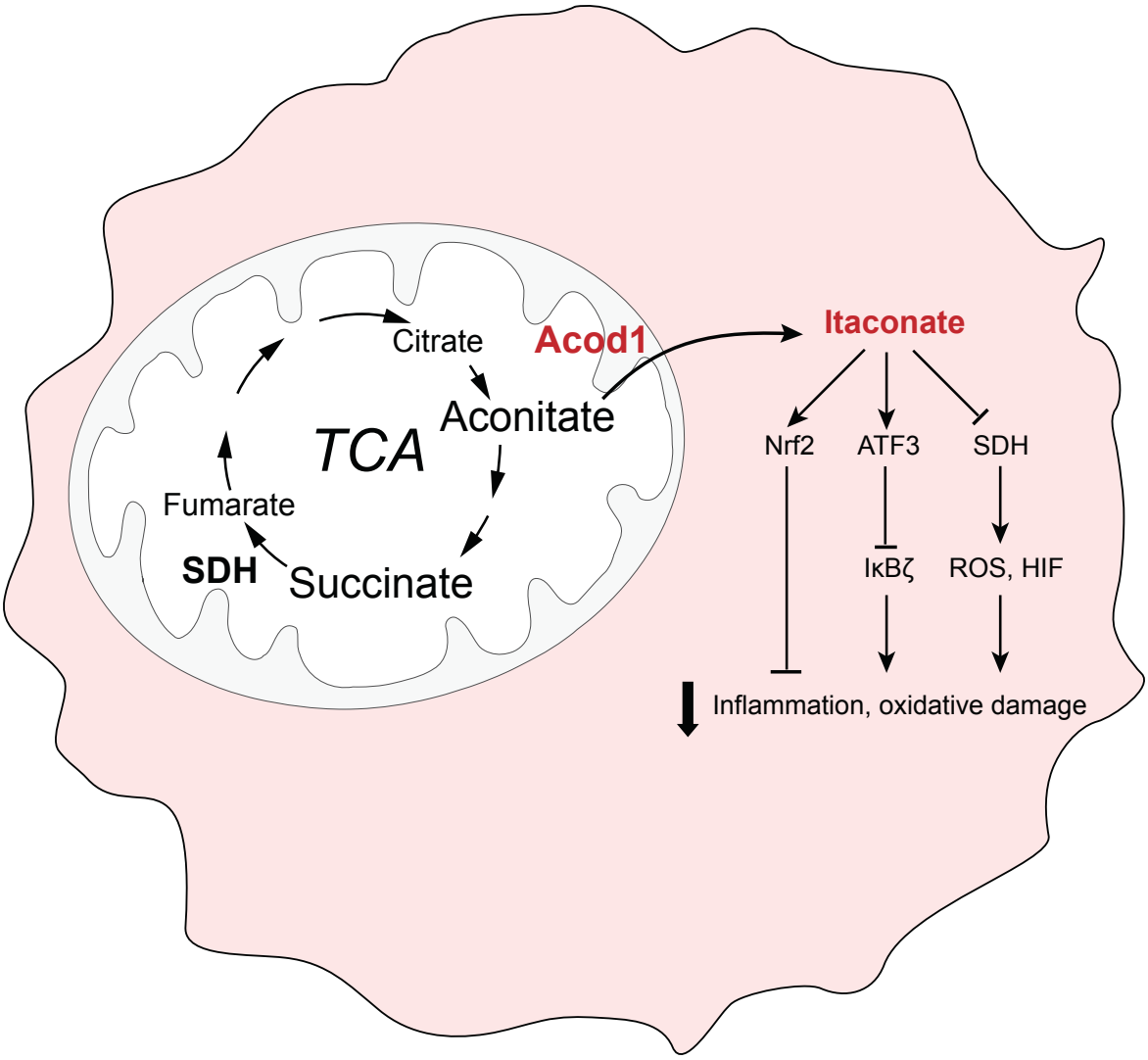

Figure 1.9. Summary of itaconates immunomodulatory mechanisms. Aconitate is used by Acod1 to produce itaconate in stimulated macrophages. Itaconate then acts as an immunomodulatory compound through several known pathways: activation of Nrf2, activation of ATF3, and/or SDH inhibition.

increased inflammation, infection, and pathology. Further studies using an Acod1 cell-specific knockout with LysM-Cre identified myeloid cells as being the responsible mediators. Taken together this group concluded that itaconate acts to decrease macrophage associated chemokine production and thus limit the influx of circulating neutrophils.

Antiviral properties of itaconate have also been recently identified. In 2019, Daniels et al. found that Acod1 expression was present in neurons following infection with Zika virus in a murine model[84]. By comparing WT and Acod1KO mice during infection, they found that mice lacking itaconate had significantly decreased survival rates post infection and increased viral burden in the

brain. Using intracranial injections of 4-OI, the group identified that itaconate was likely acting through SDH inhibition. Treatment with 4-OI significantly increased survival in Acod1KO mice after Zika virus infection. These data indicate that Acod1 expression and subsequent itaconate production inhibits viral replication by inducing an antiviral metabolic state in murine neuron cells.

The itaconate derivative 4-OI has also been found be an effective inhibitor of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) replication. A study published in 2020 by Olagnier et al. found that SARS-CoV2 patient biopsies had suppressed Nrf2 dependent gene expression[85]. They then used 4-OI along with another metabolite derivative, dimethyl fumarate (DMF), to identify whether Nrf2 activation could inhibit SARS-CoV2. Dimethyl fumarate is an approved treatment for relapsing-remitting multiple sclerosis (MS) that has anti-inflammatory effects through a Nrf2-dependent mechanism[85, 86]. They found that both 4-OI and DMF were capable of inhibiting SARS-CoV2 replication. Furthermore, 4-OI and DMF also limited the host inflammatory response to SARS-CoV2 infection. This study highlights a major potential role for itaconate as a therapeutic target for viral infections.

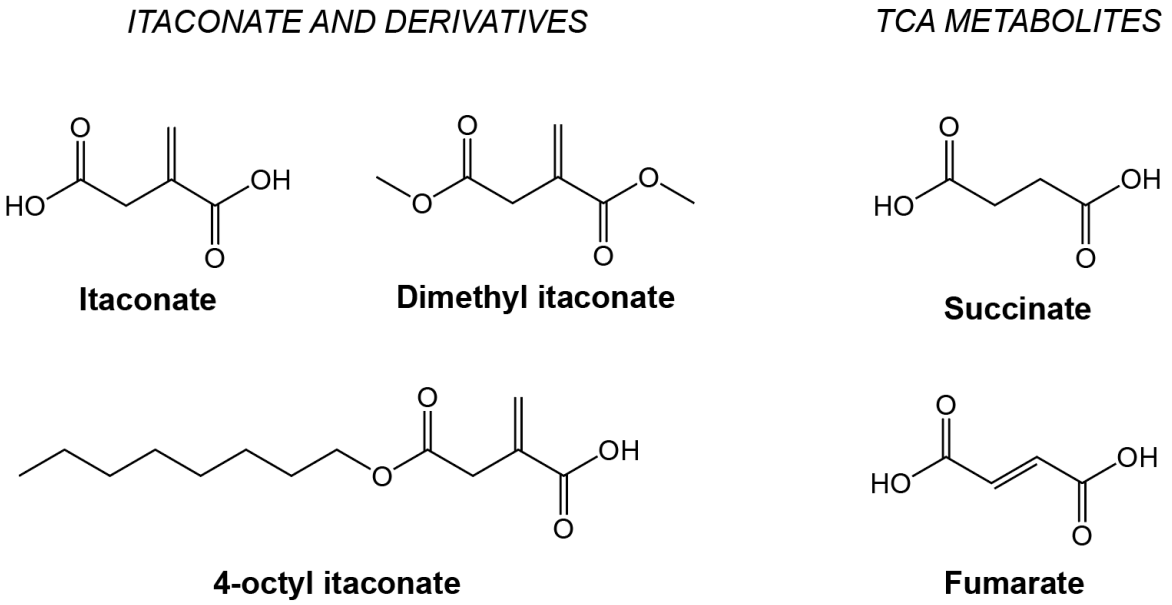

Figure 1.10 Itaconate, itaconate derivatives, and TCA cycle chemical mimetics. Itaconate is chemically similar to the TCA cycle metabolites, succinate and fumarate. Dimethyl itaconate and 4-octyl itaconate have esterified carboxyl groups reducing the charge and making them more membrane permeable.

1.11 Endogenous itaconate vs. derivative forms

Although several studies have been published detailing the mechanistic pathways for itaconate action, most of these studies have relied on derivative forms of itaconate. Initially created with the intent of increasing cellular permeability, these derivatives have played a key role in identifying many of the proposed itaconate pathways. However, recent findings call into question the mechanisms of these derivative forms compared to endogenous itaconate produced by cells expressing Acod1. Swain et al. recently found that the commonly used derivatives DMI and 4-OI do not get converted into itaconate intracellularly. Furthermore, they found that millimolar doses of exogenous itaconate treatment led to significant increases in intracellular itaconate levels, suggesting that exogenous itaconate treatment could be taken up by cells[87]. Further analysis also verified that DMI and 4-OI cause electrophilic stress, supporting previous reports of this mechanism using these itaconate derivatives. Importantly, exogenous itaconate treatment did not reproduce the electrophilic effects seen in the derivative forms, potentially highlighting a major difference between the derivative and endogenous itaconate forms. Swain et al. concluded that DMI and 4-OI likely were acting through inhibition of IkBζ by inducing a strong electrophilic stress response that is independent of the itaconate-Nrf2 pathway. DMI and 4-OI were found to inhibit pro-IL-1β induction and IL-10, IL-6 and IFN- β secretion. Exogenous itaconate treatment on the other hand had no effect on pro- IL-1β induction but did inhibit IL-1β secretion. These finding suggest that the derivative forms of itaconate are acting in part through separate mechanisms from that of endogenous itaconate. These findings also demonstrate that exogenous itaconate treatment is possible at higher doses and could be a better alternative for studying endogenous itaconate mechanisms in the future with the caveat that high doses can affect cell viability. Most recently, He et al identified that endogenously produced itaconate can be used in LPS activated macrophages to produce a separate metabolic intermediate, mesaconate[88]. Mesaconate is a metabolite that has a similar structure to itaconate and shares similar effects as well. He et al identified that both itaconate and mesaconate block the glycolytic activity in stimulated macrophages. Further analysis also revealed that mesaconate and itaconate decreased IL-6 and IL-12 protein levels through a Nrf2 and ATF3 independent mechanism. Lastly, they also found that neither itaconate nor mesaconate altered IL-1β secretion, a result that is observed with derivative forms[88]. Taken together, these findings would further strengthen the argument that

endogenous itaconate and mesaconate do alter inflammatory responses in activated macrophages, but the mechanisms are likely different than those seen with the derivative forms of itaconate.

1.12 Hypothesis and rationale of this study:

In this thesis, we sought to identify the role of Acod1 and itaconate in three separate murine models of disease: ischemic stroke, diet-induced obesity and ulcerative colitis. The commonality of these models is the role of inflammation and the immune response in disease. The role of inflammation in these disease models varies from acute, like in ischemia/reperfusion injury and ulcerative colitis, to more chronic inflammation in diet-induced obesity. We have focused on the role of macrophages in disease and along with other groups have shown that modulation of macrophage polarization away from proinflammatory (M1) activation is protective in murine disease models. Here, we hypothesized that Acod1 and itaconate are important in the pathophysiology of disease models by modulating the inflammatory response. Furthermore, we hypothesized that mice lacking Acod1 and itaconate production would have more severe disease phenotypes, and that macrophages would be the primary cell responsible for these observed phenotypes.

CHAPTER 2

Aconitate Decarboxylase 1 Suppresses Cerebral Ischemia-Reperfusion Injury in Mice

2.1 Abstract

Immunometabolic changes have been shown to be a key factor in determining the immune cell response in disease models. The immunometabolite, itaconate, is produced by aconitate decarboxylase 1 (Acod1) and has been shown to inhibit inflammatory signaling in macrophages. In this study, we explore the role of Acod1 and itaconate in cerebral ischemia/reperfusion injury. We assessed the effect of global Acod1 knockout (Acod1KO, loss of endogenous itaconate) in a transient ischemia/reperfusion occlusion stroke model. Mice received a transient 90-minute middle cerebral artery occlusion followed with 24-hours of reperfusion. Stroke lesion volume was measured by MRI analysis and brain tissues were collected for mRNA gene expression analysis. Acod1KO mice showed significant increases in lesion volume compared to control mice, however no differences in pro-inflammatory mRNA levels were observed. Cell specific knockout of Acod1 in myeloid cells (LysM-Cre), microglia cells (CX3CR1, Cre-ERT2) and endothelial cells (Cdh5(PAC), Cre-ERT2) did not reproduce lesion volume changes seen in global Acod1KO, indicating that circulating myeloid cells, resident microglia and endothelial cell populations are not the primary contributors to the observed phenotype. These effects however do not appear to be driven by changes in inflammatory gene regulation. These data suggests that endogenous Acod1 is protective in cerebral ischemia/reperfusion injury.

This chapter represents a published article: Vigil, T.M., Frieler, R.A., Kilpatrick, K.L., et al. Aconitate decarboxylase 1 suppresses cerebral ischemia-reperfusion injury in mice. *Exp Neurol.* 2022 Jan;347:113902.

2.2 Introduction

Ischemic stroke is a leading cause of mortality and permanent disability in older adults, and the only clinically effective therapeutic option is recanalization by thrombolysis or mechanical thrombectomy.[89-91] Although the utility of these therapies has increased in frequency, there is still a large percentage of patients where these therapies are contraindicated. Reperfusion injury after therapeutic or spontaneous recanalization remains a major concern and a target for new therapeutics. Although mechanisms of reperfusion injury are highly complex, the most consistently cited pathways of damage involve production of reactive oxygen species (ROS) and inflammatory injury .[92-94] ROS generated following reperfusion leads to oxidative stress that further aggravates tissue injury and has been shown to exacerbate detrimental inflammatory responses.[95, 96]

Immune cells are recruited during ischemic stroke and contribute to injury through a variety of inflammatory mechanisms. Inhibition of immune cell influx, suppression of inflammatory signaling, and myeloid cell modification to an anti-inflammatory phenotype have all been shown to mitigate cerebral ischemia/reperfusion (I/R) injury.[97-99] Metabolism and particular metabolic intermediates have been recently identified as an important determinant of inflammatory cell phenotype [100]. The metabolism in immune cells has increasingly been recognized as modulating immune function and playing an important role in pathophysiology of cardiovascular disease.

Itaconate is a TCA cycle derived metabolite and has recently been recognized to intersect with the ischemic response in modulating the TCA cycle and inflammatory responses. Itaconate is produced by aconitate decarboxylase 1 (Acod1, also called Irg1) and has anti-inflammatory and antioxidant effects through multiple mechanisms. Itaconate is an important component of macrophage phenotype and polarization. Pro-inflammatory stimuli such as LPS, dramatically induce the expression of Acod1 and increase the production of itaconate. Itaconate has been shown to have protective effects through multiple mechanisms including SDH inhibition, Nrf2 activation, and ATF3 induction.[48, 81, 82]

Succinate accumulation drives ROS production during ischemia reperfusion injury.[101] Itaconate has been identified as an inhibitor of succinate dehydrogenase (SDH), and mice lacking endogenous itaconate have increased levels of ROS and inflammatory cytokines. Administration of cell-permeable itaconate derivatives are protective in a mouse models of cardiac and cerebral I/R injury.[48, 102] Although Acod1 and itaconate have more recently become a major focus of

mechanisms of macrophage phenotype, the in vivo role of endogenous itaconate is still largely unknown.

In this study, we investigated the effects of itaconate deficiency through global Acod1 deletion in a cerebral I/R injury model of ischemic stroke. We hypothesized that mice lacking Acod1 would have exacerbated stroke injury as a result of increased inflammation. Here, we used global Acod1 knockout mice lacking endogenous itaconate to define the role of endogenous itaconate during ischemic stroke. We evaluated the effect of Acod1/itaconate on infarct size and inflammatory gene expression during cerebral ischemia-reperfusion injury.

2.3 Methods

Animals and Treatments

All animals were performed in accordance with Guidelines for the Care and Use of Laboratory Animals (8th edition). The Institutional Animal Care and Use Committee of the University of Michigan approved the use of animals. Mice were fed a standard diet of 5L0D LabDiet chow and received water ad libitum. All mice were generated on C57BL/6N background, including global and cell specific knockouts. All Acod1 global and cell specific knockout mice were acquired from the lab of Dr. Michael Diamond located at Washington University School of Medicine, St. Louis, Missouri.[83] Global Acod1 knockout and flox Acod1 mice were generated as described previously.[83] Myeloid specific Acod1 knockout (MyAcod1KO: Acod1$^{fl/fl}$-LysMCre), microglia specific Acod1 knockout (MicAcod1KO: Acod1$^{fl/fl}$- CX3CR1Cre-ERT2), endothelial specific Acod1 knockout (ECAcod1KO: Acod1$^{fl/fl}$- Cdh5(PAC)Cre-ERT2) were generated by crossing floxed Acod1 mice with the indicated Cre lines. Littermate floxed control mice were used as controls in all experiments. For CX3CR1Cre-ERT2 experiments, mice were weighed and received two administrations of 300mg/kg tamoxifen via I.P. with one day separating injections. Experiments were then performed on tamoxifen treated CX3CR1Cre-ERT2 mice six weeks following end of tamoxifen treatment. Cdh5(PAC)Cre-ERT2 mice received 40mg/kg tamoxifen via I.P. for three consecutive days.[103] Experiments were then performed on tamoxifen treated mice four weeks following end of tamoxifen treatment.

Middle cerebral artery occlusion

Transient, 90-min MCAo was performed as described previously.[98, 104] Anesthesia was induced in mice with isoflurane (5% induction, 1.5% maintenance). Core body temperature was

measured via rectal probe and maintained at 37°C with a temperature-controlled heating pad. Following anesthesia, a 8mm midline incision was made along the neck to expose the right carotid artery. Ligation of the external carotid artery was performed and a 10mm long 6-0 silicon rubber coated nylon monofilament (#602156, Doccol Corp., CA) was placed into the external carotid stump. The filament was then advanced into the internal carotid artery. Blood flow was measured via laser Doppler flowmetry (Transonic BLF22) before and during filament insertion. Occlusion was defined as a reduction in cerebral tissue perfusion at a level <20% compared to original baseline. The filament remained in the internal carotid for 90 minutes of ischemia and removed, mice were then allowed to recover and reperfusion to occur.

Infarct volume measurement

Following 24 hours of reperfusion (post-filament removal), magnetic resonance imaging (MRI) was used to measure infarct size following MCA occlusion. Mice were anesthetized with 2% isoflurane and laid prone throughout MRI examination. A 7.0T Varian Unity Inova MR scanner (183-mm horizontal bore; Varin, Palo Alto, CA) was used for MRI analysis and mice were maintained at 37°C via forced heated air. A double tuned volume radiofrequency coil was used to scan the head region of the mice. A fast spin echo sequence was used to acquire T2 weighted images set to the flowing parameters: repetition time/effective echo time- 4000/60 ms; echo spacing- 15 ms; number of echoes- 8; field of view- 20x20 mm; matrix- 256x128; slice thickness- 0.5mm; number of slices- 25; and number of scans- 1 (total scan time of 2.5 minutes). ImageJ software provided by NIH was used to analyze and measure infarcts by a blinded observer. Infarct volumes were corrected to account to brain swelling.

To measure T2 lesion volume, the following equation was used to correct for edema: TV-((CV+(IV-LV)x((TV/2)/CV)).[105] Where TV is total combined volume in both brain hemispheres, CV is contralateral volume, IV is ipsilateral volume, and LV is total measured lesion volume.

Generation and treatment of BMDMs

Mice were euthanized via CO_2 chamber and death was verified with cervical dislocation. Femur and tibia were removed from mice to flush bone marrow cells with 5ml RPMI 1640 media (Gibco) through a 27g needle and strained using a 100μm filter. Cell suspension was then centrifuged at 1250 RPM for 10 minutes to pellet cells. Supernatant was decanted and replaced

with fresh BMDM differentiation media (RPMI 1640 + 30% L929 supernatant + 10% FBS + glutamax (Gibco) + penicillin streptomycin). BMDMs were allowed to differentiate for a total of 6 days with media replacement at day 4. Differentiated macrophage cells were plated at $5x10^5$ cells in 24-well plates. In all experiments differentiated BMDMs were treated with 100 ng/ml LPS for the designated amount of time prior to collection for analysis.

BV-2 culture

BV-2 cell cultures were grown and maintained on RPMI supplemented with 5% FBS. Cells were maintained in $75cm^2$ flask and passaged every 2-3 days once 90% confluency was reached. Cells were plated at 1 x 10^6 cells/well in 24 well plates. BV-2 cells were pretreated with 2mM itaconate for 14 hours followed with 100 ng/ml LPS for 3 hours.

Flow Cytometry

Validation of CX3CR1 was performed using 8 week old male tdtomato flox mice expressing CX3CR1-CRE. Mice received two administrations of 300mg/kg tamoxifen via I.P. with one day separating injections. Six weeks following tamoxifen treatment mice were euthanized, brains were removed and homogenized. Total cell suspension was incubated in Fc Block for 10 minutes. Cells were then stained on ice for 1 hour with the following antibodies: PE anti-mouse CD45 (BioLegend, cat # 103106), and CX3CR1 (BioLegend, cat # 149021). Once staining was completed cells were washed twice with PBS and fixed with 0.1% paraformaldehyde. Flow cytometric analysis was performed on a FACSCanto II Flow Cytometer (BD Biosciences) equipped with three lasers (405-nm violet laser, 488-nm blue laser, and 640-nm red laser) and analyzed with FlowJo software (Treestar). Unstained andisotype controls were used for flow analysis.

mRNA gene expression analysis

Relative mRNA expression was measured using quantitative reverse transcription polymerase chain reaction (qPCR). RNA was isolated using TRIzol reagent. For in vitro experiments, media was removed from plates and TRIzol was immediately added. For in vivo experiments, brain tissue samples were homogenized in TRIzol. RNA was purified in RNeasy Mini Kit (Qiagen) with an on column DNase digestion. 1ug of RNA was reverse transcribed to cDNA with an Applied Biosystems kit and qPCR was performed using a 7900HT fast real time PCR system

(Applied Biosystems). The ribosomal housekeeping gene L32 was used to normalize all data and quantify relative RNA expression.

Immunoblot analysis

Cultured bone marrow macrophages were treated with 100ng/ml LPS for 6 hours to allow for sufficient increases in Acod1 mRNA levels, and subsequent Acod1 protein accumulation. Cultured cell protein was isolated by direct lysis using RIPA buffer (Sigma). Protein was quantified using Peirce BCA protein assay kit (ThermoFisher Scientific, Ref 23227) and appropriate volume of protein sample was added to produce 40ug in 5x laemmli buffer with 5% 2-mercaptoethanol. Samples were ran through SDS-page gels (Biorad, #3450027) for 50 minutes at 150V and transferred to polyvinylidene difluoride membrane using wet transfer. Transferred membranes were then blocked in 5% dried milk in TBS-Tween for 1 hour at 4°C. Primary antibody was then added at 1:1000 (Anti-IRG1, Abcam ab222411) overnight at 4°C. Secondary antibody was added for 90 minutes at room temperature. Visualization of bands was performed using chemiluminescent substrate (ThermoFisher scientific, Ref 34577) and imaged on Biorad systems.

Statistical analysis

A Shapiro-Wilk normality test was used to determine if data were normally distributed. For normally distributed data with equal variance, statistical comparison of mean values between groups was performed with the Student t test or by a two-way ANOVA with a Šídák correction for multiple comparisons, and values are presented as mean ± SEM. All statistical analysis of data was performed in GraphPad Prism (version 6; GraphPad Software, Inc). $P < 0.05$ was considered significant.

2.4 Results

Acod1 expression in macrophages and the effects of exogenous itaconate in vitro

The production of itaconate through expression of Acod1 has recently been shown to be a major hallmark of classically activated macrophages. This increased expression has been reported in both LPS and IFN-γ stimulated macrophages in vitro. To verify knockout in our mouse model, bone marrow macrophages were generated from Acod1 flox control ($Acod1^{flox/flox}$) and global Acod1 knockout mice (Acod1KO) that contain a bacterial β-galactosidase (a marker controlled by the Acod1 promoter in the knockout mice). Macrophages were treated with 100ng/mL LPS for up to 24 hours followed with mRNA collection for quantitative PCR analysis (Figures 2.1A-B).

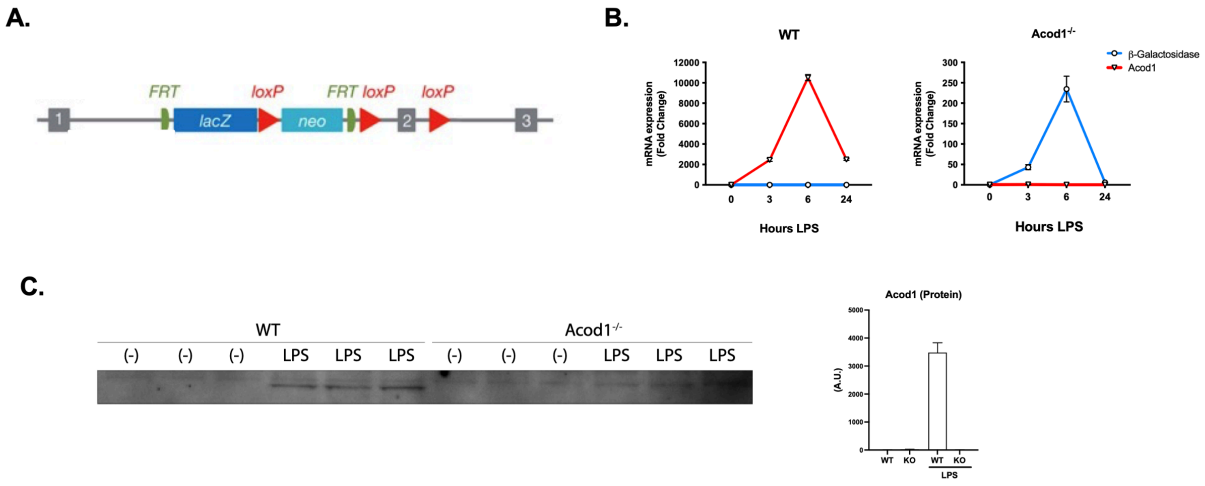

Figure 2.1 Confirmation of Acod1 gene knockout. (A-B) WT and Acod1KO bone marrow derived macrophages stimulated with LPS (100ng/ml) shows the correlation of lacZ expression (β -gal) with Acod1 gene expression. (N=3) Data are represented as SEM. (C) Stimulation of WT and Acod1KO BMDM' s with LPS for 6 hours shows decreased protein expression by western blot of Acod1 protein. Quantification of protein levels shown, data are represented as SEM.

Acod1 gene expression increased following LPS stimulation in control mice with the highest level at 6 hours post stimulation. In Acod1KO there was a similar increase in β-galactosidase gene expression and complete lack of Acod1 expression, indicating sufficient knockout of Acod1 target gene. To further verify sufficient knockout of Acod1 western blot analysis was performed to quantify Acod1 protein levels in cultured bone marrow derived macrophages (Figures 2.1C-D). Control and Acod1KO Macrophages were stimulated with LPS for 6 hours prior to protein being collected and quantified. This showed >90% reduction in Acod1 protein levels in knockout cells compared to control following LPS stimulation.

Global Acod1 deletion increases ischemic stroke damage

To assess the effect of Acod1 gene function in regulating stroke injury, we performed the filament method of transient middle cerebral artery occlusion (tMCAo) on ACODKO and control mice, as previously described [99]. Infarct size was calculated using MRI analysis performed 24 hours after ischemia/reperfusion, representative images are shown in Figure 2.2A. Acod1KO have increased I/R-induced infarct size compared to control type mice (Figure 2.2A). Edema volume was found to have a higher average in the global Acod1KO group compared to control, however this was not statistically significant (Figure 2.2B). This highlights an important role for Acod1 and itaconate during ischemic injury. We have shown a sustained increase in lesion volume in

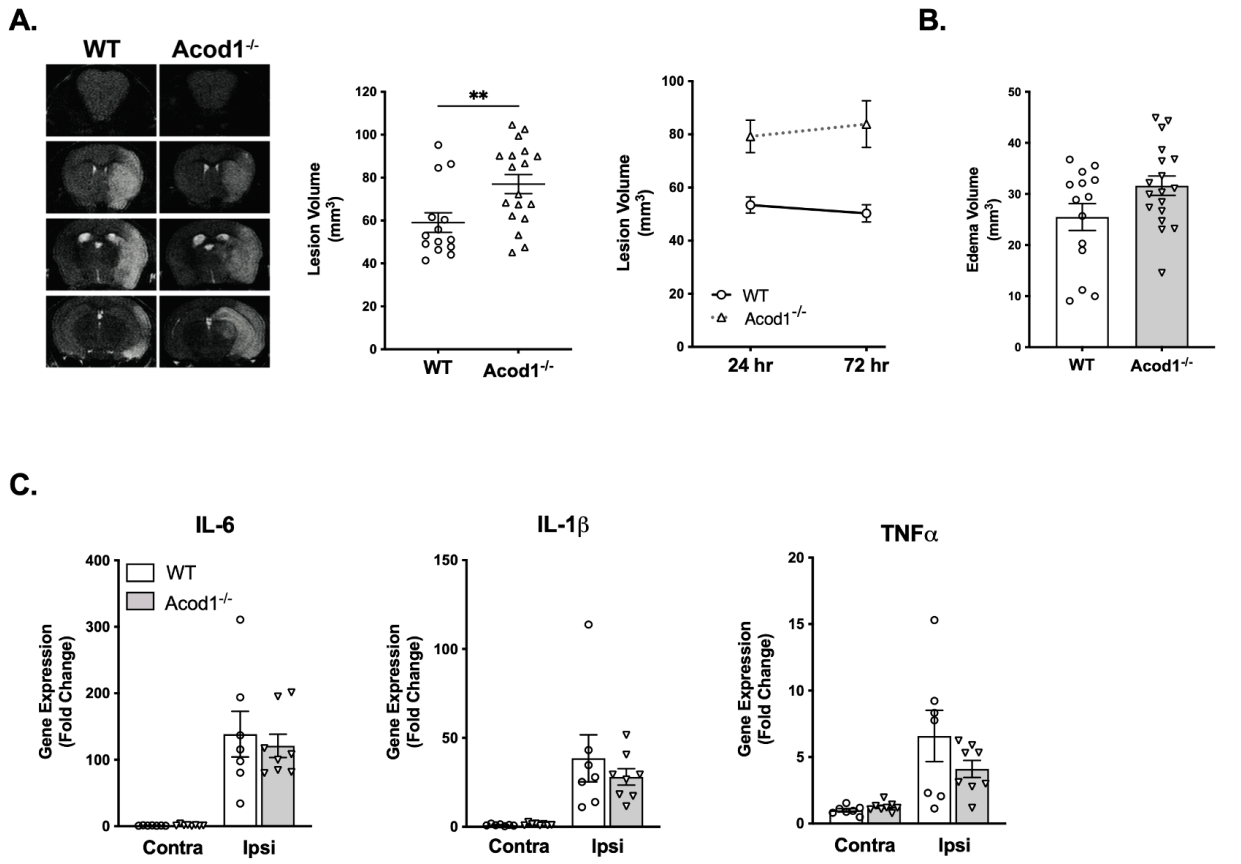

Figure 2.2. Itaconate deficiency exacerbates cerebral ischemia-reperfusion injury. (A) Lesion volume measurement of wild type and Acod1KO mice following 90-minute tMCAo. MRI analysis 24hrs after tMCAo. N=14 for WT and N=18 for Acod1KO, p=0.0006, two-tailed t-test. Lesion volume measurement of wild type and Acod1KO mice following 90-minute tMCAo. MRI analysis 24 and 72hrs after tMCAo. N=4 for WT and N=5 for Acod1KO. Data are represented as SEM. (B) Edema volume measurement of wild type and Acod1KO mice following tMCAo. MRI analysis 24hrs after tMCAo. N=14 for WT and N=18 for Acod1KO, p=0.0619, two-tailed t-test. Data are represented as SEM. (C) Gene expression of proinflammatory cytokines from contralateral and ipsilateral brain hemispheres following 90 minutes ischemia and 24hrs reperfusion. N=7 for WT and N=8 for Acod1KO, two-tailed t-test. Data are represented as SEM.

Acod1KO mice at 24 and 72 hours post-tMCAo compared to controls (Figure 2.2A). Following MRI analysis mRNA expression for inflammatory genes was performed to determine if Acod1KO had increased pro-inflammatory expression profiles compared to controls. However, there was no difference found in several key pro-inflammatory gene markers when comparing ipsilateral expression in Acod1KO compared to control mice (Figure 2.2C). This suggests an alternative pathway responsible for the observed increases in stroke lesion volume.

Deletion of Acod1 from myeloid cell population

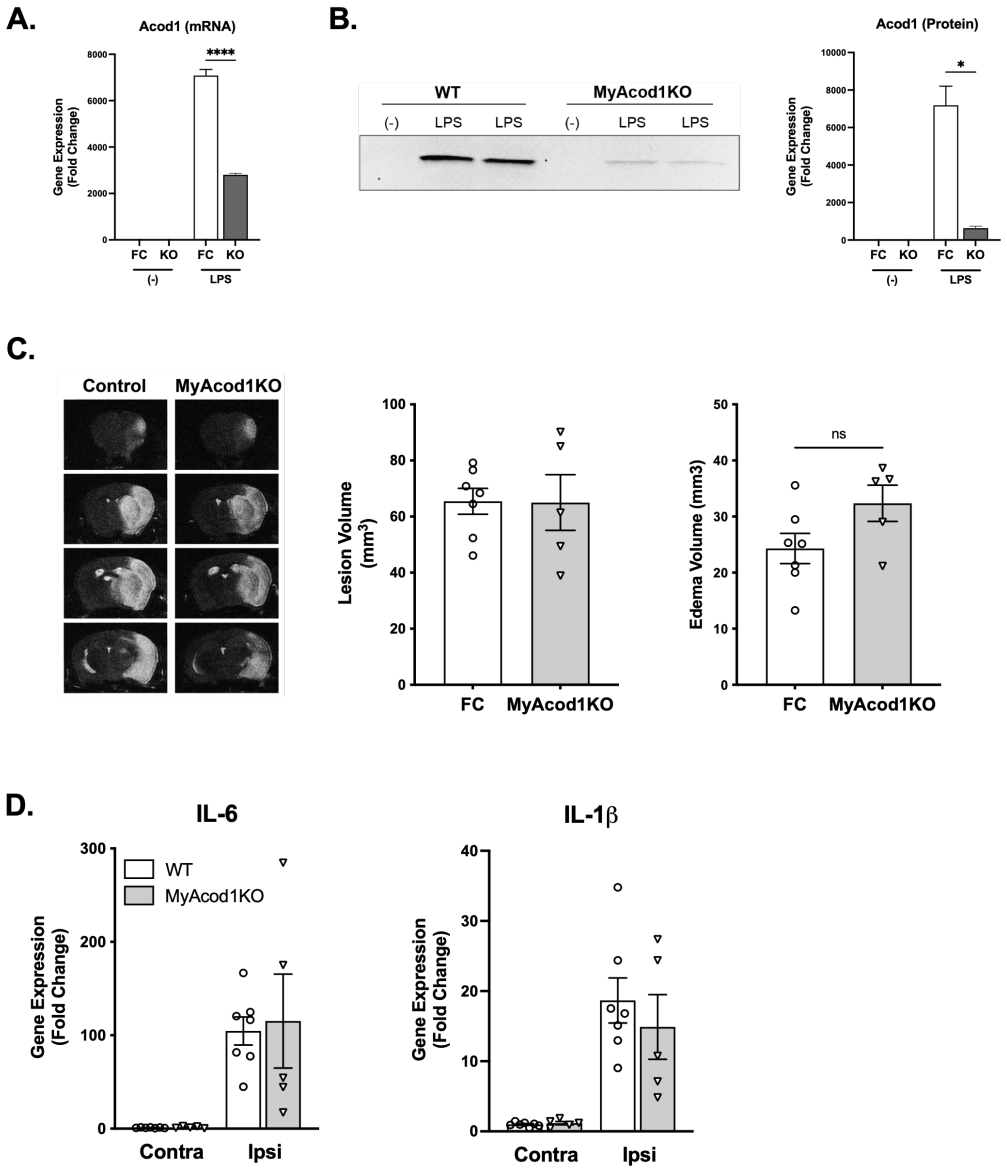

Figure 2.3. Myeloid Acod1 knockout does not phenocopy global Acod1 ablation during stroke. (A) Quantification of Acod1 mRNA levels in control and MyAcod1KO BMDM's stimulated with LPS for 3 hours. Wt and MyAcod1KO N=3 p=<0.0001, two-tailed t-test. (B) Stimulation of control and MyAcod1KO BMDM's with LPS for 6 hours shows decreased protein expression by western blot of Acod1 protein in MyAcod1KO. Wt and MyAcod1KO N=2 p=0.0236, two-tailed t-test. (C) Lesion volume and edema volume measurement of control and MyAcod1KO mice following 90-minute tMCAo. MRI analysis 24hrs after tMCAo. N=7 for control and N=5 for MyAcod1KO, p=0.9653 for Lesion volume and p=0.0838 for edema volume, two-tailed t-test. Data are represented as SEM. (D) Gene expression of proinflammatory cytokines from contralateral and ipsilateral brain hemispheres following 90 minutes ischemia and 24hrs reperfusion. N=7 for control and N=5 for MyAcod1KO, two-tailed t-test. Data are represented as SEM.

After identifying that Acod1KO had increased lesion volume in ischemic stroke, we sought to determine what cell type/types were responsible for this phenotype. Given that macrophages are known to play an important role in the pathology of stroke and are high expressers of Acod1,

myeloid cell specific knockout of Acod1 was generated using LysM-Cre (MyAcod1KO). To validate Acod1 expression in the macrophage population, bone marrow derived macrophages were generated from control and MyAcod1KO animals and treated with LPS for 3 hours for gene expression analysis and 6 hours for western blot analysis. Gene expression showed a significant decrease in Acod1 mRNA expression in KO macrophages compared to control (Figure 2.3A). Western blot analysis showed substantial decrease in Acod1 protein levels compared to flox control BMDM's (Figure 2.3B). tMCAo was performed on MyAcod1KO and flox control animals to determine the contribution of the myeloid population. Unlike global Acod1KO mice, MyAcod1KO mice did not show statistically significant differences in either lesion or edema volumes compared with control animals (Figures 2.3C). Also, there were no measurable differences in gene expression based on qPCR analysis (Figures 2.3D).

Role of resident microglia and endothelial cells

Microglia cells are a distinct group of immune cells that reside in the brain and have many similarities to macrophages in their functions and contribute to the observed pro-inflammatory response following ischemic stroke.[106, 107] Because of their role as the resident brain immune cell, we sought to identify if they had a role in stroke regarding Acod1 and itaconate. In vitro experiments were also performed on BV-2 microglia cells to identify if Acod1 was expressed following inflammatory insult. BV-2 cells were treated with LPS for 3 hours and Acod1 gene expression was measured using qPCR analysis. Acod1 expression was detected following LPS treatment in microglia cells, indicating that they are capable of expression under inflammatory responses (Figure 2.4A). Furthermore, pretreatment with itaconate prior to LPS stimulation suppressed IL-1β and TNFα gene expression (Figure 2.4A).

We performed tMCAo on microglial specific Acod1 inactivation using the Cre-lox system. Cre-ERT2 driven by the CX3CR1 promoter specifically inactivates floxed genes in microglia. Although CX3CR1 is expressed in the entire myeloid lineage including circulating monocytes, these other cells turn over quickly leaving the resident cells as the specifically recombined cells allowing for study of microglia specifically (MicAcod1KO). Mice received intraperitoneal injections of 300mg/Kg tamoxifen for two days with one day separating administrations, six weeks prior to receiving tMCAo followed with MRI analysis after 24hrs of reperfusion. Validation of CX3CR1-CRE was performed using a tdTomato reporter mouse line crossed with CX3CR1-CRE. Isolated brain cells were gated on CD45 intermediate to specifically look at microglia population.

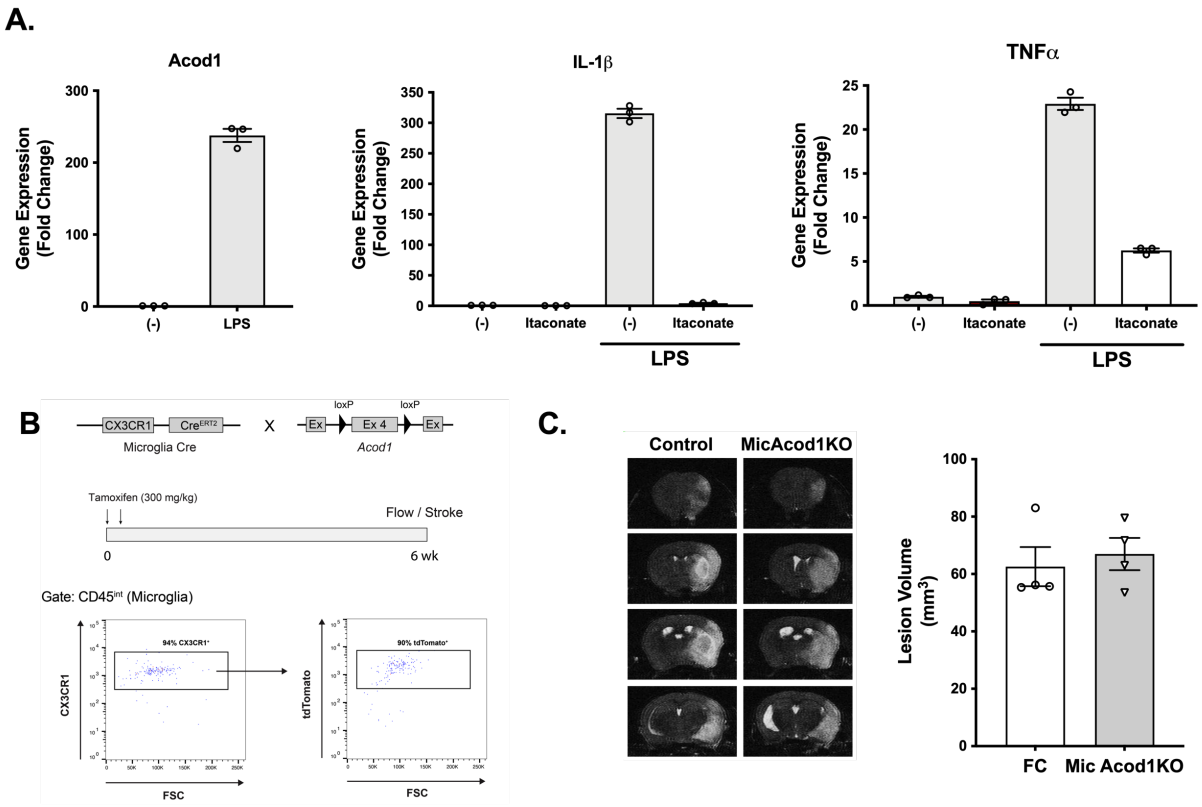

Figure 2.4. Microglia-specific Acod1 knockout does not alter infarct size. (A) Quantification of BV-2 cell gene expression. Cells were treated with 2mM itaconate for 14hrs followed with LPS stimulation for 3hrs. (B) Flow cytometry validation of CX3CR1-CRE with tdTomato reporter indicating 90% recombination in microglia cells. (C) Lesion volume measurement of control and MicAcod1KO mice following 90-minute tMCAo. MRI analysis 24hrs after tMCAo. N=4 for control and MicAcod1KO. Data are represented as SEM.

CD45 intermediate cells showed 94% co-expression of CX3CR1 and 90% expression of tdTomato reporter signal, indicating high levels of recombination in microglia cells from tamoxifen treatment (Figure 2.4B). Microglia specific Acod1KO had no effect on infarct size (Figure 2.4C), indicating that circulating immune cells or other resident brain cells expressing Acod1 are the key mediators in these experiments. It is also possible that Acod1 in microglia cells plays an important role after the 24hr time point in ischemic stroke by contributing to tissue repair processes.

Along with the resident immune cells known role in stroke, endothelial cells play an important role in maintaining blood brain barrier (BBB) integrity. The BBB is important to regulate nutrients, ions and other molecules that are critical for proper brain function. They are also sensitive to hypoxic insult and play a critical role in stroke damage and progression. Endothelial specific Acod1KO (ECAcod1KO) were generated using Cre-ERT2 driven by Cdh5(PAC) for vascular

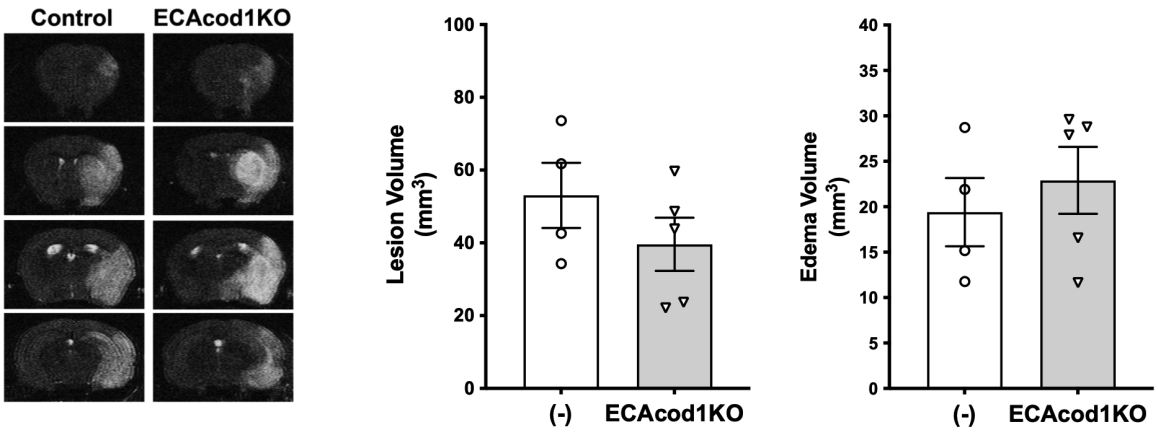

Figure 2.5. Endothelial-specific Acod1 knockout does not alter stroke infarct size. (A) Lesion volume and edema volume measurement of control and EcAcod1KO mice following 90-minute tMCAo. MRI analysis 24hrs after tMCAo. N=4 for control and N=5 for ECAcod1KO, p=0.2767 for Lesion volume and p=0.5314 for edema volume, two-tailed t-test. Data are represented as SEM.

endothelial cadherin. Mice received intraperitoneal injections of 40mg/Kg tamoxifen for three consecutive days, four weeks prior to receiving tMCAo followed with MRI analysis after 24hrs of reperfusion. Endothelial specific knockout of Acod1 had no effect on infarct or edema volume (Figures 2.5), indicating that endothelial cells are not the primary contributors to the phenotype observed in the global Acod1KO.

2.5 Discussion

The immunometabolite itaconate has been shown to be a key regulator of immune cell function. In the present study, we investigated the role of endogenous itaconate during cerebral ischemia-reperfusion injury. We identified that global knockout of Acod1 results in increased cerebral infarct size during cerebral ischemia-reperfusion, indicating that endogenous itaconate has a protective role during ischemic stroke. Itaconate and itaconate derivatives are able to very effectively suppress inflammatory signaling, however, examination of inflammatory gene expression in brain tissue showed no significant differences of pro-inflammatory signaling in Acod1 knockout mice. This suggests endogenous itaconate is mitigating infarct size through a mechanism other than regulation of inflammatory signaling. It is possible that itaconate and Acod1 are acting through modulation of cerebral ROS levels, a potential mechanism that has been shown

using derivatives of itaconate in in vitro systems [48][108]. In fact, succinate metabolism has been shown to be a major mechanism of ischemia reperfusion mediated injury, and itaconate is capable of regulating succinate through its inhibitor effect on succinate dehydrogenase.[48]

To determine the important itaconate producing and responsive cell types, we employed several cell type-specific Acod1 deficient mouse knockout lines. Acod1 and itaconate have become appreciated as key players in macrophage function acting as a major regulator of the inflammatory response[48], and they are among the most highly upregulated genes/metabolites during proinflammatory macrophage activation.[48, 60] At present, all data defining the anti-inflammatory mechanisms of itaconate has been identified in macrophages. Furthermore, myeloid specific Acod1 and itaconate has previously been shown to be critical in Mycobacterium tuberculosis infection by tempering the inflammatory response.[83] Based on this understanding, we sought to identify if circulating macrophages or resident microglia were important producers and mediators of endogenous itaconate action during stroke. Interestingly, myeloid Acod1 deficiency using Lyz2 (LysM) Cre did not phenocopy the global Acod1 knockout. This is surprising since Acod1/itaconate is largely thought to have its major effects in macrophages. Multiple publications have shown the LysM promoter has shown limited recombination in microglia[98, 109, 110], therefore we also generated microglia specific Acod1 knockout using a regulated Cre under control of the CX3CR1 promoter. Similarly, microglial specific knockout did not phenocopy the global Acod1 knockout mice, suggesting that other important non-myeloid cell lines are likely involved or that inactivation in multiple cell types are required.

Very little is known about the expression and actions of Acod1/itaconate in non-macrophage cell lines. Endothelial cells have important roles in maintaining blood brain barrier integrity, regulating vascular tone, and participating in inflammatory responses. During stroke, endothelial cell dysfunction contributes to ischemic damage and neurological deficit.[111] Acod1 is upregulated in endothelial cells during systemic inflammation, but its role is unknown.[112] We evaluated the role of Acod1 in endothelial cells during stroke using an endothelial specific knockout, but did not detect any differences as a result of gene ablation. The blood brain barrier is an important regulator of central nervous system homeostasis that is composed of endothelial cells, pericytes and astrocytes.[113-115] It remains possible that pericytes and astrocytes are critical cell types in blood brain barrier regulation that potentially express Acod1 and could be the primary cell type behind the observed global Acod1KO phenotype. Separately, Acod1 expression has been

observed in neuron cells following zika virus infection.[84] Taken together these findings would indicate the potential for Acod1 expression and itaconate production from several different resident brain cell types.

Cell permeable itaconate derivatives have been effectively employed in animal models of disease to effectively suppress inflammatory responses. The itaconate derivative, DMI was found to decrease neurological deficit during cerebral ischemia-reperfusion injury,[102] suggesting that supplementation with exogenous itaconate could have important beneficial effects. However, the mechanisms regulating endogenous itaconates protective effects have recently been shown to diverge from that of the derivative forms of itaconate. Treatment with exogenous itaconate has been shown to be readily taken up into treated cells, whereas the derivative form dimethyl itaconate has shown to not be converted into itaconate intracellularly. Furthermore, dimethyl itaconate has been shown to decrease levels of the antioxidant glutathione (GSH).[87] This is in direct contrast to treatment with exogenous itaconate treatment, where it has been shown to protect against depletion of GSH and increased neurological function following in vivo cerebral ischemia. [108] This would suggest the potential role of endogenous itaconate being protective through maintaining cerebral GSH levels and limiting oxidative damage caused by I/R insult.

LIMITATIONS OF THE STUDY

Itaconate has been shown to have anti-inflammatory in macrophages through multiple mechanisms including SDH, ATF3, and Nrf2. In this study we evaluated infarction size and inflammatory signaling but have not addressed other mechanisms. Since succinate metabolism is a major contributor to ROS generation during ischemia-reperfusion injury, and Nrf2 actions has been shown to be protective during stroke, these other mechanisms could be important targets for itaconate action during stroke. Further studies need to evaluate these other inflammatory mechanisms to determine if they mediate the protective actions of endogenous itaconate during stroke.

CONCLUSIONS

This study identifies that endogenous itaconate plays a key role in infarct development during stroke. The effects of itaconate do not appear to be through inflammatory gene regulation and could be through a separate mechanism, such as oxidative stress regulation. Furthermore, we identify that neither circulating macrophages nor resident brain microglia are responsible for the effects observed in global deletion of Acod1. This indicates that another cell type(s) are potentially

responsible and capable of Acod1 expression during cerebral stroke. Although we were not able to identify the cell-specific origin or action of itaconate, we demonstrate that Acod1 and that actions of endogenous itaconate extend beyond just macrophages.

CHAPTER 3

Aconitate Decarboxylase 1 and Itaconate Protect Against Ferroptosis by Modulating Cellular Glutathione Levels in Macrophages

3.1 Abstract

Objective: The immunometabolite itaconate regulates immune cell function through modulation of inflammatory signaling and reactive oxygen species formation. Ferroptosis has recently been identified as a major regulator of cell death during ischemic damage through generation of lipid peroxides. Using bone marrow derived macrophages (BMDMs), we examined the role of Acod1 and itaconate in RSL3-induced ferroptosis.

Method: WT and Acod1$^{-/-}$ BMDMs were treated with LPS for 24 hours to induce Acod1 expression. Following LPS stimulation, ferroptosis was induced with RAS-selective lethal 3 (RSL3) for 24, 48, and 72hrs. Cell viability was measured by MTT assay, intracellular ATP levels, and cell culture media lactate dehydrogenase (LDH) levels. Separately, treatment with exogenous itaconate was used to increase cellular itaconate levels.

Results: LPS treatment was protective to both WT and Acod1$^{-/-}$ BMDMs after 24hrs of RSL3 treatment. However, at 48hrs and 72hrs WT BMDMs showed sustained protection, whereas Acod1$^{-/-}$ BMDMs showed significant increases in cell death as measured by LDH. Further analysis showed that Acod1$^{-/-}$ BMDMs had significantly lower glutathione levels at 48hrs. Treatment with exogenous itaconate was found to significantly increase intracellular glutathione (GSH) levels in BMDMs. Furthermore, exogenous itaconate treatment in Acod1$^{-/-}$ BMDMs was found to restore protection from ferroptosis and GSH levels.

Conclusion: Our data shows that LPS induction is highly protective against ferroptosis in stimulated macrophages, and cells lacking Acod1 have diminished protection over time. LPS-induced Acod1 and itaconate are important regulators of ferroptosis, which may be a direct result

of modulating GSH levels. Furthermore, treatment with exogenous itaconate is sufficient to restore protection in cells lacking Acod1.

3.2 Introduction

Ferroptosis is a novel form of iron-induced programmed cell death that differs from other types of programmed cell death such as apoptosis, autophagy, and necroptosis[116, 117]. A unique characteristic of ferroptosis is the accumulation of iron dependent ROS that primarily occurs through Fe(II)-dependent lipid peroxidation, leading to loss of cell membrane integrity and programmed cell death[118, 119]. Induction of ferroptosis involves several factors including cellular iron handing, redox homeostasis, and mitochondrial activity[116]. One of the most well understood pathways known to govern ferroptosis is the System xc^-/GSH/GPX4 axis. The System xc^-/GSH/GPX4 pathway functions as a major regulator of oxidative stress, where System xc^- acts as an antiporter to take up cystine. Cystine taken up into cells is reduced to cysteine, which is then used to create glutathione (GSH). GSH is a potent antioxidant that is used by glutathione peroxidase 4 (GPX4) to decrease lipid peroxidation[120]. The first identified inducer of ferroptosis was erastin, an inhibitor of System xc^-[117, 121, 122]. By inhibiting System xc^-, erastin effectively decreases available intracellular cystine, which leads to decreased GSH levels available for GPX4. It is through this capacity that erastin was identified as a known inducer of ferroptosis. Since the identification of erastin, several compounds have been identified as potent inducers of ferroptosis, with the majority of them also targeting the System xc^-/GSH/GPX4 pathway at one of the three major locations[116]. RSL3 was identified in 2014 as a ferroptosis inducer that directly inhibit GPX4, effectively inhibiting its anti-lipid peroxidation activity [123]. Although the term ferroptosis was only coined in 2012, extreme interest in its role in pathophysiology has led to numerous publications in the last decade. Ferroptosis has been of particular interest in the field of cancer biology where tumor cells have shown susceptibility to ferroptosis[124, 125]. Recently however, the role of ferroptosis in ischemic injury has become of particular interest where inhibitors of ferroptosis have been found to be highly protective in a model of transient ischemia/reperfusion [116, 126].

The immunometabolite itaconate is a strong regulator of immune cell function through regulation of inflammatory signaling and oxidative stress responses[127]. Itaconate is produced

by the enzyme aconitate decarboxylase 1 (Acod1) [9] and itaconate has an integral role in the ischemic response with several anti-inflammatory mechanisms already identified[48, 81, 82]. Acod1 expression and subsequent itaconate production is highly upregulated in macrophages with proinflammatory stimuli, including LPS and IFN's. Furthermore, treatment with cell permeable itaconate derivatives has also been shown to be protective in murine models of cardiac and cerebral ischemia and decreases in vitro ROS levels[48, 102].

In this study, we investigated the effects of Acod1 deletion and subsequent loss of itaconate in ferroptosis using BMDMs. We hypothesized that cells lacking Acod1 expression would have increased susceptibility to RSL3-induced ferroptosis from lack of itaconate production. Here, we use global Acod1 knockout mice ($Acod1^{-/-}$) to explore the role of endogenous itaconate, as well as treatment with high level doses of exogenous itaconate. We evaluated the effect of Acod1 and itaconate by measuring cell death and viability following ferroptosis induction.

3.3 Methods

Generation and treatment of BMDMs

WT and $Acod1^{-/-}$ mice were euthanized via isoflurane overdose and femur and tibia were excised. Bones marrow cells were flushed from bones with 5ml RPMI 1640 media (Gibco) using a 27g needle and then strained using a 100 μm strainer. Cell suspensions were then centrifuged at 350 x g for 10 minutes to pellet cells. Supernatant was decanted and replaced with fresh BMDM differentiation media (RPMI 1640 + 30% L929 supernatant + 10% FBS + glutamax (Gibco) + penicillin streptomycin). BMDMs were allowed to differentiate for a total of 6 days with media replacement at day 4. Differentiated macrophage cells were plated at $1x10^5$ cells in 48-well plates. For LPS experiments, differentiated BMDMs were pretreated with 100 ng/ml LPS for 24hrs. RSL3 (Cayman chemical, item No. 19288) was dissolved in dimethyl formamide (DMF, Sigma, item No. 270547) to make a 113.4mM stock solution. Further dilutions to 0.01, 0.1, 1, and 10mM were generated using DMF.

Cell death and viability measurements

Cell death measurements were taken from cell culture supernatant following end of RSL3 treatment. 100μl of culture media was collected, and lactate dehydrogenase (LDH) was measured (TakaRa, Cat. #MK401) following manufacturer's instructions.

Cell viability was assessed by measuring ATP with CellTiter-Glo (Promega, G9241) following manufacturer's instructions or with thiazolyl blue tetrazolium bromide (MTT) assay. 5X MTT was made in PBS to generate MTT stock solution (Alfa Aesar, L11939). At end of treatment, cells were treated with 1X MTT solution for 45 minutes at 37°C. Supernatant was then removed, 300 μl of DMSO was added to each well, and the plate was placed back in 37°C for five minutes. Absorbance was then measured at 570nm.

Intracellular glutathione level measurements

Intracellular glutathione level measurements were completed (Promega, V6911) following manufacturer's instructions at the end of treatment. Media was removed from culture plate wells at the end of treatment and 100μl of GSH-Glo reagent was added for 30 minutes at 37°C. Following incubation, 100μl luciferin detection reagent was added and incubated for 15 minutes. At the end of incubation, luminescence was measured on a luminometer and GSH concentration was quantified.

Extracellular nitrite measurements

Nitrite levels were assessed (Sigma-Aldrich, MAK367) after 24 and 96 hours of LPS stimulation following manufacturer's instructions. 100μl of culture media was collected after 24hrs and 96hrs of LPS treatment. Equal volume of 1x Griess reagent was added to sample and incubated for 15 minutes at room temperature. Absorbance was then measured at 540nm.

3.4 Results

Acod1 knockout enhances ferroptosis in macrophages

To define the role of Acod1 and endogenous itaconate in ferroptosis, we used BMDMs as a model cell type capable of producing high levels of Acod1 and itaconate. WT and Acod1$^{-/-}$ BMDMs were pretreated with 100ng/μL LPS for 24hrs to allow for expression of Acod1 and subsequent itaconate production. Following LPS pretreatment, BMDMs were then subjected to ferroptosis induction with increasing doses of RSL3. Lactate dehydrogenase in culture media was used as a measurement of cell death to determine susceptibility to ferroptosis-induced cell death. At 24hrs of RSL3 treatment, WT and Acod1$^{-/-}$ cells pretreated with LPS showed high levels of

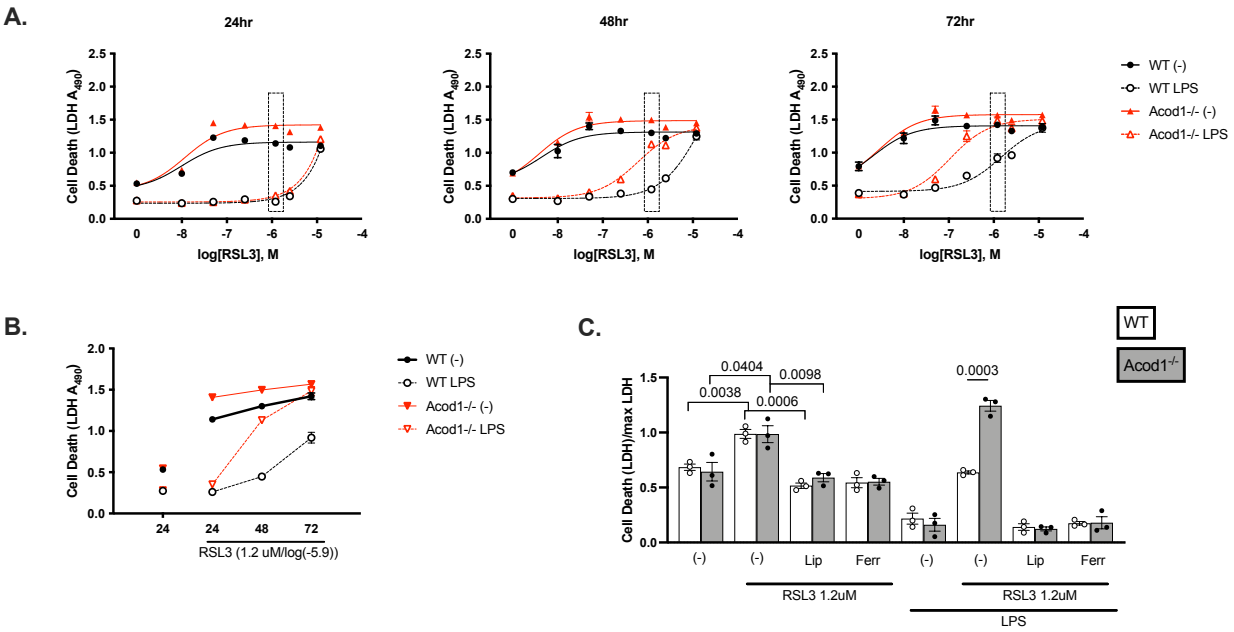

Figure 3.1. Lack of Acod1 leads to increased sensitivity to RSL3-induced ferroptosis in cultured BMDMs. (A) Dose response analysis of RSL3-induced ferroptosis at 24, 48 and 72 hours in WT and Acod1$^{-/-}$ BMDMs using LDH as a measure of cell death. BMDMs received 24hrs of 100ng/μL LPS prior to RSL3 treatment. (B) Quantification of LDH at 24, 48 and 72 hours following 1.2μM RSL3 treatment (log (-5.9), indicated by grey rectangle in Figure 3.1A). (C) Quantification of cell death following 72hr 1.2μM RSL3 treatment. BMDMs received 24hr LPS pretreatment followed with 1.2μM RSL3 treatment or cotreatment with 0.5 μM liproxstatin or ferrostatin. N=3 for control and Acod1$^{-/-}$, two-tailed t-test. Data are represented as SEM.

protection compared to non-LPS controls, indicating LPS stimulation leads to protection from ferroptosis. However, Acod1$^{-/-}$ BMDMs showed decreased protection at 48 and 72hr compared to WT controls. Increased LDH levels observed in Acod1$^{-/-}$ BMDMs at these timepoints indicate a potential protective role for Acod1 and itaconate in ferroptosis (Figure 3.1A). Treatment with 1.2μM of RSL3 showed increases in ferroptosis susceptibility in Acod1$^{-/-}$ BMDMs (Figure 3.1B). To test whether the enhanced cell death at 1.2μM RSL3 treatment was occurring specifically through ferroptosis, we used inhibitors of ferroptosis, liproxstatin and ferrostatin. WT and Acod1$^{-/-}$ BMDMs were pretreated with LPS for 24hrs followed with 1.2μM of RSL3 or co-treatment of 1.2μM of RSL3 with 0.5 μM of liproxstatin or ferrostatin. Cells treated with RSL3 showed significant increases in LDH, indicating increased cell death. Acod1$^{-/-}$ BMDMs pretreated with LPS again showed significantly increased cell death compared to WT cells. Importantly, WT and Acod1$^{-/-}$ BMDMs co-treated with liproxstatin or ferrostatin showed significant protection from ferroptosis (Figure 3.1C). This indicates that RSL3 is inducing cell death through ferroptosis and not another form of cell death.

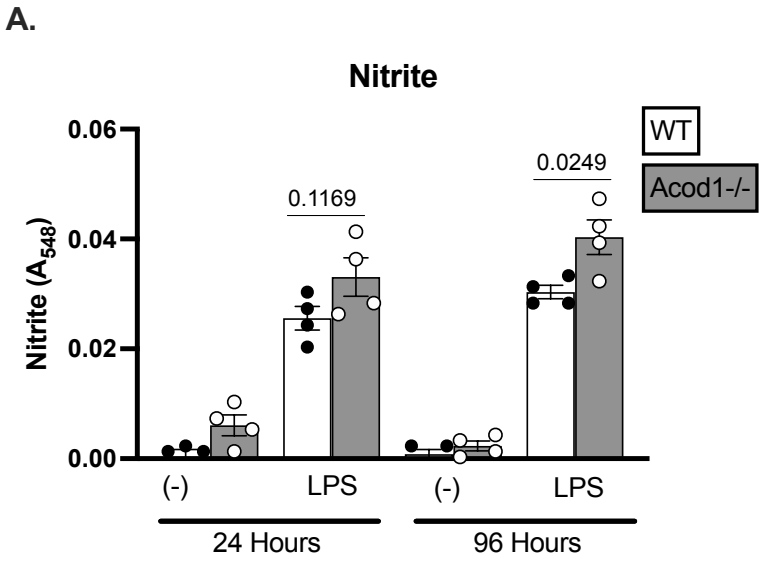

Figure 3.2. Acod1 deficiency leads to elevated nitrite levels in LPS stimulated BMDMs. (A) Quantification of extracellular nitrite levels in WT and Acod1$^{-/-}$ BMDMs at 24 and 96hrs of 100ng/μL LPS treatment. N=4 for control and Acod1$^{-/-}$, two-tailed t-test. Data are represented as SEM.

Ferroptosis susceptibility in Acod1$^{-/-}$ is not caused by decreases in nitrogen levels

Recently, inducible nitric oxide synthase (iNOS) has been identified as the major driver of protection from ferroptosis in LPS stimulated macrophages. Yang et al. identified that nitric oxide production from iNOS was upregulated in LPS stimulated macrophages and led to protection against ferroptosis. To determine if Acod1$^{-/-}$ BMDMs had decreased levels of nitric oxide, WT and Acod1$^{-/-}$ BMDMs were stimulated with LPS for 24 and 96hrs and nitrite levels were measured in culture media. Interestingly, Acod1$^{-/-}$ BMDMs showed increased levels of nitrite at the 96hr measurement (Figure 3.2A). This would suggest that increased susceptibility to ferroptosis observed in Acod1$^{-/-}$ cells is likely not caused by changes in iNOS activity or nitic oxide levels.

Acod1 deficiency leads to decreased glutathione levels following ferroptosis induction

To determine if Acod1 and itaconate modulate GSH during ferroptosis, we measured intracellular GSH levels following induction of ferroptosis in WT and Acod1$^{-/-}$ BMDMs. Cells were pretreated with LPS for 24hrs followed with 0.25 or 1.2μM RSL3 treatment for 24 and 48hrs. GSH levels were found to be significantly lower in Acod1$^{-/-}$ BMDMs compared to WT at 48hrs in both 0.25 and 1.2μM RSL3 treatment (Figure 3.3A). To determine if itaconate alone was sufficient to increase GSH levels, we treated WT BMDMs with high doses of itaconate followed with GSH quantification. WT BMDMs treated with 20mM itaconate for 72hrs showed significantly elevated

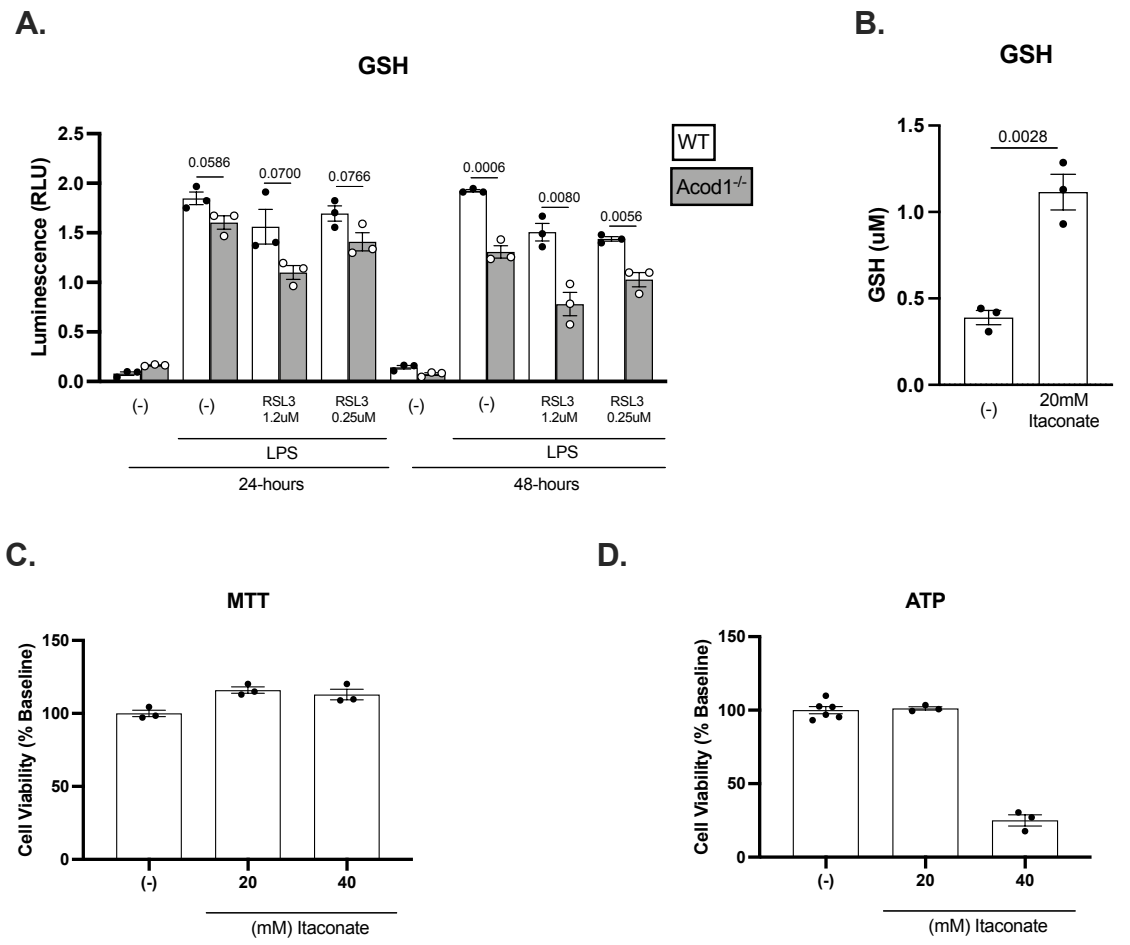

Figure 3.3. Acod1 deficiency causes decreased GSH levels following RSL3-induced ferroptosis in LPS stimulated BMDMs. Quantification of intracellular GSH levels in (A) WT and Acod1$^{-/-}$ BMDMs. Cells received 24hrs of 100ng/μL LPS pretreatment prior to 0.25 μM or 1.2μM RSL3 treatment. GSH measurements were taken at 24 and 48 hrs of RSL3 treatment. (B) Quantification of GSH in WT BMDMs following 72hr treatment with 20mM itaconate. Cell viability measured by (C) MTT or (D) ATP following 24hrs of 20mM or 40mM itaconate treatment. N=3 for control and Acod1$^{-/-}$, two-tailed t-test. Data are represented as SEM.

levels of GSH (Figure 3.3B), indicating that itaconate alone is capable of regulating GSH. Further analysis was performed to test if high doses of itaconate were not toxic to BMDMs. WT BMDMs were treated with 20 and 40mM itaconate for 24hrs followed by cell viability analysis with MTT and ATP measurements. Cells treated with itaconate showed significant increases in MTT values in both doses compared to control (Figure 3.3C). ATP levels were not affected by 20mM itaconate, but 40mM itaconate resulted in a significant decrease in ATP levels (Figure 3.3D). This suggests that 20mM itaconate is a viable dose to modulate GSH in BMDMs.

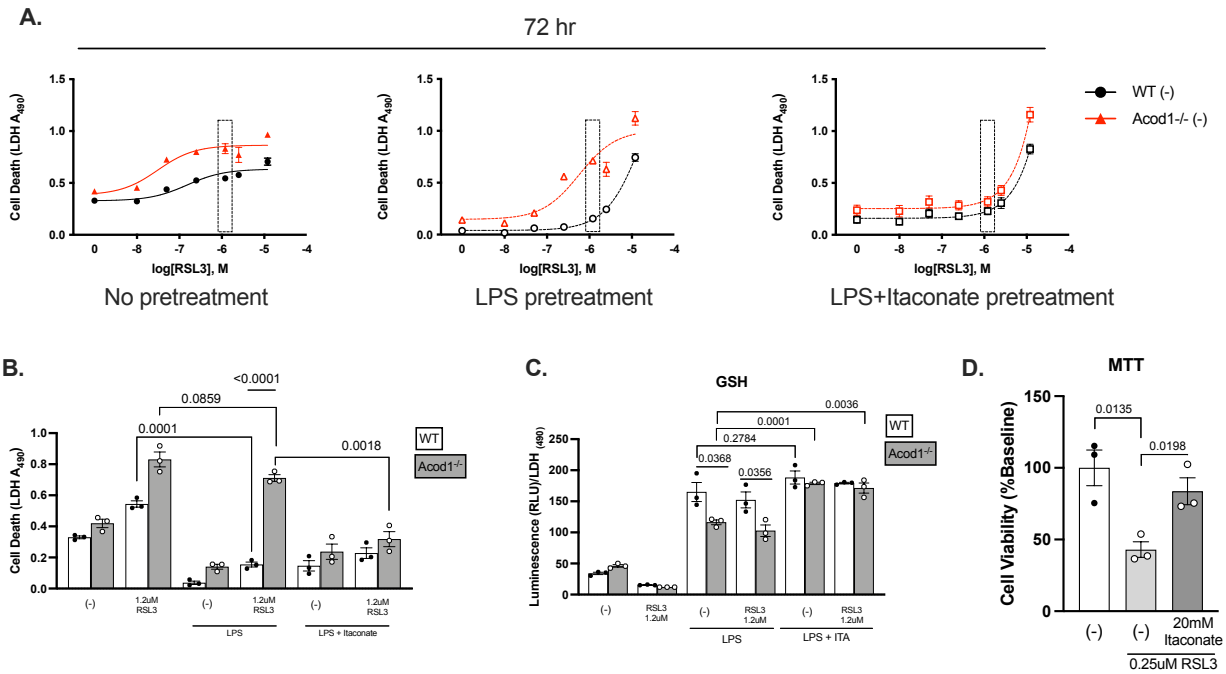

Figure 3.4. Exogenous itaconate treatment restores ferroptosis protection and increases GSH levels. (A) Dose response analysis of RSL3-induced ferroptosis at 72 hours in WT and Acod1$^{-/-}$ BMDMs using LDH as a measure of cell death. BMDMs received 24hrs of 100ng/μL LPS (LPS pretreatment) or 6hrs of LPS treatment followed with an additional 18hrs (24hrs total pretreatment) of 20mM itaconate pretreatment (LPS+Itaconate pretreatment) prior to RSL3. (B) Quantification of cell death by LDH at 72 hours following 1.2μM RSL3 treatment (log (-5.9), indicated by grey rectangle in Figure 3.4A). (C) Quantification of GSH at 72hrs following 1.2μM RSL3 treatment. (D) Quantification of cell viability by MTT analysis. WT BMDMs received 24hrs of 20mM itaconate pretreatment followed with 5hr treatment with 0.25μM RSL3. N=3 for control and Acod1$^{-/-}$, two-tailed t-test. Data are represented as SEM.

Itaconate treatment restores protection in Acod1$^{-/-}$ BMDMs and GSH levels

Since Acod1$^{-/-}$ decreases levels of GSH during ferroptosis, we next assessed whether treatment with exogenous itaconate could rescue the loss of protection in knockout cells. WT and Acod1$^{-/-}$ BMDMs were pretreated with LPS for 24hrs or pretreated with 6hrs of LPS followed by 18hrs of 20mM itaconate. BMDMs were then treated with increasing doses of RSL3 to induce ferroptosis. As previously observed, Acod1$^{-/-}$ cells pretreated with LPS alone showed increased LDH release compared to WT at 72hrs of RSL3 treatment, indicative of increased cell death from ferroptosis induction. However, this effect was reversed in Acod1$^{-/-}$ cells treated with itaconate (Figure 3.4A). This indicates that supplemental administration with exogenous itaconate in Acod1$^{-/-}$ cells is sufficient to restore protection from ferroptosis-induced death. Specifically looking at 1.2μM RSL3 treatment (log (-5.9)), we again find that following LPS pretreatment, WT cell show significantly decreased LDH levels compared to Acod1$^{-/-}$ cells. Treatment with LPS+itaconate

decreases LDH levels in Acod1$^{-/-}$ to levels similar in WT cells, indicating itaconate treatment is conferring protection in Acod1$^{-/-}$ BMDMs (Figure 3.4B). To determine if GSH was responsible for the observed changes following exogenous itaconate treatment, we measured intracellular GSH levels. As previously observed, we again found that Acod1$^{-/-}$ BMDMs had significantly decreased GSH levels compared to WT cells following LPS pretreatment. GSH levels were restored with the addition of itaconate (Figure3.4C), indicating that exogenous itaconate supplementation was sufficient to also restore GSH levels in Acod1$^{-/-}$ BMDMs. Lastly, by measuring cell viability with MTT quantification, we found that exogenous treatment of itaconate significantly increased viability in WT BMDMs following low dose RSL3 treatment for 5hrs (Figure 3.4D).

3.5 Discussion

In this study, we investigated the role of Acod1 and endogenous itaconate during ferroptosis-induced cell death. We found that BMDMs lacking Acod1 had increased susceptibility to RSL3-induced ferroptosis following LPS stimulation compared to WT controls. Further analysis identified that GSH levels were significantly decreased following LPS stimulation in Acod1$^{-/-}$ cells. Recent findings have identified iNOS and and nitric oxide as a major regulator of ferroptosis protection in LPS stimulate macrophages[128]. In this capacity, it is believed that iNOS expression and nitric oxide formation is increased in LPS stimulated macrophages, and that this nitric oxide is a major regulator of ferroptosis protection. To determine if nitric oxide levels were altered in Acod1$^{-/-}$ cells, we measured extracellular nitrite in culture media. If nitric oxide levels were responsible for the increased ferroptosis-induced death, we would expect to see decreased nitrite levels in Acod1$^{-/-}$ BMDMs. However, after quantification of nitrite levels, we found significantly elevated levels of nitrite in Acod1$^{-/-}$ cells at 96hrs compared to WT control. This finding indicated that iNOS activity and nitric oxide levels were likely not responsible for the observed effects in Acod1$^{-/-}$ BDMDs.

GSH plays a major role in the regulation of ferroptosis by acting as an antioxidant through GPX4 activity. Treatment with high dose levels of itaconate (20mM) was found to not effect BMDM cell viability, and importantly was also found to increase intracellular GSH levels. To determine if itaconate supplementation would restore protection in Acod1$^{-/-}$ cells, we treated BMDMs with high dose itaconate. We found that supplementation with exogenous itaconate restored protection in Acod1$^{-/-}$ cells back to levels seen in WT BMDMs. Similarly, GSH levels

were also restored in Acod1$^{-/-}$ cells, indicating that Acod1 produced itaconate is capable of modulating GSH levels at late timepoints following stimulation. These finds together would indicate that Acod1, and itaconate produced by Acod1, plays an important role in LPS-induced protection from ferroptosis in macrophages.

Although we have a role for itaconate in LPS-induced protection from ferroptosis, it is important to note that Acod1 and itaconate are not solely responsible for this protection. LPS stimulation has been noted as a potent driver of protection from ferroptosis and likely acts through a variety of mechanisms to achieve this protection. Specifically, iNOS and nitric oxide have been identified as a major regulator of this LPS-induced protection from ferroptosis[128]. It remains likely that iNOS is a major player in is current study and contributes to LPS protection from ferroptosis. However, our findings indicate that itaconate is acting separately from iNOS, where we found that Acod1$^{-/-}$ BMDMs have elevated nitrite levels at later time-points of LPS stimulation. This suggests that iNOS activity and nitric oxide levels are not decreased with Acod1 knockout and could potentially be elevated. This study highlights a separate pathway of ferroptosis protection through Acod1 and endogenous itaconate.

CHAPTER 4

Itaconate Regulates Glucose Homeostasis and Obesity in Mice

4.1 Abstract

Objective: The intersection between immunology and metabolism contributes to the pathogenesis of obesity-associated metabolic diseases as well as molecular control of inflammatory responses. The metabolite, itaconate and cell permeable derivatives have robust anti-inflammatory effects, therefore we hypothesize that Acod1-produced itaconate has a protective, anti-inflammatory effect during diet-induced obesity and metabolic disease.

Methods: Wild type and Acod1$^{-/-}$ mice were subjected to diet-induced obesity. Glucose metabolism was analyzed by glucose tolerance test (GTT), insulin tolerance test (ITT), and indirect calorimetry. Gene expression and transcriptome analysis were performed using qRT-PCR and RNA-Seq.

Results: WT and Acod1$^{-/-}$ mice on high fat (HF) diet had equivalent weight gain, but Acod1$^{-/-}$ mice had impaired glucose metabolism. ITT and GTT at 12 weeks on high fat diet revealed significantly higher blood glucose levels in Acod1$^{-/-}$ mice. This was associated with significant enrichment of inflammatory gene sets and a reduction in genes related to adipogenesis and fatty acid metabolism. Analysis of naïve Acod1$^{-/-}$ mice showed a significant increase in fat deposition at 3 and 6 months age and obesity and insulin resistance by 12 months.

Conclusions: Our data show that Acod1-produced itaconate has an important role in the regulation glucose homeostasis and obesity under normal and high fat diet conditions that worsens significantly with age.

4.2 Introduction

The obesity epidemic represents a major challenge to public health because of its role in the development of type 2 diabetes mellitus and its association with adverse health consequences. Chronic, low-grade systemic inflammation is a widely recognized aspect of obesity and is a contributing factor in the development of insulin resistance and other associated co-morbidities. Adipose tissue is one of the major metabolic tissues driving systemic inflammation during obesity, and this is largely due to a significant influx of inflammatory cells as well as activation of resident immune cells. Adipose tissue macrophages are present in lean, visceral white adipose tissue depots, but are further recruited and undergo activation and proinflammatory signaling in response to excessive adipose tissue expansion during obesity.[129] This occurs in coordination with a network of other innate and adaptive immune cells, and this proinflammatory response impairs adipocyte function and contributes to insulin resistance.

Metabolic regulation and immune responses during obesity and adipose tissue inflammation are critically intertwined. There is a body of literature demonstrating that proinflammatory cytokines and chemokines can promote inflammation and induce both localized and systemic insulin resistance and metabolic dysfunction.[130, 131] Nutrient metabolism and intracellular metabolic pathways also have critical roles in governing immune cell function and metabolic responses in non-immune cells.[132] Metabolism, particularly mitochondrial metabolism, and the production of metabolites has been shown to be critical in regulating inflammatory signaling and macrophage phenotype.[133] Itaconate is a TCA-cycle derived metabolite produced by the enzyme cis-aconitate decarboxylase 1 (Acod1/Irg1), and Acod1 and itaconate are among the most highly upregulated genes and metabolites during proinflammatory macrophage activation.[48] Itaconate has been shown to have anti-inflammatory and anti-oxidative effects in vitro in macrophages through multiple mechanisms including succinate dehydrogenase (SDH) inhibition, induction of ATF3 and Nrf2, and inhibition of NLRP3 inflammasome activity.[48, 81, 87, 134-136]

Augmentation of itaconate signaling has also been shown to be effective in mitigating inflammation in various animal models of disease. The cell-permeable itaconate derivatives, dimethyl itaconate (DMI) and 4-octyl itaconate (4-OI) have been employed as a strategy to target immunometabolism to regulate hypoxia and inflammation in disease. Itaconate has been shown to decrease oxidative phosphorylation and ROS production by both diverting cycle intermediates

towards itaconate production and by inhibiting SDH. Itaconate derivatives have been shown to be effective in mitigating injury during hypoxia responses such as cardiac ischemia,[48] cerebral ischemia,[102] and experimental colitis.[137] Hypoxia and oxidative responses mediated by HIF1a and ROS are also thought to play a role in the pathophysiology of adipose dysfunction and insulin resistance during obesity.[138, 139] In addition, itaconate derivatives have are effective in a wide array of inflammatory and autoimmune models of disease.[140-148]

Although Acod1 and itaconate have been the focus of many studies on immune metabolism and immunoregulation, none have addressed the in vivo role in glucose metabolism, obesity or diabetes mellitus. Because of the anti-inflammatory effects of itaconate in immune cells, we hypothesized that itaconate would have a beneficial effect on adiposity and metabolic dysfunction during DIO by dampening adipose tissue inflammatory responses. In the present study, we examined the role of endogenous itaconate in the development of obesity and diabetes using Acod1 deficient mice as a model of itaconate deficiency in the context of diet-induced obesity. Here we defined the effects of itaconate on adiposity and glucose homeostasis in both obese and aging mice.

4.3 Methods

Animals

Male Acod1$^{-/-}$ mice (Acod1$^{tm1a(KOMP)Wtsi}$) and littermate controls were on a C57BL/6N background, and myeloid Acod1 knockout mice (MyAcod1KO: Acod1$^{fl/fl}$-*LysM*-Cre) and littermate floxed controls were on a C57BL/6J background as described previously.[48, 83] Mice were housed in static cages in a temperature controlled room (21-23°C) with a light:dark cycle of 12:12 h (lights on at 6 A.M.). Mice were maintained on standard laboratory chow (5L0D, LabDiet) or high fat diet (60 kcal% fat, [D12492, Research Diets]) and water ad libitum. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Food Consumption

Food intake was repeatedly measured in both standard static cages and metabolic cages over 2-3 day periods.

LPS-induced systemic inflammation

Mice were injected intraperitoneally with LPS (5 mg/kg, *Escherichia coli* serotype O127:B8,

Sigma-Aldrich, L3129) dissolved in PBS. Mice were euthanized after 6 h and tissues were harvested for gene expression analysis.

RNA-Seq

Total RNA was extracted from tissues using TRIzol reagent and purified using an RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. RNA sequencing was performed by the University of Michigan Advanced Genomics Core, using the Lexogen QuantSeq library prep and sequenced on an Illumina NextSeq system (~10 million raw reads per sample). Data were trimmed using TrimGalore (v 0.5.0) and Aligned using STAR (v 2.6.0). Differential expression analysis was performed using the DESeq2 package in R.

Body Composition

Body composition analysis of body fat, lean mass, and free fluid was determined using an NMR-based analyzer (EchoMRI 1100). Conscious mice were placed individually in a measuring tube with measurements lasting less than 2 minutes. The EchoMRI analyzer was checked daily using a reference sample (canola oil) as recommended by the manufacturer.

Glucose and Insulin Tolerance Tests

For glucose tolerance tests (GTT), mice were fasted for 6 h (5:00 A.M.–11:00 A.M.) before receiving an intraperitoneal injection of glucose. Glucose dose (1.25 mg/g lean mass) was determined from lean body mass to avoid confounding effects from obesity.[149, 150] Blood glucose was measured at 0, 15, 30, 60, and 120 min after glucose injection using a Contour (Bayer) glucometer. For insulin measurements, plasma was collected at 0, 30 and 60 min after glucose injection and plasma insulin was assayed using an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem). For insulin tolerance tests (ITT), mice were injected with insulin (0.5 U/kg lean mass) (Humulin R; Lilly) and blood glucose was measured at 0, 15, 30, 60, and 120 min.

Gene expression analysis

Relative mRNA expression was determined using quantitative reverse transcription–polymerase chain reaction. Total RNA was extracted from tissues using TRIzol reagent and purified using an RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. RNA (1 ug) was reverse transcribed to cDNA with an Applied Biosystems kit and quantitative reverse transcription–polymerase chain reaction was performed using a 7900HT fast real-time PCR system (Applied Biosystems). The relative mRNA expression was quantified by the comparative method

and normalized to the housekeeping gene L32.

Indirect Calorimetry

Whole body oxygen consumption (VO2), carbon dioxide production (VCO2), spontaneous motor activity and food intake were measured using an integrated open-circuit calorimeter (CLAMS, Columbus Instruments). Mice were weighed before measurements and were placed in individual sealed chambers with free access to food and water and measured continuously for 72 hours.

3T3-L1 Adipocyte Culture

3T3-L1 cells were cultured to confluency and treated with preadipocyte expansion media (DMEM + 10% calf serum). After 48 h, cells were then cultured in differentiation media (DMEM + 10% FBS, 1.0 mM dexamethasone, 0.5 mM methylisobutylxanthine [IBMX], 1.0 ug/mL bovine insulin) for 10 days. Differentiated 3T3-L1 adipocyte cultures were then pretreated with dimethyl itaconate (250 mM) and 4-ocytyl itaconate (250 μM) for 18 h and then stimulated with LPS (100 ng/mL) for 3 h.

Statistical analysis

A Shapiro-Wilk normality test was used to determine if data were normally distributed. For normally distributed data with equal variance, values are presented as mean ± SEM, and statistical comparison of mean values between multiple groups was performed by Student's t test, one-way ANOVA with a Tukey's post-test, or two-way ANOVA with a Tukey's post-test as indicated in the text. For normally distributed data with unequal variance, a Welch's t test was used. Data that were not normally distributed were analyzed with the nonparametric Mann-Whitney test. All statistical analysis of data was performed in GraphPad Prism (version 7; GraphPad Software, Inc). $P < 0.05$ was considered significant.

4.4 Results

Acod1 expression is increased during DIO but does not affect weight gain or fat deposition

To delineate the role of itaconate during DIO, we used Acod1$^{-/-}$ mice from KOMP which employ the tm1a cassette to produce global gene inactivation. We confirmed using BMDMs a total inhibition of LPS-induced Acod1 expression in Acod1$^{-/-}$ mice (Figure 4.1A). Systemic inflammation by LPS treatment showed significant induction of Acod1 expression in mouse fat

depots (Figure 4.1B). Similarly, mice on high fat diet also had significantly elevated expression of Acod1 in gonadal white adipose tissue (gWAT) (Figure 4.1C).

To determine the role of Acod1 in the pathogenesis of diet-induced obesity and metabolic disease, WT and Acod1$^{-/-}$ mice were placed on a HF diet for 12 wk. WT and Acod1$^{-/-}$ mice gained weight at an equivalent rate and displayed similar levels of food consumption (Figure 4.1D-E). Although body weights of Acod1$^{-/-}$ mice were marginally greater at the end of the study (Figure 4.1E), this was due to a higher baseline body weight prior to administration of HF diet (discussed below). Both fat mass and lean body mass were similar between WT and Acod1$^{-/-}$ mice on HF diet (Figure 4.1F). Subsequent analysis of major WAT depots revealed a similar degree of gWAT and inguinal white adipose tissue (iWAT) expansion in WT and Acod1$^{-/-}$ mice on HF diet (Figure

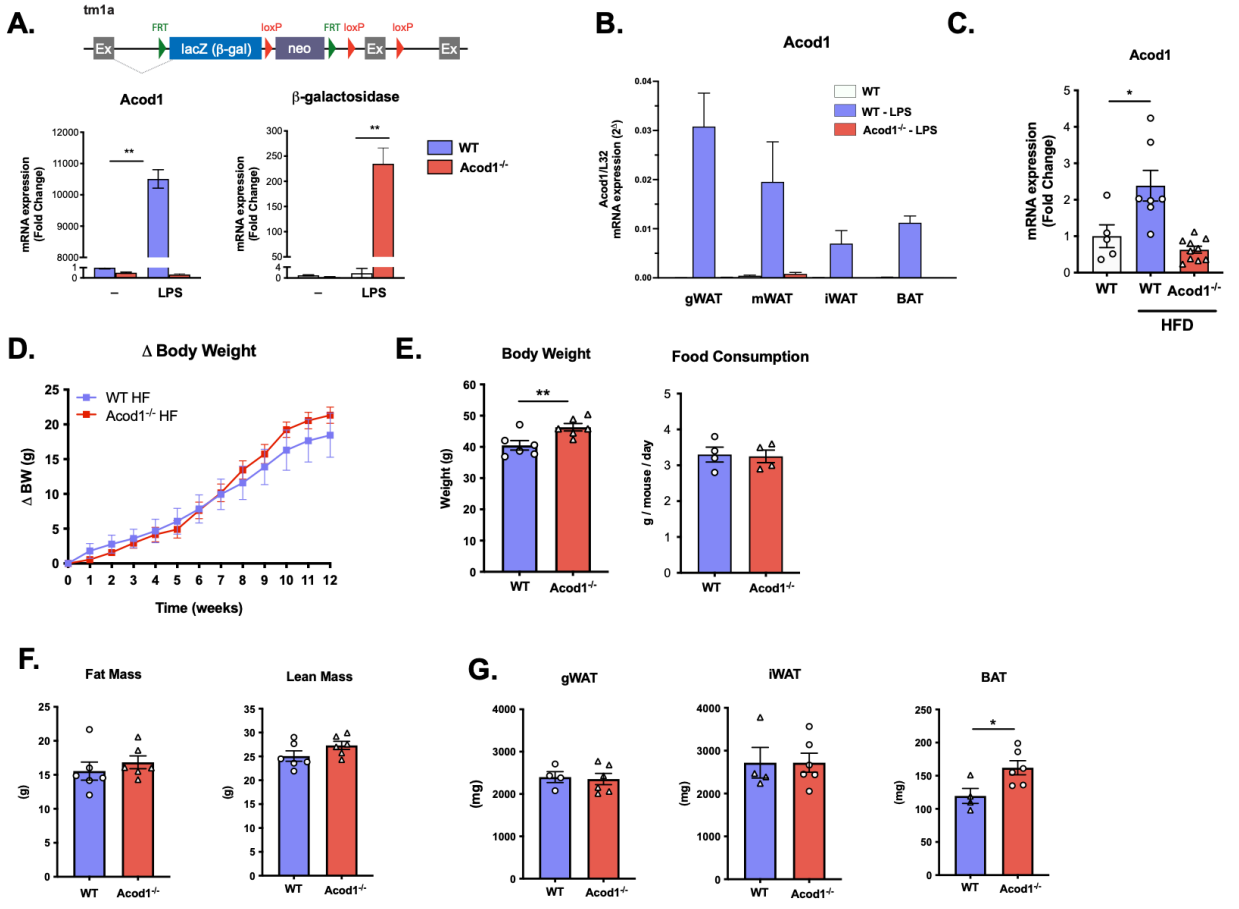

Figure 4.1. Acod1 is increased in adipose tissue during inflammation and obesity. (A) tm1a construct from KOMP used to generate Acod1$^{-/-}$ mice. BMDMs from Acod1$^{-/-}$ mice treated with LPS show efficient knockout. Effect of LPS (B) and HFD (C) on Acod1 expression by qPCR in AT. (D) Acod1$^{-/-}$ mice on HFD gain the same amount of weight. (E) Body weight after 12 wk. on high fat diet, and food consumption. (F) Total fat mass and lean mass measured by MRI at 12 wk. on HF diet (G) Fat depot mass at 12 wk. on HF diet. N = 3-6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

4.1G), although Acod1$^{-/-}$ mice had a significant increase in brown adipose tissue (BAT) depot compared to WT mice.

Acod1 is important in maintaining glucose homeostasis and insulin sensitivity during obesity.

To assess whether disruption of Acod1 and itaconate function are involved in glucose metabolism during obesity, we performed GTT and ITT on WT and Acod1$^{-/-}$ mice. After 2 wk. on

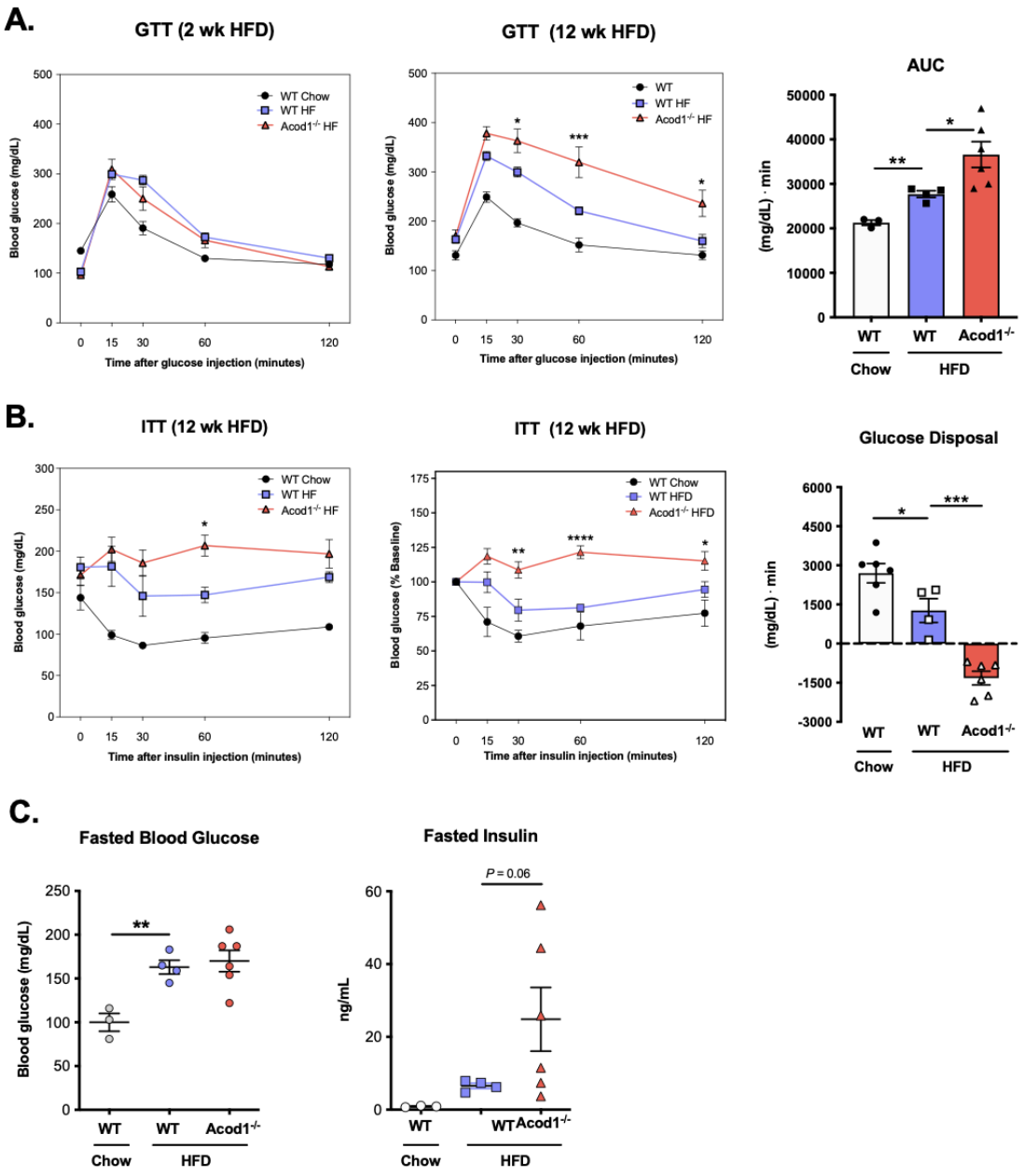

Figure 4.2. Acod1 deficiency impairs glucose metabolism during obesity. (A) GTT at 2 and 12 wk. on HF diet. (B) ITT and glucose disposal at 12 wk. on HF diet (C) Fasted blood glucose and insulin levels. N = 3-6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

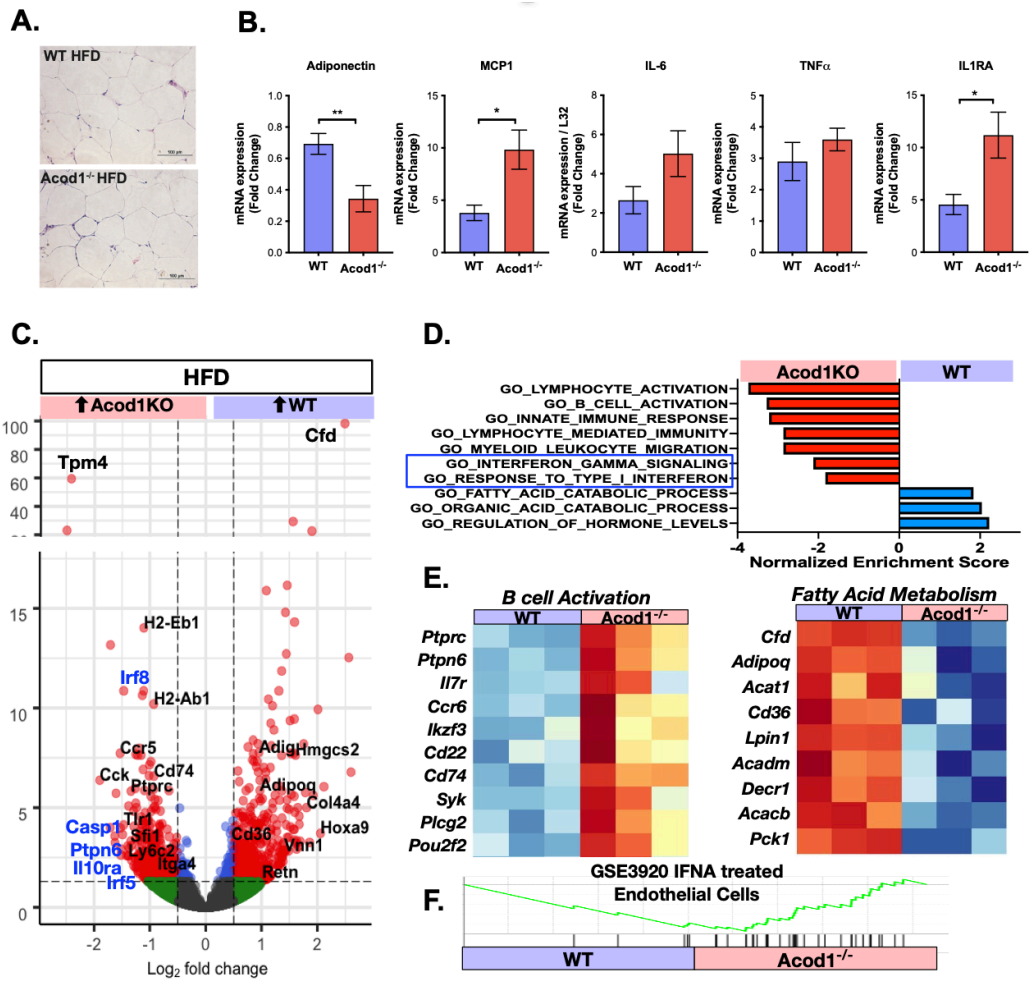

Figure 4.3. Increased inflammatory gene expression in eWAT of obese Acod1$^{-/-}$ **mice.** (A) eWAT histology at 12 wk. HF diet. (B) qRT-PCR analysis of inflammatory genes from eWAT. RNA seq (Quantseq) was performed on eWAT from DIO WT and Acod1$^{-/-}$ mice. (C) Volcano plots of DE genes. Inflammatory (black) and Interferon responsive (blue) genes are highlighted. (D) Enriched pathways in HFD eWAT (GSEA analysis; all padj<0.05). (E) Heatmaps of B cell and FA metabolic genes. (F) GSEA of IFNA dependent genes. N = 4-6. *$P < 0.05$, **$P < 0.01$.

HF diet, no differences were detected between WT and Acod1$^{-/-}$ mice on HF diet (Figure 4.2A). By week 12, Acod1$^{-/-}$ mice had significantly elevated blood glucose levels during GTT compared to WT controls (Figure 4.2A). Acod1$^{-/-}$ mice also displayed signs of insulin resistance during ITT where they showed increased glucose levels with significant impairment of glucose disposal (Figure 4.2B). No statistically significant differences were detected in fasting glucose or insulin, although the mean fasting insulin levels were greater and suggestive of insulin impairment (Figure 4.2C).

Acod1$^{-/-}$ mice have increased inflammatory genes adipose tissue

Histological analysis of adipose tissue after high fat diet did not reveal any structural or morphological differences between WT and Acod1$^{-/-}$ mice (Figure 4.3A). Since itaconate has anti-inflammatory effects, and Acod1 is significantly upregulated in inflammatory macrophages, we analyzed the expression of inflammatory genes in adipose tissue by qRT-PCR. Acod1 deficiency selectively regulated inflammatory gene expression during DIO. We detected increases in the expression of the chemokine MCP1/CCL2 and IL1RA, but not other inflammatory cytokines such as TNFa or IL6 (Figure 4.3B). Acod1$^{-/-}$ mice also had a significant decrease in the insulin-sensitizing adipokine adiponectin.

We next perform RNA sequencing on epididymal white adipose tissue (eWAT) from HFD fed Acod1$^{-/-}$ and WT mice and identified 1033 differentially expressed (DE) genes. Gene set enrichment analysis (GSEA) analysis showed that Acod1$^{-/-}$ mice had significant enrichment of inflammatory gene sets related to B cell activation, lymphocyte activation, interferon responses, and myeloid responses (Fig 4.3 C-E). Enrichment of B cell related genes in the Acod1$^{-/-}$ mice was prominent and supported by enrichment analysis in the Immgen database. WT mice had increased expression of adipogenic (Adipoq, Cd36) and fatty acid (FA) catabolic genes (Lpin1, Acat1) suggesting Acod1 may play a role in enhancing FA metabolism and limiting fat expansion.

Myeloid Acod1 deficiency does not contribute to diet-induced metabolic dysregulation

Since Acod1 is highly expressed in macrophages and has been established as an important immunomodulatory mechanism, we tested whether macrophages and other myeloid cell were critical Acod1 expressing cell types during obesity and metabolic dysfunction. We verified sufficient knockout of Acod1 expression in BMDMs from MyAcod1KO mice at both the mRNA and protein level (Figure 4.4A-B). Injection of mice with LPS showed an attenuation of Acod1 expression in MyAcod1KO spleen, liver, and eWAT, but Acod1 induction was not completely lost suggesting activation of Acod1 in LysM negative cells (Figure 4.4C). MyAcod1KO and controls were placed on a HF diet for 12 wk. and no differences were detected in body weight gain (Figure 4D). In contrast to what was seen in global Acod1$^{-/-}$ mice, MyAcod1KO mice did not show any impairment of glucose control during GTT or ITT during HF diet when compared to controls (Figure 4.4E-F). Similarly, no differences were detected in fasted or fed blood glucose levels (Figure 4.4G).

Adipocytes express Acod1 and have attenuated inflammatory signaling with itaconate treatment

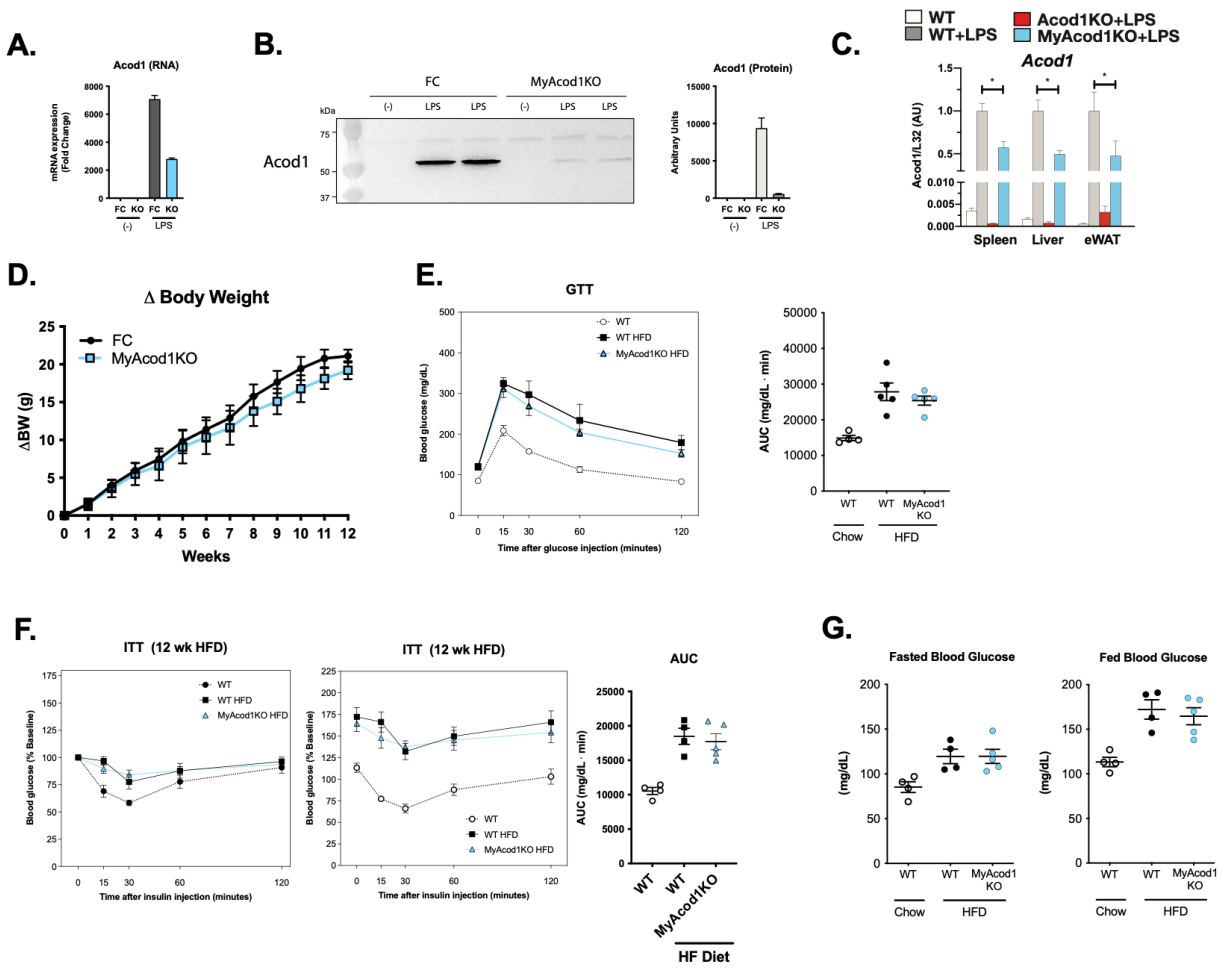

Figure 4.4. Myeloid Acod1 deficiency does not phenocopy global Acod1 knockout. (A) mRNA expression and (B) Western blot of Acod1 in macrophages showing loss of Acod1 expression in MyAcod1KO. (C) *Acod1* mRNA expression from tissues of mice treated with LPS for 6h (D) Change in body weight gain during HF diet (E) GTT at 12 on HF diet (F) ITT at 12 wk. on HF diet (G) Fasted and fed blood glucose at 12 wk. on HF diet. N = 4-5 per group. $*P < 0.05$.

To further assess the possibility that itaconate has a role in regulating adipocyte function, we cultured 3T3-L1 adipocytes and stimulated them with LPS. Acod1 expression was significantly upregulated with the addition of LPS (Figure 4.5A). This is consistent with a previous report showing that treatment of 3T3-L1 adipocytes with TNFa can induce Acod1expression.[151] To evaluate if the cell permeable itaconate derivatives 4-OI and DMI (Figure 4.5B) can influence adipocyte differentiation, 3T3-L1 cells were differentiated with or without the itaconate analogues however no differences in cell proliferation or morphology were observed (Figure 4.5C-D). To further evaluate the effect of itaconate derivatives on inflammation, 3T3-L1 adipocytes were treated with LPS in the presence or absence of 4-OI or DMI. Both 4-OI and DMI significantly

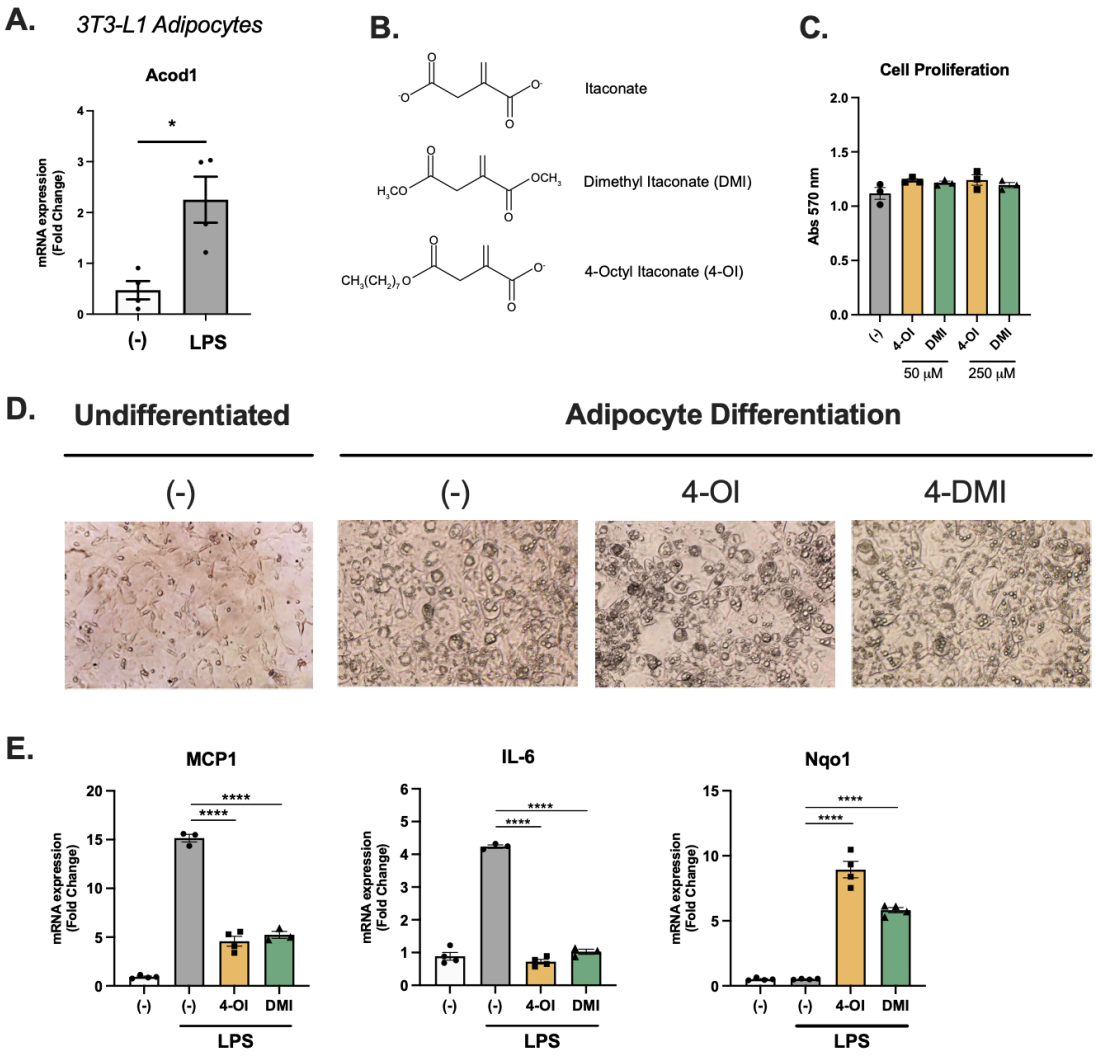

Figure 4.5. Acod1$^{-/-}$ **mice develop spontaneous obesity and impaired glucose metabolism with age.** (A) Body weight time course in WT and Acod1$^{-/-}$ mice (B) Time course of eWAT, iWAT, and BAT fat pad mass (C) GTT and (D) ITT in naïve 12 mo. Acod1$^{-/-}$ mice. (E) Adipocyte size in 12 mo. Acod1$^{-/-}$ mice. N = 3-7 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

suppressed LPS-induced MCP1 and IL-6 expression (Figure 4.5E). The Nrf2 dependent gene Nqo1 (NAD(P)H Quinone Dehydrogenase 1) was also induced by DMI and 4-OI in adipocytes suggesting Nrf2 dependent suppression of inflammatory genes in adipocytes.

Acod1$^{-/-}$ mice develop spontaneous obesity, glucose intolerance, and adipose tissue inflammation with age

While analyzing multiple cohorts of mice, we observed that the body weights of the male Acod1$^{-/-}$ mice were significantly larger than littermate WT control mice. Body weights began to diverge at 6 months of age and 12 month old Acod1$^{-/-}$ mice were markedly larger in body weight

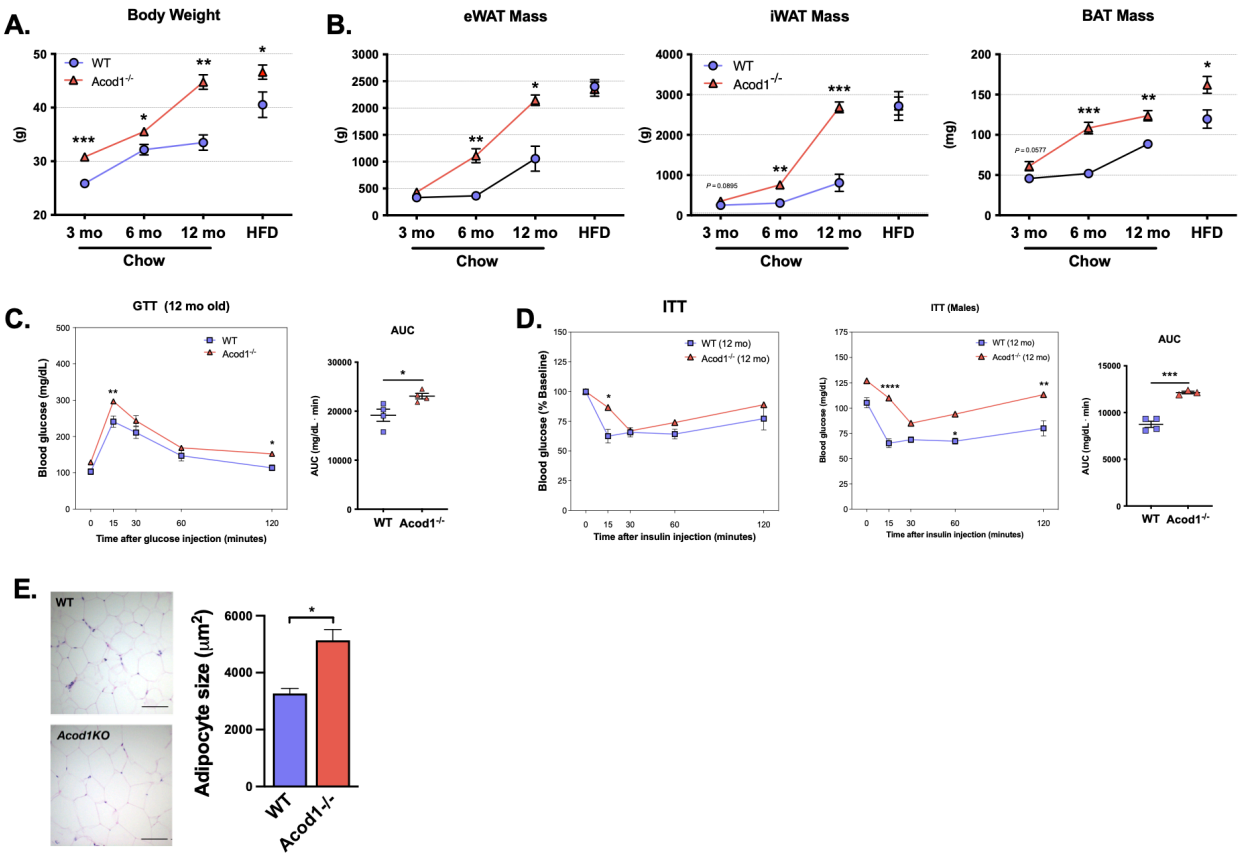

Figure 4.6. Acod1$^{-/-}$ **mice develop spontaneous obesity and impaired glucose metabolism with age.** (A) Body weight time course in WT and Acod1$^{-/-}$ mice (B) Time course of eWAT, iWAT, and BAT fat pad mass (C) GTT and (D) ITT in naïve 12 mo. Acod1$^{-/-}$ mice. (E) Adipocyte size in 12 mo. Acod1$^{-/-}$ mice. N = 3-7 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

compared to WT controls (Figure 4.6A). The difference in body weight was due to increased fat mass in Acod1$^{-/-}$ mice based on body composition analysis (MRI) and weight of fat depots (Figure 4.6B). Analysis of glucose metabolism by GTT and ITT revealed that old Acod1$^{-/-}$ mice were glucose intolerant and insulin resistant compared to age matched WT controls (Figure 4.6C-D). This was associated with significantly increased adipocyte size in eWAT from 12mo old Acod1$^{-/-}$ mice (Figure 4.6E). Further analysis of Acod1$^{-/-}$ mice revealed a significant increase in both cardiac and kidney mass (Figure 4.7A-B). Cardiac hypertrophy in Acod1$^{-/-}$ mice was associated with increased cardiomyocyte size (Figure 4.7C).

RNA sequencing was performed on eWAT from 12 month old mice and PCA analysis demonstrated that the old Acod1$^{-/-}$ mice gene profiles clustered with HFD fed mice (Figure 4.8A). Compared to age matched controls, old Acod1$^{-/-}$ mice had significant enrichment of inflammatory genes related to macrophage activation (Itgax, Trem2, Spp1), lymphocyte activation, and genes in

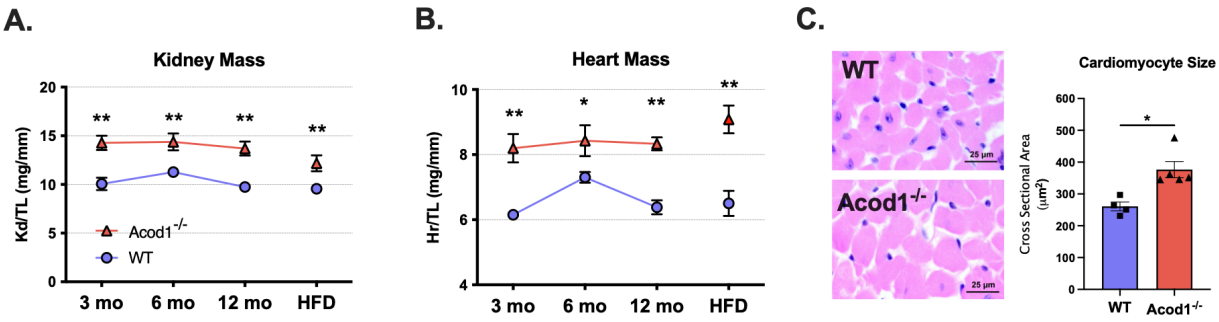

Figure 4.7. Organomegaly in Acod1 knockout mice. Time course of (A) kidneys mass and (B) heart mass in WT and Acod$^{-/-}$ mice. (C) Photomicrograph of cardiomyocytes and quantification of cardiomyocyte size in 12 mo old WT and Acod$^{-/-}$ mice. n = 3-7 per group. *$P < 0.05$, **$P < 0.0001$.

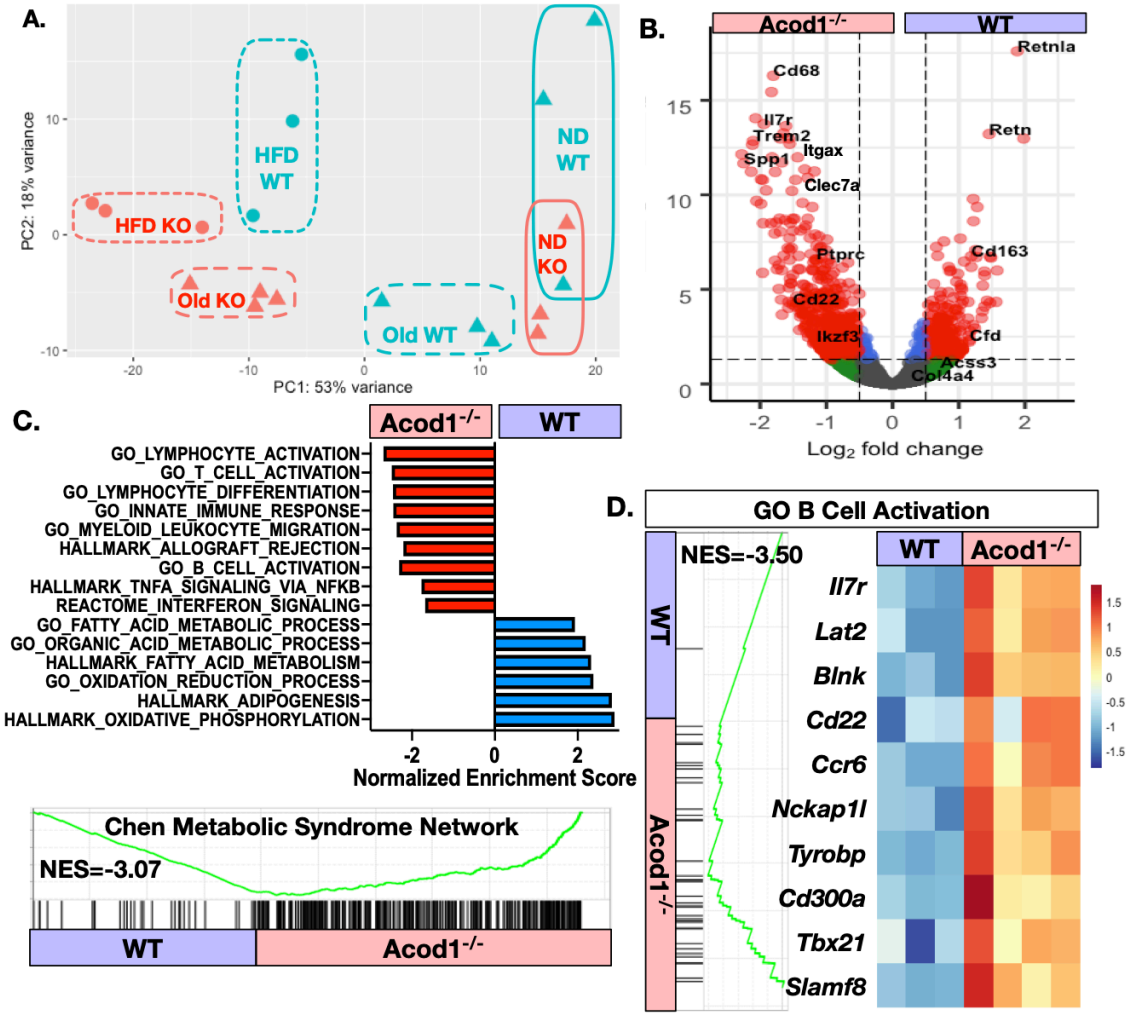

Figure 4.8. Old Acod1$^{-/-}$ **mice have increased inflammatory gene expression.** RNA seq of eWAT from 12 mo. old mice. (A) PCA analysis of ND, HFD, and old WT and Acod1$^{-/-}$ mice. (B) Volcano plot of DE genes between 12 mo. old WT and Acod1$^{-/-}$ mice. (C) Most enriched pathways from DE genes (GSEA) in 12 mo. old WT and Acod1$^{-/-}$ mice. (padj<0.05). (D) B cell activation module (all padj<0.05). N = 3-4 per group.

the Chen Metabolic Syndrome Network (Figure 4.8B-C).19 Similar to DIO mice, old Acod1$^{-/-}$

mice had increased B cell activation genes (Figure 4.8D) These data demonstrate that Acod1$^{-/-}$ mice develop obesity with age that leads to features of unhealthy WAT expansion and exaggerated myeloid inflammation.

Acod1$^{-/-}$ mice have increased fatty acid (FA) synthetic gene expression in adipose tissue To better understand the mechanisms of the increased weight gain in the 12 month old Acod1$^{-/-}$ mice, we used CLAMS to evaluate an independent cohort of weight matched 3 month old mice before body weight between genotypes diverged. During the CLAMS, Acod1$^{-/-}$ mice had increased food intake and increased resting energy expenditure per mouse that disappeared when normalized for lean body mass (Figure 4.9A-B). Activity and RER were not different between KO and WT mice. Assessment of substrate utilization demonstrated a decrease in fat oxidation and an increase in glucose oxidation in Acod1$^{-/-}$ mice compared to controls (Figure 4.9C). RNAseq was performed in eWAT from weight matched 3 month old chow fed Acod1$^{-/-}$ and WT mice and 1279 DE genes

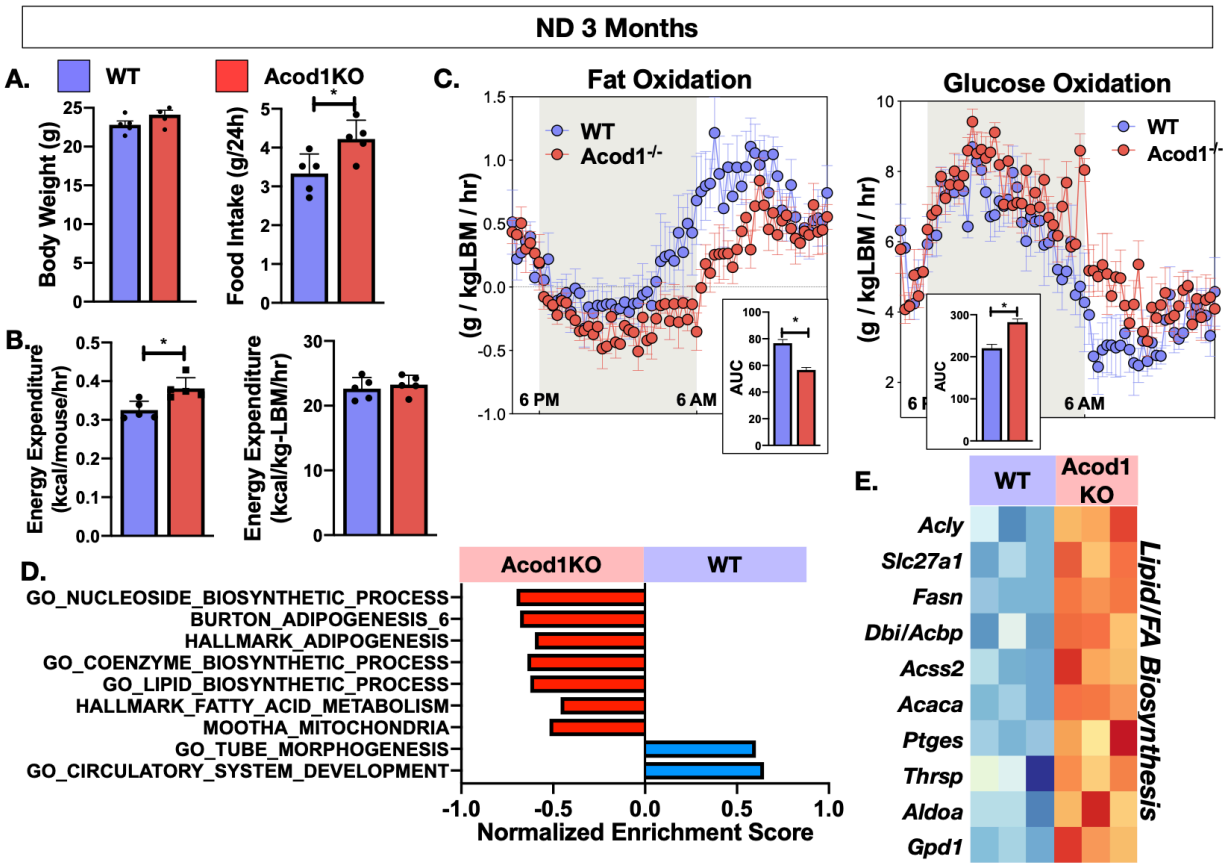

Figure 4.9. Non-obese Acod1$^{-/-}$ mice have altered energy substrate utilization. 3 mo. old chow diet fed weight matched WT and Acod1$^{-/-}$ mice analyzed by (A) CLAMS for food intake, (B) energy expenditure, (C) fat, and glucose oxidation. (D) Enriched pathways in chow fed WT and Acod1$^{-/-}$ mice (GSEA; padj<0.05). (E) Heatmap of DE genes related to FA synthesis. N = 3-5 per group. *$P < 0.05$.

were identified (Figure 4.9D-E). eWAT from Acod1$^{-/-}$ mice had a significant increase in the expression of pathways related to adipogenesis, lipid biosynthesis, and FA synthesis (Acly, Fasn, Slc27a1, Acbp).

4.5 Discussion

The intersection between immune and metabolic responses is a widely recognized feature of metabolic dysfunction that occurs during both obesity and aging. Although many proinflammatory pathway which contribute to adipose tissue inflammation and insulin resistance have been identified, many of the intracellular metabolic pathways that govern immune cell function and metabolism are largely undefined.

In this study, we investigated the role of the anti-inflammatory TCA-cycle metabolite, itaconate, during the pathogenesis of obesity and metabolic dysfunction. We found that itaconate has a critical role in glucose homeostasis and adiposity during obesity and aging. We have shown that Acod1$^{-/-}$ mice have impaired glucose homeostasis during obesity, and this is associated with increased inflammatory signaling in visceral adipose tissue. Naïve Acod1$^{-/-}$ mice also developed obesity, insulin resistance, and enhanced inflammatory signatures with age, highlighting an important endogenous, immunometabolic mechanism that protects against obesity and glucose dysregulation. To our knowledge, this is the first investigation to link Acod1 and itaconate to glucose metabolism and obesity.

Although global deletion of Acod1 did not have any effect on weight gain during DIO, it did result in increased glucose intolerance and insulin resistance. Acod1$^{-/-}$ / itaconate deficiency was also associated with increased inflammation in Acod1$^{-/-}$ mice as determined by gene expression and transcriptomic analysis using qPCR and RNA-Seq. These findings are consistent with the hypothesis that Acod1 has a protective, anti-inflammatory effect during DIO through suppression of the immune response. In vitro and in vivo studies using both genetic (Acod1$^{-/-}$) and pharmacological (itaconate derivatives: DMI, 4-OI) approaches have shown that itaconate has potent anti-inflammatory effects.

Surprisingly, knockout of Acod1 in myeloid cells did not phenocopy the global knockout. MyAcod1KO mice displayed similar weight gain and metabolic dysfunction (GTT/ITT) as WT mice. Acod1 has been shown to be most highly upregulated in macrophages, and the in vitro characterization of itaconates' anti-inflammatory effects has been defined exclusively in

macrophages. Since macrophages are central mediators of adipose tissue inflammation, we hypothesized that macrophages would be critical for Acod1 and itaconate responses. However, these results suggest an important role for Acod1/itaconate from non-LysM expressing cell types. GSEA from RNA-Seq data did show significant enrichment of genes related to myeloid leukocyte response. Although the *LysM/Lyz2* promoter is often considered to be myeloid-specific and has high recombination efficiency in most ATMs, neutrophils, and dendritic cells, the recombination efficiency can vary depending on the flox locus and it is possible that minor leukocyte populations not targeted in $Lyz2^{cre}$ generate sufficient itaconate to protect MyAcod1KO mice from metabolic inflammation.

There was also enrichment of inflammatory gene sets related to lymphocyte and B-cell activation. Although there are limited reports of Acod1 effects outside of activated macrophages, there is a growing body of literature implicating B-cells in the pathogenesis of obesity and aging.[152-155] Similar to obesity, aging is also associated with chronic inflammation with B-cell infiltration that drives insulin resistance and metabolic dysfunction. This would be consistent with our findings showing significant enrichment of B-cell related genes in $Acod1^{-/-}$ mice with both DIO and aging. Our data in 3T3-L1 cells also implicates a possible role for Acod1 in adipocytes as either a potential producer of itaconate or as an effector cell. Overall, our results suggest a novel role for Acod1 in a non-LysM expressing cells with evidence to implicate B cells and adipocytes as effectors or primary responders to the action of Acod1-itaconate.

$Acod1^{-/-}$ mice had alterations in energy substrate utilization with a decrease in fatty acid oxidation and increase in glucose oxidation. These observations suggest that loss of Acod1/itaconate relieves inhibition of glycolysis and promotes glucose oxidation at the expense of fat oxidation. Changes in substrate utilization were associated with increases in genes related to adipogenesis, lipid biosynthesis, and fatty acid synthesis. This suggests a tonic role for Acod1 limiting fatty acid deposition in adipose tissue under chow diet conditions as a potential mechanism for the age-induced obesity. Thus, in lean mice, tonic Acod1 expression and itaconate production protects against age-induced obesity by counteracting lipid deposition in adipocytes either by promoting lipolysis, suppressing lipogenesis, or promoting adipogenesis. With DIO or aging, the expression of Acod1 in B cells, LysM-negative DCs, and/or adipocytes limits the inflammatory response to obesity in adipose tissue and protects against insulin resistance.

LIMITATIONS OF THE STUDY AND FUTURE DIRECTIONS:

We have identified a new connection between immunometabolism and metabolic dysfunction, but it remains to be determined whether this pathway can be manipulated to protect against disease. Numerous studies have employed the use of cell permeable itaconate derivatives (DMI and 4-OI) to augment itaconate signaling and modulate and suppress inflammation during various models of disease. Future studies will be focused on determining if supplementation with exogenous itaconate derivatives and suppress inflammation and mitigate metabolic dysfunction. However, these derivatives are esterified to enhance cell permeability, and they do not fully recapitulate the endogenous effects of itaconate as they do not get metabolized to itaconate. Therefore, while beneficial and potentially useful as a therapeutic agent, it is still critical to understand the role of endogenous itaconate signaling using genetic tools to manipulate the metabolic system. It will also be important to delineate the itaconate-responsive cell types. Acod1-itaconate effects be a result of intracellular action or may reflect a more complex intercellular communication involving itaconate secretion and uptake.

SUMMARY

In summary, our data advance our understanding of the Acod1/itaconate pathway in immunometabolism and show that it protects against metabolic dysfunction in DIO and age. This is critical to our understanding of the pathogenesis of metabolic dysfunction that occurs with obesity and aging, because while many of the pro-inflammatory mechanisms are well defined, we know very little about anti-inflammatory pathways that can be targeted for therapeutic intervention. Attenuating low-grade, systemic inflammation during obesity through modulation of immunometabolism may have therapeutic potential to regulate glucose homeostasis and prevent metabolic dysfunction.

CHAPTER 5

Itaconate Suppresses Inflammation and Ameliorates Experimental Colitis

5.1 Abstract

Background

Inflammation is a hallmark of inflammatory bowel disease, and alterations in tricarboxylic acid cycle (TCA) metabolism have been identified as major regulators of immune cell phenotype during inflammation and hypoxia. The TCA cycle metabolite, itaconate, is produced by the enzyme aconitate decarboxylase 1 (Acod1) and is highly upregulated during classical macrophage activation and during experimental colitis. Itaconate and cell permeable derivatives have robust anti-inflammatory effects on macrophages, therefore we hypothesized that Acod1-produced itaconate has a protective, anti-inflammatory effect during experimental colitis.

Methods and Results

Wild type (WT) control and Acod1$^{-/-}$ mice were administered 3% Dextran Sulfate Sodium (DSS) in water for 7 days to induce experimental colitis. After DSS was discontinued, Acod1$^{-/-}$ mice had significantly reduced body weight recovery with increased macroscopic disease severity, and upon dissection had decreased colon length and more severe inflammation. To determine if myeloid cells are the critical Acod1/itaconate-producing cell type, we generated myeloid-specific Acod1 deficient mice, however no differences in weight loss, colon length or inflammatory gene expression were detected compared to WT controls. To test whether supplementation with exogenous itaconate could ameliorate colitis, WT mice were treated with the cell-permeable form of itaconate, dimethyl itaconate (DMI). Administration of DMI significantly improved recovery after 7 days of DSS treatment and significantly reduced inflammatory gene expression in the colon.

Conclusion

Our data suggest that Acod1-produced itaconate has an important role in the regulation of inflammation during experimental colitis. Although myeloid cells have been thought to be major producers of Acod1 and itaconate, our data indicate that other cell types are involved. These results highlight the importance of this immunometabolic pathway and suggest that preservation or enhancement of this pathway with natural metabolites or metabolite derivatives could have beneficial effects during colitis.

5.2 Introduction

Ulcerative colitis is a chronic inflammatory disease with unknown etiology that affects more than 900,000 people in the US each year. Inflammation is a critical feature in the pathophysiology of ulcerative colitis and has been linked to localized oxygen depletion, or hypoxia [156]. In the intestine, there exists a complex oxygen gradient that is necessary in maintaining normal intestinal homeostasis. During colitis and related IBDs, there is a dysregulation in cellular oxygen dynamics, which contributes to the pathophysiology. Importantly, immune cells have been linked to these pathophysiological changes. Immune cells can increase oxygen consumption and decrease oxygen supply through various mechanisms to induce localized hypoxia during colitis. It has recently been shown that transmigrating neutrophils rapidly consume oxygen during respiratory burst and thereby deplete microenvironmental oxygen and induce hypoxia in colonic epithelia[157]. Moreover, localized inflammation can induce vasculitis, which also decreases oxygen availability in affected tissues[158]. Therapeutic strategies targeted at blocking inflammation and inflammatory signaling during IBD have shown moderate success but are limited in efficacy and do not provide therapeutic benefit in all patients. There is an important need for the development of novel therapies to treat ulcerative colitis and other IBDs[159, 160].

Recently, metabolism and specific metabolic intermediates have been shown to control aspects of inflammation and responses to hypoxia. The TCA cycle and its intermediate compounds have been shown to be key in macrophage polarization and play an important part in the production of inflammatory cytokines in macrophages stimulated by LPS. The enzyme aconitate decarboxylase (Acod1) has recently been found to produce itaconate from the TCA cycle intermediate aconitate. Acod1 originally identified as immune responsive gene 1 (Irg1) because it is highly induced in

macrophages activated with LPS[60]. Itaconate is a major product of LPS activated macrophages and exerts anti-inflammatory action and inhibits ROS production through several potential mechanisms, including SDH inhibition, ATF3 induction, and Nrf2 accumulation[48, 81, 82].

In the present study, we tested whether Acod1-mediated production of itaconate is important in the pathophysiology of colitis. We hypothesized that mice lacking Acod1 and itaconate production would have increased disease severity following induction of experimental colitis. Here we subjected Acod1$^{-/-}$ mice to dextran sulfate sodium (DSS)-induced colitis and evaluated colitis disease severity and inflammation.

5.3 Methods

Animals

Male and female Acod1$^{-/-}$ mice (Acod1tm1a(KOMP)Wtsi) and littermate controls were on a C57BL/6N background, and myeloid Acod1 knockout mice (MyAcod1KO: Acod1fl/fl-LysM-Cre) and littermate floxed controls were on a C57BL/6J background as described previously (6, 26). Mice were multi-housed in static cages in a temperature controlled SPF room (21-23°C) with a light:dark cycle of 12:12 h (lights on at 6 A.M.). Mice were maintained on standard laboratory chow (5L0D, LabDiet) and water ad libitum. Estimation of required sample size for all experiments were based on A priori power analysis calculations using expected standard deviations based on previous experiments and published literature. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Food Consumption

Food intake was repeatedly measured in both standard static cages and metabolic cages over 2-3 day periods.

LPS-induced systemic inflammation

Mice were injected intraperitoneally with LPS (5 mg/kg, Escherichia coli serotype O127:B8, Sigma-Aldrich, L3129) dissolved in PBS. Mice were euthanized after 6 h and tissues were harvested for gene expression analysis.

Induction and Assessment of Colitis

Mice were administered 3% (wt/vol) DSS (MW ca, 40,000; Alpha Aesar) in the drinking water for 7 days. Daily changes in body weight were assessed daily. For macroscopic and microscopic assessment of colitis, all evaluators were blinded to the experimental groups. Macroscopic disease severity was scored as follows: 0, normal stool consistency and negative for presence of blood; 1, soft stool and positive for presence of blood; 2, very soft stool with traces of blood; 3, watery stool with visible rectal bleeding. For microscopic assessment of colonic damage, colons were excised and cut longitudinally, rinsed with PBS, and fixed in 10% buffered formalin. The colons were Swiss-rolled to examine entire length of the colonand processed in paraffin. Colitis was scored on routine hematoxylin and eosin-stained section, according to previously described morpho-logical criteria (13). A total injury score was assigned based on histological assessment of 4 criteria: 1) immune cell infiltration 2) depth of injury 3) crypt damage 4) percentage of tissue involved as previously described.

Gene expression analysis

Relative mRNA expression was determined using quantitative reverse transcription–polymerase chain reaction. Total RNA was extracted from tissues using TRIzol reagent and purified using an RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. RNA (1 ug) was reverse transcribed to cDNA with an Applied Biosystems kit and quantitative reverse transcription–polymerase chain reaction was performed using a 7900HT fast real-time PCR system (Applied Biosystems). The relative mRNA expression was quantified by the comparative method and normalized to the housekeeping gene L32.

Statistical analysis

A Shapiro-Wilk normality test was used to determine if data were normally distributed. For normally distributed data with equal variance, values are presented as mean ± SEM, and statistical comparison of mean values between multiple groups was performed by Student's t test, one-way ANOVA with a Tukey's post-test, or two-way ANOVA with a Tukey's post-test as indicated in the text. For normally distributed data with unequal variance, a Welch's t test was used. Data that were not normally distributed were analyzed with the nonparametric Mann-Whitney test. All statistical analysis of data was performed in GraphPad Prism (version 7; GraphPad Software, Inc). $P < 0.05$ was considered significant.

5.4 Results

Acod1 deficiency exacerbates severity of DSS-induced colitis in male and female mice

To understand the role of Acod1 and itaconate in colitis, we administered 3% DSS to WT and Acod1$^{-/-}$ mice for seven days. Following seven days of DSS treatment, DSS water was replaced with regular drinking water until the conclusion of the study. Body weight was measured each day to calculate body weight change. At conclusion of the study, colon length was measured, and colon tissue samples were collected for further analysis. Acod1$^{-/-}$ mice showed increased weight loss compared to WT. WT male and female mice showed a decrease in body weight caused by DSS treatment, followed with weight recover after end of DSS treatment. This was not observed in male or female Acod1$^{-/-}$ mice, where they continued to show decreases in body weight and showed no sign of recovery after the end of DSS treatment (Figure 5.1A and 5.2A). At termination of the study mice were euthanized, and colon length was measured. Both male and female Acod1$^{-/-}$ mice

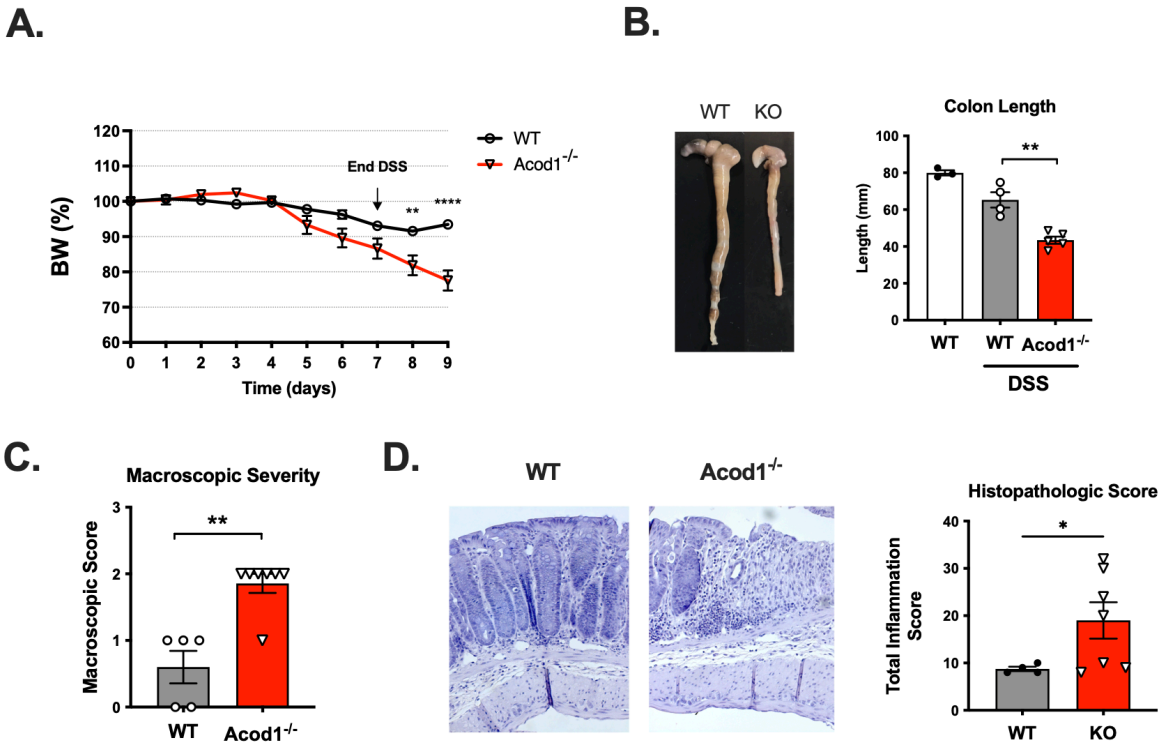

Figure 5.1. Acod1$^{-/-}$ **deficiency exacerbates DSS-induced colitis in male mice.** WT and Acod1$^{-/-}$ male mice received 3% DSS in water for seven days followed with regular drinking water for two days. (A) % BW analysis measured daily. (B) Colon length measured at day nine. (C) Disease severity day nine. (D) Representative images and histopathologic scoring of colon in WT and Acod1$^{-/-}$. N=5 for WT and N=6 for Acod1$^{-/-}$.

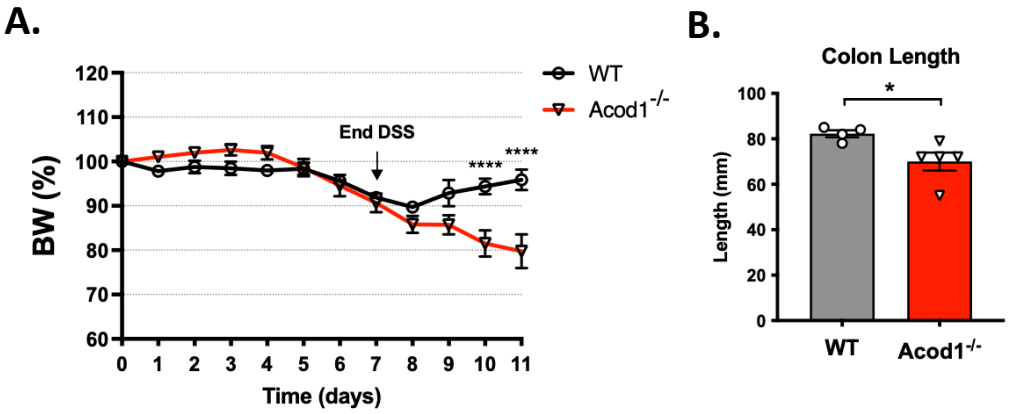

Figure 5.2. Acod1$^{-/-}$ **deficiency exacerbates DSS-induced colitis in female mice.** WT and Acod1$^{-/-}$ female mice received 3% DSS in water for seven days followed with regular drinking water for an additional four days. (A) % Body weight analysis measured daily. (B) Colon length measured at day nine. N=4 for WT and N=5 for Acod1$^{-/-}$.

had decreased colon length compared to WT counterparts, indicating increased colitis severity (Figure 5.1B and 5.2B). Disease severity was also measured using histopathological scoring in male mice (Figure 5.1D). To determine if Acod1 was acting through modulation of proinflammatory signaling we analyzed gene expression from descending colon tissue samples in WT and Acod1$^{-/-}$. Analysis revealed that male and female Acod1$^{-/-}$ mice showed elevated proinflammatory gene expression compared to WT control tissues (Figure 5.3A and 5.3B).

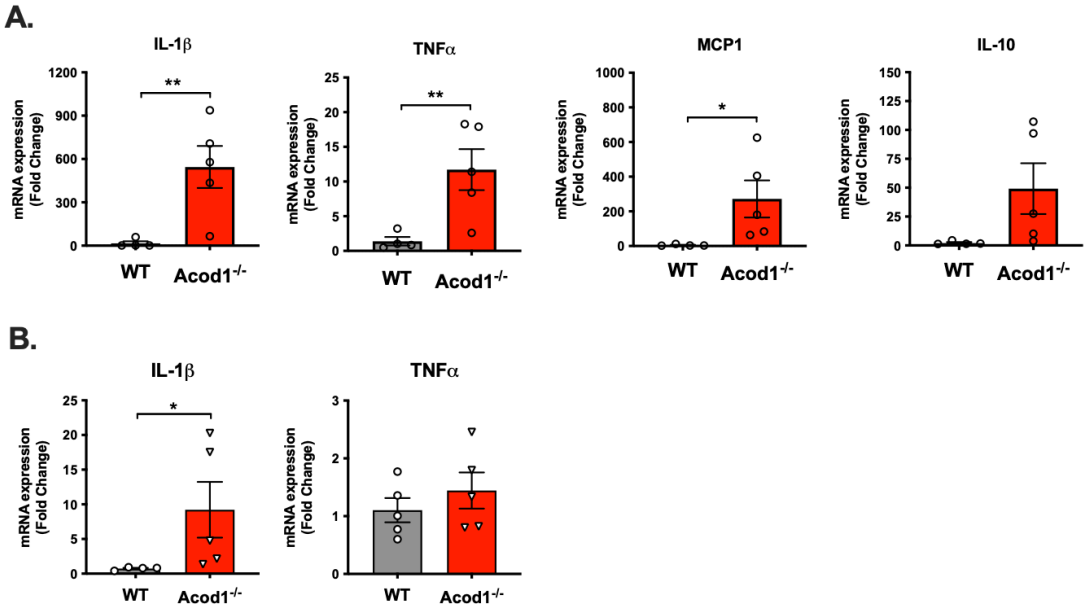

Figure 5.3. Acod1$^{-/-}$ **deficiency exacerbates DSS-induced proinflammatory gene expression.** Gene expression analysis of (A) male and (B) female descending colon tissue from WT and Acod1$^{-/-}$ mice. N=4 for WT and N=5 for Acod1$^{-/-}$ for males, and N=4 for WT and N=5 for Acod1$^{-/-}$ for females.

Itaconate derivative DMI decreases DSS-induced colitis severity and proinflammatory gene expression

After finding that deletion of itaconate lead to increased colitis severity, we sought to determine if treatment with exogenous itaconate derivatives would be protective in DSS-induced colitis. WT mice were given either a regular chow diet or chow diet supplemented with 1g/Kg dimethyl itaconate (chow + DMI) starting seven days prior to 3% DSS treatment and continued throughout DSS treatment. Body weight analysis showed WT mice treated with DMI had significantly less body weight loss compared to WT mice fed chow diet alone (Figure 5.4A). To ensure food consumption was consistent across chow and chow + DMI groups we measured chow weight prior to the start of DSS treatment and found no differences (Figure 5.4B). Analysis of colon length found that chow + DMI treated mice also showed increased colon length compared to chow diet fed mice (Figure 5.4C). Chow + DMI fed mice had significantly decreased proinflammatory gene expression. Furthermore, DMI treated mice also had increased expression of antioxidant Nrf2 target gene Nqo1, as well as decreased expression levels of Hif1a and Hmox1 (Figure 5.4D).

Myeloid specific knockout of Acod1 does not modulate DSS-induced colitis severity

After identifying that Acod1$^{-/-}$ had increased DSS-induced colitis severity, we sought to determine what cell type/types were responsible for this phenotype. Immune cells are known to play an important role in the pathology of colitis, and macrophages specifically are believed to play a key role as gatekeepers of intestinal immune homeostasis and promoting inflammation resolution. Given that macrophages are known to express high levels Acod1 and produce itaconate, we sought to determine if macrophages/myeloid cells were the primary contributors to the observed phenotype in global Acod1$^{-/-}$. To achieve this, myeloid cell specific knockout of Acod1 was generated using LysM-Cre (MyAcod1KO). Flox control (FC) and MyAcod1KO mice were subjected to 3% DSS treatment for seven days, followed with five additional days of treatment with drinking water. MyAcod1KO mice showed no difference in body weight change over the course of DSS treatment compared to FC mice (Figure 5.5A). Similarly, MyAcod1KO mice also showed no differences in colon length compared to FC mice at conclusion of treatment on day twelve (Figure 5.5B).

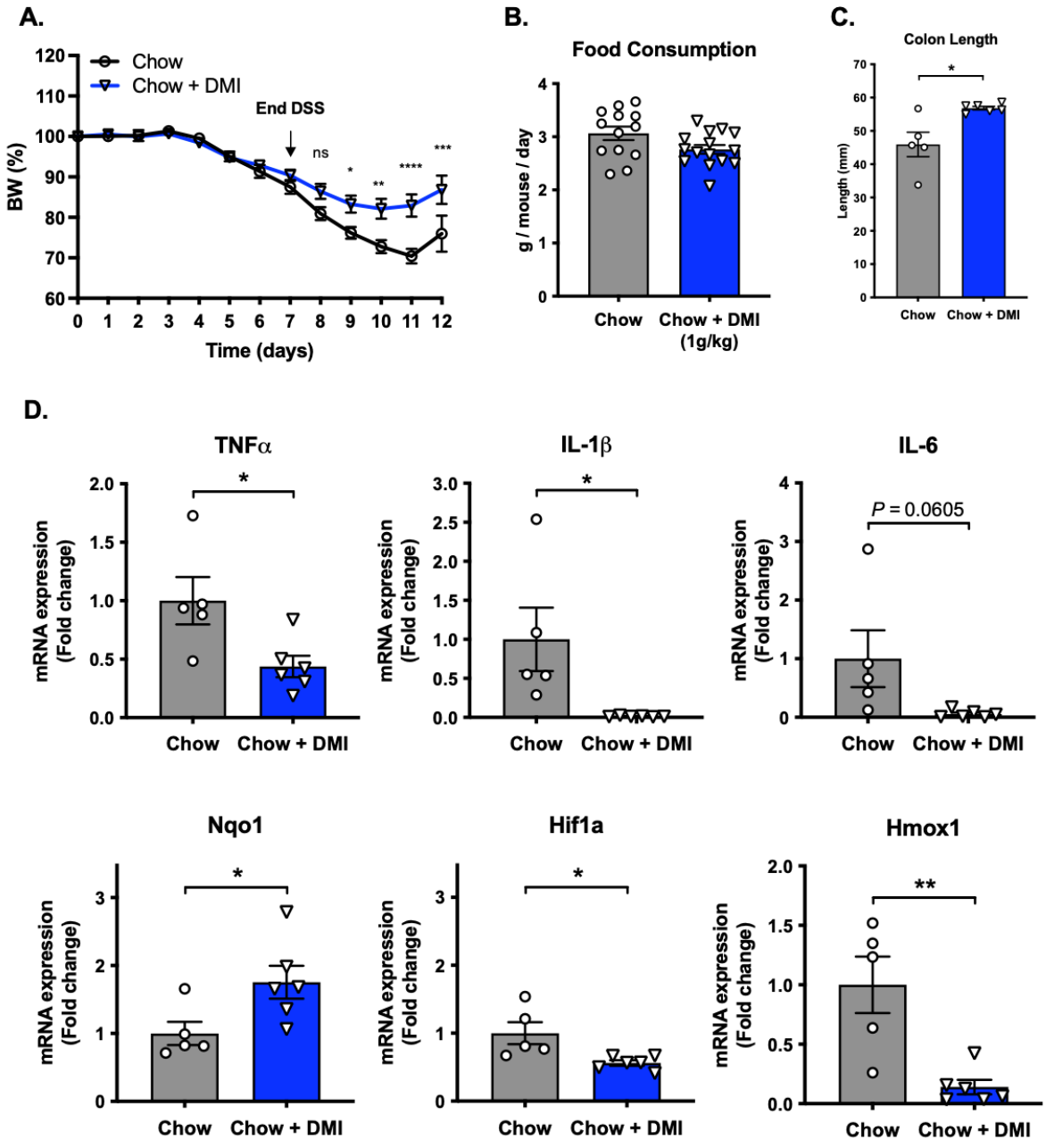

Figure 5.4. DMI treatment in chow is protective in DSS-induced colitis. Male WT mice received regular chow or chow+DMI (1g/Kg) along with 3% DSS for seven days followed with 5 days of drinking water. (A) % body weight analysis measured daily. (B) Food consumption measurement of chow and chow+DMI. (C) Colon length measured at day twelve. (D) Gene expression analysis of descending colon tissue from day twelve. N=5 for chow diet and N=6 for chow+DMI diet.

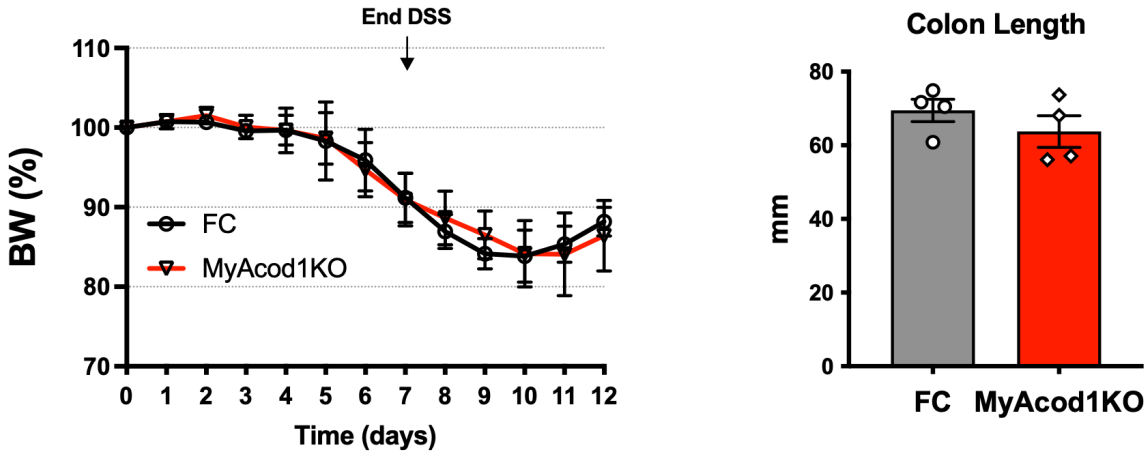

Figure 5.5. Myeloid cell specific knockout of Acod1 does not affect DSS-induced colitis severity. Male WT and MyAcod1KO mice received 3% DSS in drinking water for seven days followed with five days of drinking water. (A) % body weight changes measured daily. (B) Colon length at day twelve. N=4 for control and N=4 for MyAcod1KO.

Bone marrow transplant of Acod1$^{-/-}$ cells does not alter DSS-induced colitis severity

To further analyze the role of myeloid and circulating hematopoietic immune cells in general, we performed bone marrow transplants in WT mice. WT and Acod1$^{-/-}$ bone marrow was isolated from femur and tibia bones. After bone marrow collection, irradiated WT mice received bone marrow transplant via tail vein injection of either WT (WT → WT) or Acod1$^{-/-}$ bone marrow (Acod1$^{-/-}$ → WT). Mice then received 3% DSS treatment as before followed with 5 additional days of drinking water. To verify sufficient engraftment of bone marrow transplant, we performed genotype analysis of Acod1 on bone marrow isolated from femurs of recipient mice at conclusion of the study. Genotype analysis revealed that appropriate engraftment of bone marrow was received in both WT and Acod1$^{-/-}$ recipient groups (Figure 5.6A). Body weight analysis of WT → WT and Acod1$^{-/-}$ → WT mice showed significant increases in weight loss in Acod1$^{-/-}$ recipient mice (Figure 5.6B). However, colon length analysis showed no significant differences compared to WT recipient mice (Figure 5.6C). Similarly, proinflammatory gene expression analysis found no differences between groups (Figure 5.6D). To understand if circulating immune cells were potentially responsible for the observed differences in WT → WT and Acod1$^{-/-}$ → WT mice, we

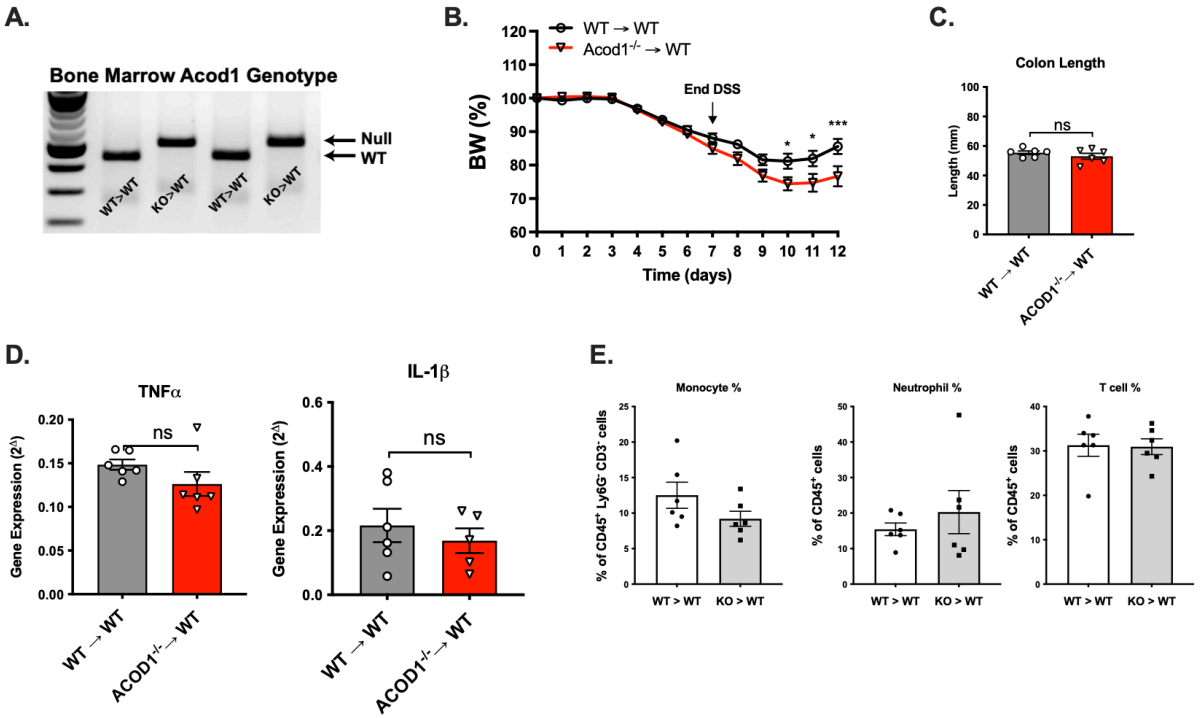

Figure 5.6. Transplant of $Acod1^{-/-}$ bone marrow increases BW severity, but not inflammatory markers. WT mice were subjected to irradiation followed with bone marrow transplant via tail veil injection of either WT or $Acod1^{-/-}$ bone marrow. (A) Acod1 genotype analysis of bone marrow from recipient mice at end of study. (B) % body weight changes measured daily. (C) Colon length measured at day twelve. (D) Gene expression analysis of descending colon tissue from day twelve. (E) Flow cytometry analysis of circulating immune cells. N=6 for WT → WT and $Acod1^{-/-}$ → WT.

performed flow cytometry analysis to quantify monocytes, neutrophils, and T cells. Consistent with the finding in gene expression, there were no observable differences in these immune cell types (Figure 5.6E). These findings indicate that macrophages and circulating myeloid cells are not the primary cell type responsible for the observed global $Acod1^{-/-}$ phenotype.

5.5 Discussion

Immune cell metabolism has been identified as a major driver of inflammation and disease. Acod1 and itaconate are highly upregulated in response to proinflammatory stimuli and have been implicated as targets of ischemic and inflammatory disease. In the present study, we sought to identify the potential role of Acod1 and itaconate in ulcerative colitis. We found that global $Acod1^{-/-}$ mice showed significantly worse signs of disease severity by having significant increases in body weight loss, decreased colon length, and increased pro-inflammatory gene expression

compared to WT mice. This was true in both male and female mice, where Acod1$^{-/-}$ weight loss was sustained even after 3% DSS treatment was ended. This was in stark contrast with the recovery of lost BW in WT mice once DSS was replaced with drinking water. This would strongly indicate the Acod1 and itaconate are playing a significant role in colitis disease progression. Importantly, there were no observed differences in BW% in either group during 3% DSS treatment. Significant differences in BW% only appeared once DSS treatment was replaced with regular drinking water. This could indicate sustained inflammation and increased disease severity even after DSS had been removed.

To better understand the role of itaconate itself, we then used exogenous treatment in chow with the cell permeable derivative DMI. We found that mice readily ate chow supplemented with 1g/kg DMI, as there were no differences in food consumption prior to DSS treatment. We found that DMI treatment showed significant protection by significantly decreasing body weight % change, increasing colon length, and decreasing proinflammatory gene expression. DMI has been identified as a potent inhibitor of proinflammatory gene expression. Furthermore, DMI treatment also increased Nqo1 gene expression levels. Nqo1 is a major part of the antioxidant response and is one the main targets of Nrf2. One of the main proposed mechanisms of DMI is through Nrf2, were DMI has been found to modulate KEAP1 cysteine residues which then allow Nrf2 translocate to the nucleus to act as a transcription factor. Previous studies have implicated Nrf2 as playing a significant role in protection intestinal integrity by modulation of proinflammatory cytokines [161]. Separate studies focused on the role of Nrf2 in colitis-associated colorectal cancer have further reinforced that Nrf2 appears to play a protective role in inflammation-associated intestinal disease[162]. Our current findings would imply that DMI is potentially acting through regulation of intestinal Nrf2 with potential therapeutic implications.

Given that most studies regarding Acod1 and itaconate have focused primarily on its effects in macrophages, we sought to identify if circulating macrophages and myeloid cells were responsible for the observed global Acod1$^{-/-}$ we used cell specific LysM-Cre and bone marrow transplant. In these studies, we did not see the same effect of cell specific Acod1$^{-/-}$ in the MyAcod1$^{-/-}$ or in the bone marrow transplant that was observed in global Acod1$^{-/-}$. In fact, except for significantly decreased BW% in the Acod1$^{-/-}$ bone marrow recipient mice, no significant differences were observed. Furthermore, the decrease seen in the Acod1$^{-/-}$ bone marrow transplant, although significant, did not show the sustained lack of BW% recovery that was seen in the global

Acod1$^{-/-}$. This finding could indicate that a subset of transplant cells may play a role in the Acod1$^{-/-}$ phenotype, but likely that they are not fully responsible for them. To further support that circulating myeloid cells were not the primary drivers, we also observed no changes in colon length compared to control, and not increases in proinflammatory gene expression. Taken together, these two experiments would indicate that another, potentially non-immune cell type, may be responsible for the global Acod1$^{-/-}$ phenotype.

In summary, our data advance our understanding of the Acod1 and itaconate and show that it protects against DSS-induced ulcerative colitis. Furthermore, the derivative form DMI is shown to be protective, potentially by modulation of Nrf2-induced inhibition of proinflammatory signaling. These findings highlight the potential therapeutic properties of DMI in this disease. Lasty, our data strongly suggests that Acod1 and itaconate are not acting through circulating myeloid cells. This finding highlights the need for further studies in other non-immune cell types to identify potential Acod1 expression under stress and inflammatory conditions.

CHAPTER 6

Discussion

Overview

The work presented in this dissertation identifies Acod1 and itaconate as important players in multiple models of diseases. Inflammation and the immune system are known to play a significant role in several diseases, and chronic inflammation is believed to exacerbate disease progression. Our lab has previously identified that modulation of macrophage polarization is a major driver of cardiac damage and ischemic stroke[97, 98, 163, 164]. Acod1 has recently been identified as a highly upregulated gene in proinflammatory stimulated macrophages, and itaconate has been identified as its primary product. Itaconate is an immunometabolite that is now understood to be a potent immunomodulator capable of limiting inflammatory gene expression and ROS through several potential mechanisms[48, 81, 82, 165-167].

Based on the previous findings, we hypothesized that Acod1 and itaconate in myeloid/macrophage cells would play a protective role in these diseases through modulation the inflammatory response. To test this hypothesis, we used global Acod1$^{-/-}$ and cell specific knockout of Acod1 in several murine models: transient ischemic stroke, diet-induced obesity (DIO), and DSS-induced colitis. This work demonstrates that Acod1 plays a novel role in disease regulation.

In this present work, we demonstrate several important findings across these multiple disease types: 1) Acod1 is an important regulator of disease as observed by global Acod1$^{-/-}$ mice showing increased severity compared to WT mice, 2) Myeloid/macrophage cells do not appear to be the major cell type involved in any of the three disease models, 3) The mechanism of Acod1 may depend on the cell types involved depending on the disease model, where we did not see alterations in inflammatory gene expression in ischemic stroke, but did in diet-induced obesity and DSS-induced colitis, 4) Acod1 alters ferroptosis sensitivity, and could play a major role in ferroptosis induction in ischemic disease models, 5) Exogenous itaconate is capable of modulating inflammatory gene expression and alter severity in ferroptosis.

In support of our stated hypothesis, we identified that Acod1$^{-/-}$ was more severe in all three murine models studied by increasing stroke lesion volume following tMCA occlusion, altering glucose homeostasis and obesity in HFD, and increasing DSS-induced colitis severity. To address if myeloid/macrophage cells were the primary cell type responsible, we used cell specific knockout of Acod1 in LysM-Cre expressing cells. Across all three animal models we did not see a replication of the global Acod1$^{-/-}$ phenotype, indicating that circulating macrophages are likely not the cell type responsible. Further analysis using a knockout of resident microglia cells in the tMCA occlusion model further highlighted that the effect was not through resident immune cells in this specific model. These finding would strongly indicate that another, potentially non-immune, cell type is responsible for the observed phenotype.

Our initial hypothesis stated that the effects of Acod1 and itaconate would be through modulation of the inflammatory response. In this regard, we saw a disease specific regulation of inflammatory gene expression. In tMCA occlusion, there were no observable changes in proinflammatory gene expression in the ipsilateral hemisphere post-stroke, indicating that in the model of tMCA ischemia/reperfusion, Acod1 is likely working through a separate mechanism. In DIO and DSS-induced colitis, we did find significant changes in inflammatory gene expression. Specifically in DIO, Acod1$^{-/-}$ mice showed elevated inflammatory gene expression and a reduction in genes regulating adipogenesis and fatty acid metabolism. In DSS-induced colitis, Acod1$^{-/-}$ mice showed significant elevations in proinflammatory gene expression in both male and female mice. These findings together would highlight that Acod1 and itaconate can regulate disease severity through inflammatory modulation depending on the disease model, and potentially dependent on the cell types involved. However, regulation of inflammatory gene expression is likely not the major mechanism for all disease models. Acod1 and itaconate are known to be regulators of oxidative stress, this could be a separate mechanism of action in our model of tMCA occlusion stroke and other diseases that feature high levels of oxidative stress driving disease progression.

Ferroptosis has recently been identified as a major driver of lipid-peroxidation and cell death/damage in ischemic disease[126, 168]. Itaconate is known to modulate oxidative stress and ROS through several potential pathways[48, 81, 82]. In the present study we sought to identify if Acod1 and itaconate modulated sensitivity to ferroptosis induction. Using macrophages as a model cell type that expresses high levels of Acod1 and itaconate following LPS stimulation, we used RSL3 to induce ferroptosis in vitro. Cell death measurements were performed by measuring lactate

dehydrogenase (LDH) released by dead/dying cells in culture supernatant. Surprisingly, we found that LPS induction was highly protective in both WT and Acod1$^{-/-}$ bone marrow derived macrophages (BMDMs), although protection in Acod1$^{-/-}$ cells significantly decreased at the later timepoints of 48 and 72hrs of RSL3 treatment. This observation would suggest that Acod1 and itaconate promotes a protective effect in WT cells at later time-points of RSL3-induced ferroptosis, and that this sustained protection effect is lacking in Acod1$^{-/-}$ BDMDs that do not produce endogenous itaconate. Further analysis revealed that Acod1$^{-/-}$ BMDMs also had decreased levels of intracellular glutathione (GSH) compared to WT controls following LPS stimulation. GSH is a key antioxidant compound and is a significant part of the System xc^{-}/GSH/GPX4 axis. The System xc^{-}/GSH/GPX4 axis is a key player in limiting lipid-peroxidation and oxidative damage caused by ferroptosis[169, 170]. This finding would suggest that Acod1 and itaconate are potentially acting through modulation of GSH levels to aid cells in a sustained protective response from ferroptosis. To further support this finding, we sought to identify if treatment with exogenous itaconate would restore protection in Acod1$^{-/-}$ BMDMs. We found that supplementing Acod1$^{-/-}$ BMDMs with exogenous itaconate was sufficient to restore protection to WT levels. Along with this restored protection, we also observed restoration of GSH levels in Acod1$^{-/-}$ BMDMs back to levels observed in WT cells. Taken together, these findings suggest that Acod1 and itaconate are capable of modulating ferroptosis induction at late stages through sustained GSH levels in BMDMs. Ferroptosis has recently been identified as a major driver of damage in cerebral ischemia/reperfusion, and inhibition of ferroptosis has been found to be highly protective[171]. This mechanism is a potential pathway in our model of tMCA occlusion stroke that requires further study, where mice lacking Acod1$^{-/-}$ and subsequent itaconate production could have increased cell death through ferroptosis induction.

Future studies to identify Acod1 expressing cell types and mechanisms of Acod1

In the current studies we have identified that Acod1 and itaconate are important regulators of disease. However, our initial hypothesis of Acod1 acting through myeloid/macrophage cells was not supported in our cell specific knockout studies. Publications focused on Acod1 and itaconate have heavily focused on macrophages due to the fact that they express Acod1 at such high levels and are known to create mM levels of itaconate intracellularly[48, 81, 82]. Because of this most of the literature has largely ignored Acod1 in other cell types, especially in non-immune cell types.

Our work strongly suggests that Acod1 in other cell types may be responsible for the observed phenotypes in our studies. Much of the current literature surrounding Acod1 has yet to specifically identify the cell types responsible and focuses heavily on in vitro macrophage studies to determine potential pathways. As relevant as Acod1 and itaconate may be in macrophage polarization and inflammation in general, there remain serious questions if these same pathways are the primary drivers in other cell types that have yet to be identified. Future studies are needing to determine disease specific cellular expression of Acod1. Far fewer studies have been performed on non-immune resident tissue cell types[84, 165]. Although macrophages express high levels of Acod1, it remains a possibility that other cell types may express Acod1 at far lower levels in a stressed or stimulated state. This could lead to challenges in identifying potential expressors of Acod1. There remain alternative approaches for identification of Acod1 for future studies, specifically RNAscope in situ hybridization. This technique would allow for Acod1 gene expression to be identified, and potentially could identify cells expressing lower levels. Doing this would allow for a more focused approach to identifying future cell types for cell specific knockout models, or potentially show that a variety of cell types express Acod1 in a given tissue. Acod1 expression has been identified in a murine viral infection model, indicating that Acod1 expression is possible in resident brain cells[84]. Furthermore, cultured primary astrocytes and neuron have shown enhanced protection against oxidative stress through antioxidant effects of itaconate treatment following reperfusion injury[165]. This would highlight the potential of itaconates protective effects in non-immune resident cells in cerebral disease.

In our current studies we sought to identify the primary mechanism of Acod1 and itaconate in several disease models and hypothesized that Acod1 would be acting through modulation of proinflammatory signaling. As previously mentioned, there appears to be a varied outcome and a disease specific response to global Acod1$^{-/-}$, where we did see significant increases in inflammatory markers in the models of DIO and DSS-induced colitis but not in the model of tMCA occlusion. It remains possible that Acod1 and itaconate are acting through different mechanisms in these disease models. Acod1 and itaconate have been implicated in regulation of oxidative stress and inflammation through several different pathways. This opens the possibility of Acod1 acting through multiple pathways to promote its protective effects. Separately, we identified Acod1 and itaconate as inhibitors of ferroptosis-induced cell death. Ferroptosis is a novel programmed cell death that was only just described within the last decade and has been found to be a major driver

of cerebral and cardiac ischemic/reperfusion (I/R) damage [171-176]. Inhibition with ferroptosis specific inhibitors has been found to be highly protective in a variety of I/R models and has recently been viewed as a potential therapeutic target for future studies. In our current studies, we identified Acod1 and endogenous itaconate as promotors of ferroptosis protection through sustained intracellular GSH levels. Furthermore, treatment with exogenous itaconate was found to restore protection in Acod1$^{-/-}$ cells and have protective effects in WT cells. This finding strongly suggests regulation of ferroptosis as a novel pathway of action by Acod1 and itaconate.

Previous publications have relied heavily on identification of Acod1/itaconate mechanisms using cell permeable derivative forms of itaconate, including dimethyl itaconate and 4-octyl itaconate. Initially, these derivative forms were created with the hope of increased cellular uptake. Although these derivative forms have been reported to have protective effects in several disease models, a recent study has called into question the similarities and differences that derivative forms may have from endogenous itaconate[87]. Although the protective effects of these derivatives are consistently being published and hold promising therapeutic potential, it remains a strong possibility that their mechanisms do not align with the endogenously produced form of itaconate. In our current study, we identify mM doses of itaconate as safe and effective for in vitro studies. Separate groups have reported similar findings and found protective effects in in vivo murine models as well with exogenous itaconate treatment[87, 165]. These findings provide a strong case for future studies to rely on exogenous itaconate, not derivative forms, to identify potential mechanisms in animal models.

Therapeutic potential of itaconate in disease modulation

The recent appreciation that immune cell metabolism is a major driver of inflammation has sparked great interest in the past decade and has led to a greater understanding of the connection between the immune response and cellular metabolism. This newly studied field has the potential to identify novel therapeutic compounds and novel pathways in disease. Itaconate specifically represents an immunometabolite with potent protective effects in multiple disease models ranging from ischemia/reperfusion injury to antiviral effects in SARS-CoV2[48, 85]. Furthermore, itaconate has been found to influence several pathways to enact its protective immunomodulating effects. This ability underscores the therapeutic potential of itaconate in a variety of disease. Other metabolites have already been approved by the FDA for treatment of diseases. Specifically

dimethyl fumarate (DMF), another TCA cycle metabolite, is currently approved for treatment of active relapsing multiple sclerosis[86, 177].

Ischemia/reperfusion injury is an area of great interest for research into future therapeutics[178, 179]. Itaconate and its derivatives represents promising compounds in this area of research. Future studies will need to be conducted to better differentiate the mechanisms governing endogenous itaconate and its derivate forms, but it remains clear that they have potent effects in a variety of disease models in animal studies.

Conclusions

In this dissertation, we have identified an important role of Acod1 and itaconate in several disease models. These findings further advance the understanding of the role of Acod1 and the immunometabolite itaconate. Here, we also find that myeloid cell specific Acod1 is not responsible for ameliorating disease severity during ischemic stroke, diet-induced obesity, and ulcerative colitis. This finding calls into question the cell types that are producing itaconate and suggest that Acod1 expression in non-immune cell types is critical in these disease models. This indicates further studies are needed with a specific focus on identification of other potential cell types expressing Acod1.

REFERENCES

1. Marshall, J.S., et al., *An introduction to immunology and immunopathology.* Allergy Asthma Clin Immunol, 2018. **14**(Suppl 2): p. 49.
2. Turvey, S.E. and D.H. Broide, *Innate immunity.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S24-32.
3. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
4. Kuby, J., *Immunology*. 3rd ed. 1997, New York: W.H. Freeman. xxiv, 664 p.
5. Alberts, B., *Molecular biology of the cell*. 4th ed. 2002, New York: Garland Science. xxxiv, 1548 p.
6. Gasteiger, G., et al., *Cellular Innate Immunity: An Old Game with New Players.* J Innate Immun, 2017. **9**(2): p. 111-125.
7. Okabe, Y. and R. Medzhitov, *Tissue biology perspective on macrophages.* Nat Immunol, 2016. **17**(1): p. 9-17.
8. Elkhal, A., H. Rodriguez Cetina Biefer, and M.A. de la Fuente, *Impact of Metabolism on Immune Responses.* J Immunol Res, 2018. **2018**: p. 5069316.
9. Wu, R., et al., *ACOD1 in immunometabolism and disease.* Cell Mol Immunol, 2020. **17**(8): p. 822-833.
10. Kim, E. and S. Cho, *Microglia and Monocyte-Derived Macrophages in Stroke.* Neurotherapeutics, 2016. **13**(4): p. 702-718.
11. Swirski, F.K., et al., *Identification of splenic reservoir monocytes and their deployment to inflammatory sites.* Science, 2009. **325**(5940): p. 612-6.
12. Bao, Y., et al., *A role for spleen monocytes in post-ischemic brain inflammation and injury.* J Neuroinflammation, 2010. **7**: p. 92.
13. Sahota, P., et al., *Changes in spleen size in patients with acute ischemic stroke: a pilot observational study.* Int J Stroke, 2013. **8**(2): p. 60-7.
14. Seyfried, A.N., J.M. Maloney, and K.C. MacNamara, *Macrophages Orchestrate Hematopoietic Programs and Regulate HSC Function During Inflammatory Stress.* Front Immunol, 2020. **11**: p. 1499.
15. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm.* J Immunol, 2000. **164**(12): p. 6166-73.
16. Nathan, C.F., et al., *Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity.* J Exp Med, 1983. **158**(3): p. 670-89.
17. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.* J Exp Med, 1992. **176**(1): p. 287-92.
18. Orecchioni, M., et al., *Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages.* Front Immunol, 2019. **10**: p. 1084.

19. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
20. Kelly, B. and L.A. O'Neill, *Metabolic reprogramming in macrophages and dendritic cells in innate immunity.* Cell Res, 2015. **25**(7): p. 771-84.
21. Atri, C., F.Z. Guerfali, and D. Laouini, *Role of Human Macrophage Polarization in Inflammation during Infectious Diseases.* Int J Mol Sci, 2018. **19**(6).
22. Zhao, K., et al., *Induction of inducible nitric oxide synthase increases the production of reactive oxygen species in RAW264.7 macrophages.* Biosci Rep, 2010. **30**(4): p. 233-41.
23. Panday, A., et al., *NADPH oxidases: an overview from structure to innate immunity-associated pathologies.* Cell Mol Immunol, 2015. **12**(1): p. 5-23.
24. Canton, M., et al., *Reactive Oxygen Species in Macrophages: Sources and Targets.* Front Immunol, 2021. **12**: p. 734229.
25. Yap, J., et al., *Role of Macrophages in Cardioprotection.* Int J Mol Sci, 2019. **20**(10).
26. Chernykh, E.R., et al., *Safety and Therapeutic Potential of M2 Macrophages in Stroke Treatment.* Cell Transplant, 2016. **25**(8): p. 1461-71.
27. Liu, X., et al., *Interleukin-4 Is Essential for Microglia/Macrophage M2 Polarization and Long-Term Recovery After Cerebral Ischemia.* Stroke, 2016. **47**(2): p. 498-504.
28. Yunna, C., et al., *Macrophage M1/M2 polarization.* Eur J Pharmacol, 2020. **877**: p. 173090.
29. Medzhitov, R. and C. Janeway, Jr., *Innate immune recognition: mechanisms and pathways.* Immunol Rev, 2000. **173**: p. 89-97.
30. Park, B.S. and J.O. Lee, *Recognition of lipopolysaccharide pattern by TLR4 complexes.* Exp Mol Med, 2013. **45**: p. e66.
31. Fujihara, M., et al., *Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex.* Pharmacol Ther, 2003. **100**(2): p. 171-94.
32. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways.* Front Immunol, 2014. **5**: p. 461.
33. Sakai, J., et al., *Lipopolysaccharide-induced NF-kappaB nuclear translocation is primarily dependent on MyD88, but TNFalpha expression requires TRIF and MyD88.* Sci Rep, 2017. **7**(1): p. 1428.
34. Krawczyk, C.M., et al., *Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation.* Blood, 2010. **115**(23): p. 4742-9.
35. Gais, P., et al., *TRIF signaling stimulates translation of TNF-alpha mRNA via prolonged activation of MK2.* J Immunol, 2010. **184**(10): p. 5842-8.
36. Warburg, O., *The Metabolism of Carcinoma Cells1.* The Journal of Cancer Research, 1925. **9**(1): p. 148-163.
37. Warburg, O.H., *Uber den stoffwechsel der tumoren.* 1926.
38. Sbarra, A.J. and M.L. Karnovsky, *The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes.* J Biol Chem, 1959. **234**(6): p. 1355-62.
39. Hard, G.C., *Some biochemical aspects of the immune macrophage.* Br J Exp Pathol, 1970. **51**(1): p. 97-105.
40. Luan, H.H. and R. Medzhitov, *Food Fight: Role of Itaconate and Other Metabolites in Antimicrobial Defense.* Cell Metab, 2016. **24**(3): p. 379-387.

41. Alabduladhem, T.O. and B. Bordoni, *Physiology, Krebs Cycle*, in *StatPearls*. 2022: Treasure Island (FL).
42. Janson, L.W. and M.E. Tischler, *CARBOHYDRATE METABOLISM*, in *The Big Picture: Medical Biochemistry*. 2018, McGraw-Hill Education: New York, NY.
43. Bender, D.A. and P.A. Mayes, *The Citric Acid Cycle: The Central Pathway of Carbohydrate, Lipid, & Amino Acid Metabolism*, in *Harper's Illustrated Biochemistry, 31e*, V.W. Rodwell, et al., Editors. 2018, McGraw-Hill Education: New York, NY.
44. O'Neill, L.A.J. and M.N. Artyomov, *Itaconate: the poster child of metabolic reprogramming in macrophage function.* Nat Rev Immunol, 2019. **19**(5): p. 273-281.
45. Pearce, E.L. and E.J. Pearce, *Metabolic pathways in immune cell activation and quiescence.* Immunity, 2013. **38**(4): p. 633-43.
46. Peace, C.G. and L.A. O'Neill, *The role of itaconate in host defense and inflammation.* J Clin Invest, 2022. **132**(2).
47. Aktan, F., *iNOS-mediated nitric oxide production and its regulation.* Life Sci, 2004. **75**(6): p. 639-53.
48. Lampropoulou, V., et al., *Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation.* Cell Metab, 2016. **24**(1): p. 158-66.
49. Vijayan, V., et al., *Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis.* Redox Biol, 2019. **22**: p. 101147.
50. Tannahill, G.M., et al., *Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha.* Nature, 2013. **496**(7444): p. 238-42.
51. Rubic, T., et al., *Triggering the succinate receptor GPR91 on dendritic cells enhances immunity.* Nat Immunol, 2008. **9**(11): p. 1261-9.
52. McFadden, B.A. and S. Purohit, *Itaconate, an isocitrate lyase-directed inhibitor in Pseudomonas indigofera.* J Bacteriol, 1977. **131**(1): p. 136-44.
53. McKinney, J.D., et al., *Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase.* Nature, 2000. **406**(6797): p. 735-8.
54. Michelucci, A., et al., *Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production.* Proc Natl Acad Sci U S A, 2013. **110**(19): p. 7820-5.
55. Naujoks, J., et al., *IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid.* PLoS Pathog, 2016. **12**(2): p. e1005408.
56. Baup, S., *Ueber eine neue Pyrogen-Citronensäure, und über Benennung der Pyrogen-Säuren überhaupt.* 1836. **19**(1): p. 29-38.
57. Turner, E., J. Liebig, and W. Gregory, *Elements of Chemistry: Including the Actual State and Prevalent Doctrines of the Science*. 1842.
58. Okabe, M., et al., *Biotechnological production of itaconic acid and its biosynthesis in Aspergillus terreus.* Appl Microbiol Biotechnol, 2009. **84**(4): p. 597-606.
59. Steiger, M.G., et al., *Biochemistry of microbial itaconic acid production.* Front Microbiol, 2013. **4**: p. 23.
60. Lee, C.G., et al., *Cloning and analysis of gene regulation of a novel LPS-inducible cDNA.* Immunogenetics, 1995. **41**(5): p. 263-70.

61. Shin, J.H., et al., *(1)H NMR-based metabolomic profiling in mice infected with Mycobacterium tuberculosis.* J Proteome Res, 2011. **10**(5): p. 2238-47.
62. Sugimoto, M., et al., *Non-targeted metabolite profiling in activated macrophage secretion.* Metabolomics, 2012. **8**.
63. Strelko, C.L., et al., *Itaconic acid is a mammalian metabolite induced during macrophage activation.* J Am Chem Soc, 2011. **133**(41): p. 16386-9.
64. Chen, F., et al., *Crystal structure of cis-aconitate decarboxylase reveals the impact of naturally occurring human mutations on itaconate synthesis.* Proc Natl Acad Sci U S A, 2019. **116**(41): p. 20644-20654.
65. Lattin, J.E., et al., *Expression analysis of G Protein-Coupled Receptors in mouse macrophages.* Immunome Res, 2008. **4**: p. 5.
66. Cordes, T., et al., *Immunoresponsive Gene 1 and Itaconate Inhibit Succinate Dehydrogenase to Modulate Intracellular Succinate Levels.* J Biol Chem, 2016. **291**(27): p. 14274-14284.
67. Yu, X.H., et al., *Itaconate: an emerging determinant of inflammation in activated macrophages.* Immunol Cell Biol, 2019. **97**(2): p. 134-141.
68. Chouchani, E.T., et al., *Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS.* Nature, 2014. **515**(7527): p. 431-435.
69. Valls-Lacalle, L., et al., *Succinate dehydrogenase inhibition with malonate during reperfusion reduces infarct size by preventing mitochondrial permeability transition.* Cardiovasc Res, 2016. **109**(3): p. 374-84.
70. Kohlhauer, M., et al., *Protection against cardiac ischemia-reperfusion injury by hypothermia and by inhibition of succinate accumulation and oxidation is additive.* Basic Res Cardiol, 2019. **114**(3): p. 18.
71. Baird, L. and M. Yamamoto, *The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway.* Mol Cell Biol, 2020. **40**(13).
72. Kensler, T.W., N. Wakabayashi, and S. Biswal, *Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 89-116.
73. Yoh, K., et al., *Nrf2-deficient female mice develop lupus-like autoimmune nephritis.* Kidney Int, 2001. **60**(4): p. 1343-53.
74. Ma, Q., L. Battelli, and A.F. Hubbs, *Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2.* Am J Pathol, 2006. **168**(6): p. 1960-74.
75. Motohashi, H. and M. Yamamoto, *Nrf2-Keap1 defines a physiologically important stress response mechanism.* Trends Mol Med, 2004. **10**(11): p. 549-57.
76. Walters, D.M., H.Y. Cho, and S.R. Kleeberger, *Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: a potential role for Nrf2.* Antioxid Redox Signal, 2008. **10**(2): p. 321-32.
77. Dinkova-Kostova, A.T., et al., *Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4584-9.
78. Talalay, P., et al., *Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation.* Proc Natl Acad Sci U S A, 2007. **104**(44): p. 17500-5.

79. Ma, Q. and K. Kinneer, *Chemoprotection by phenolic antioxidants. Inhibition of tumor necrosis factor alpha induction in macrophages.* J Biol Chem, 2002. **277**(4): p. 2477-84.
80. Kobayashi, E.H., et al., *Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription.* Nat Commun, 2016. **7**: p. 11624.
81. Bambouskova, M., et al., *Electrophilic properties of itaconate and derivatives regulate the IkappaBzeta-ATF3 inflammatory axis.* Nature, 2018. **556**(7702): p. 501-504.
82. Mills, E.L., et al., *Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1.* Nature, 2018. **556**(7699): p. 113-117.
83. Nair, S., et al., *Irg1 expression in myeloid cells prevents immunopathology during M. tuberculosis infection.* J Exp Med, 2018. **215**(4): p. 1035-1045.
84. Daniels, B.P., et al., *The Nucleotide Sensor ZBP1 and Kinase RIPK3 Induce the Enzyme IRG1 to Promote an Antiviral Metabolic State in Neurons.* Immunity, 2019. **50**(1): p. 64-76 e4.
85. Olagnier, D., et al., *Author Correction: SARS-CoV2-mediated suppression of NRF2-signaling reveals potent antiviral and anti-inflammatory activity of 4-octyl-itaconate and dimethyl fumarate.* Nat Commun, 2020. **11**(1): p. 5419.
86. Montes Diaz, G., et al., *Dimethyl fumarate treatment in multiple sclerosis: Recent advances in clinical and immunological studies.* Autoimmun Rev, 2018. **17**(12): p. 1240-1250.
87. Swain, A., et al., *Comparative evaluation of itaconate and its derivatives reveals divergent inflammasome and type I interferon regulation in macrophages.* Nat Metab, 2020. **2**(7): p. 594-602.
88. He, W., et al., *Mesaconate is synthesized from itaconate and exerts immunomodulatory effects in macrophages.* Nat Metab, 2022. **4**(5): p. 524-533.
89. Chen, Q., et al., *Radiomics in Stroke Neuroimaging: Techniques, Applications, and Challenges.* Aging Dis, 2021. **12**(1): p. 143-154.
90. Cheng, Z., et al., *NIHSS Consciousness Score Combined with ASPECTS is a Favorable Predictor of Functional Outcome post Endovascular Recanalization in Stroke Patients.* Aging Dis, 2021. **12**(2): p. 415-424.
91. Tao, X., et al., *Models of poststroke depression and assessments of core depressive symptoms in rodents: How to choose?* Exp Neurol, 2019. **322**: p. 113060.
92. Hallenbeck, J.M. and A.J. Dutka, *Background review and current concepts of reperfusion injury.* Arch Neurol, 1990. **47**(11): p. 1245-54.
93. Pundik, S., K. Xu, and S. Sundararajan, *Reperfusion brain injury: focus on cellular bioenergetics.* Neurology, 2012. **79**(13 Suppl 1): p. S44-51.
94. White, B.C., et al., *Brain ischemia and reperfusion: molecular mechanisms of neuronal injury.* J Neurol Sci, 2000. **179**(S 1-2): p. 1-33.
95. Shao, A., et al., *Oxidative Stress at the Crossroads of Aging, Stroke and Depression.* Aging Dis, 2020. **11**(6): p. 1537-1566.
96. Adav, S.S. and S.K. Sze, *Hypoxia-Induced Degenerative Protein Modifications Associated with Aging and Age-Associated Disorders.* Aging Dis, 2020. **11**(2): p. 341-364.
97. Usher, M.G., et al., *Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice.* J Clin Invest, 2010. **120**(9): p. 3350-64.

98. Frieler, R.A., et al., *Myeloid-specific deletion of the mineralocorticoid receptor reduces infarct volume and alters inflammation during cerebral ischemia.* Stroke, 2011. **42**(1): p. 179-85.
99. Frieler, R.A., et al., *Myeloid mineralocorticoid receptor during experimental ischemic stroke: effects of model and sex.* J Am Heart Assoc, 2012. **1**(5): p. e002584.
100. Mills, E.L. and L.A. O'Neill, *Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal.* Eur J Immunol, 2016. **46**(1): p. 13-21.
101. Chouchani, E.T., et al., *Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS.* Nature, 2014. **515**(7527): p. 431-5.
102. Zhang, D., et al., *A likely protective effect of dimethyl itaconate on cerebral ischemia/reperfusion injury.* Int Immunopharmacol, 2019. **77**: p. 105924.
103. Sorensen, I., R.H. Adams, and A. Gossler, *DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries.* Blood, 2009. **113**(22): p. 5680-8.
104. Frieler, R.A., et al., *Myeloid mineralocorticoid receptor during experimental ischemic stroke: effects of model and sex.* Journal of the American Heart Association, 2012. **1**(5): p. e002584.
105. Frieler, R.A., et al., *Genetic neutrophil deficiency ameliorates cerebral ischemia-reperfusion injury.* Exp Neurol, 2017. **298**(Pt A): p. 104-111.
106. Xue, Y., et al., *Microglial Polarization: Novel Therapeutic Strategy against Ischemic Stroke.* Aging Dis, 2021. **12**(2): p. 466-479.
107. Wu, J., et al., *Genome-Wide Screen and Validation of Microglia Pro-Inflammatory Mediators in Stroke.* Aging Dis, 2021. **12**(3): p. 786-800.
108. Cordes, T., et al., *Itaconate modulates tricarboxylic acid and redox metabolism to mitigate reperfusion injury.* Mol Metab, 2020. **32**: p. 122-135.
109. Orthgiess, J., et al., *Neurons exhibit Lyz2 promoter activity in vivo: Implications for using LysM-Cre mice in myeloid cell research.* Eur J Immunol, 2016. **46**(6): p. 1529-32.
110. Cho, I.H., et al., *Role of microglial IKKbeta in kainic acid-induced hippocampal neuronal cell death.* Brain, 2008. **131**(Pt 11): p. 3019-33.
111. Andjelkovic, A.V., et al., *Endothelial Targets in Stroke: Translating Animal Models to Human.* Arterioscler Thromb Vasc Biol, 2019. **39**(11): p. 2240-2247.
112. Chen, L.S., et al., *Transcriptional Analysis of Sepsis-Induced Activation and Damage of the Adrenal Endothelial Microvascular Cells.* Front Endocrinol (Lausanne), 2019. **10**: p. 944.
113. Daneman, R. and A. Prat, *The blood-brain barrier.* Cold Spring Harb Perspect Biol, 2015. **7**(1): p. a020412.
114. Shimizu, Y., et al., *Neuroprotective Effects of Endogenous Secretory Receptor for Advanced Glycation End-products in Brain Ischemia.* Aging Dis, 2020. **11**(3): p. 547-558.
115. Li, W., et al., *Serum Occludin as a Biomarker to Predict the Severity of Acute Ischemic Stroke, Hemorrhagic Transformation, and Patient Prognosis.* Aging Dis, 2020. **11**(6): p. 1395-1406.
116. Chen, Y., et al., *Ferroptosis: A Novel Therapeutic Target for Ischemia-Reperfusion Injury.* Front Cell Dev Biol, 2021. **9**: p. 688605.
117. Dixon, S.J., et al., *Ferroptosis: an iron-dependent form of nonapoptotic cell death.* Cell, 2012. **149**(5): p. 1060-72.

118. Xie, Y., et al., *Interplay Between Lipid Metabolism and Autophagy.* Front Cell Dev Biol, 2020. **8**: p. 431.
119. Stockwell, B.R., et al., *Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease.* Cell, 2017. **171**(2): p. 273-285.
120. Seibt, T.M., B. Proneth, and M. Conrad, *Role of GPX4 in ferroptosis and its pharmacological implication.* Free Radic Biol Med, 2019. **133**: p. 144-152.
121. Dolma, S., et al., *Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells.* Cancer Cell, 2003. **3**(3): p. 285-96.
122. Yang, W.S. and B.R. Stockwell, *Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells.* Chem Biol, 2008. **15**(3): p. 234-45.
123. Yang, W.S., et al., *Regulation of ferroptotic cancer cell death by GPX4.* Cell, 2014. **156**(1-2): p. 317-331.
124. Chen, X., et al., *Broadening horizons: the role of ferroptosis in cancer.* Nat Rev Clin Oncol, 2021. **18**(5): p. 280-296.
125. Zhang, C., et al., *Ferroptosis in cancer therapy: a novel approach to reversing drug resistance.* Mol Cancer, 2022. **21**(1): p. 47.
126. Jiang, X., B.R. Stockwell, and M. Conrad, *Ferroptosis: mechanisms, biology and role in disease.* Nat Rev Mol Cell Biol, 2021. **22**(4): p. 266-282.
127. Li, R., et al., *Itaconate: A Metabolite Regulates Inflammation Response and Oxidative Stress.* Oxid Med Cell Longev, 2020. **2020**: p. 5404780.
128. Kapralov, A.A., et al., *Redox lipid reprogramming commands susceptibility of macrophages and microglia to ferroptotic death.* Nat Chem Biol, 2020. **16**(3): p. 278-290.
129. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.
130. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance.* J Clin Invest, 2003. **112**(12): p. 1821-30.
131. de Luca, C. and J.M. Olefsky, *Inflammation and insulin resistance.* FEBS Lett, 2008. **582**(1): p. 97-105.
132. Lee, Y.S., J. Wollam, and J.M. Olefsky, *An Integrated View of Immunometabolism.* Cell, 2018. **172**(1-2): p. 22-40.
133. O'Neill, L.A., R.J. Kishton, and J. Rathmell, *A guide to immunometabolism for immunologists.* Nat Rev Immunol, 2016. **16**(9): p. 553-65.
134. Mills, E.L., et al., *Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1.* Nature, 2018.
135. Hooftman, A., et al., *The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation.* Cell Metabolism, 2020. **32**(3): p. 468-478.e7.
136. Bambouskova, M., et al., *Itaconate confers tolerance to late NLRP3 inflammasome activation.* Cell Rep, 2021. **34**(10): p. 108756.
137. Wang, Q., et al., *The anti-inflammatory drug dimethyl itaconate protects against colitis-associated colorectal cancer.* Journal of Molecular Medicine, 2020. **98**(10): p. 1457-1466.
138. Trayhurn, P., *Hypoxia and adipose tissue function and dysfunction in obesity.* Physiol Rev, 2013. **93**(1): p. 1-21.

139. Takikawa, A., et al., *HIF-1alpha in Myeloid Cells Promotes Adipose Tissue Remodeling Toward Insulin Resistance.* Diabetes, 2016. **65**(12): p. 3649-3659.
140. Zhang, S., et al., *Dimethyl Itaconate Alleviates the Inflammatory Responses of Macrophages in Sepsis.* Inflammation, 2020.
141. Tang, C., et al., *4-Octyl Itaconate Activates Nrf2 Signaling to Inhibit Pro-Inflammatory Cytokine Production in Peripheral Blood Mononuclear Cells of Systemic Lupus Erythematosus Patients.* Cell Physiol Biochem, 2018. **51**(2): p. 979-990.
142. Kuo, P.C., et al., *Dimethyl itaconate, an itaconate derivative, exhibits immunomodulatory effects on neuroinflammation in experimental autoimmune encephalomyelitis.* J Neuroinflammation, 2020. **17**(1): p. 138.
143. Xin, Y., L. Zou, and S. Lang, *4-Octyl itaconate (4-OI) attenuates lipopolysaccharide-induced acute lung injury by suppressing PI3K/Akt/NF-kappaB signaling pathways in mice.* Exp Ther Med, 2021. **21**(2): p. 141.
144. Li, Y., et al., *4-Octyl Itaconate Alleviates Lipopolysaccharide-Induced Acute Lung Injury in Mice by Inhibiting Oxidative Stress and Inflammation.* Drug Des Devel Ther, 2020. **14**: p. 5547-5558.
145. Gu, L., et al., *Dimethyl itaconate protects against fungal keratitis by activating the Nrf2/HO-1 signaling pathway.* Immunol Cell Biol, 2020. **98**(3): p. 229-241.
146. Shan, Q., et al., *Protective effects of dimethyl itaconate in mice acute cardiotoxicity induced by doxorubicin.* Biochem Biophys Res Commun, 2019. **517**(3): p. 538-544.
147. Xu, M., et al., *Dimethyl itaconate protects against lipopolysaccharide-induced endometritis by inhibition of TLR4/NF-kappaB and activation of Nrf2/HO-1 signaling pathway in mice.* Iran J Basic Med Sci, 2020. **23**(9): p. 1239-1244.
148. Zhao, C., et al., *Dimethyl itaconate protects against lippolysacchride-induced mastitis in mice by activating MAPKs and Nrf2 and inhibiting NF-kappaB signaling pathways.* Microb Pathog, 2019. **133**: p. 103541.
149. Ayala, J.E., et al., *Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice.* Dis Model Mech, 2010. **3**(9-10): p. 525-34.
150. McGuinness, O.P., et al., *NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse.* Am J Physiol Endocrinol Metab, 2009. **297**(4): p. E849-55.
151. Ruan, H., et al., *Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory.* Diabetes, 2002. **51**(5): p. 1319-36.
152. Camell, C.D., et al., *Aging Induces an Nlrp3 Inflammasome-Dependent Expansion of Adipose B Cells That Impairs Metabolic Homeostasis.* Cell Metab, 2019. **30**(6): p. 1024-1039 e6.
153. Nishimura, S., et al., *Adipose Natural Regulatory B Cells Negatively Control Adipose Tissue Inflammation.* Cell Metab, 2013. **18**(5): p. 759-766.
154. DeFuria, J., et al., *B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile.* Proc Natl Acad Sci U S A, 2013. **110**(13): p. 5133-8.
155. Winer, D.A., et al., *B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies.* Nat Med, 2011. **17**(5): p. 610-7.

156. Shah, Y.M., *The role of hypoxia in intestinal inflammation.* Mol Cell Pediatr, 2016. **3**(1): p. 1.
157. Campbell, E.L., et al., *Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation.* Immunity, 2014. **40**(1): p. 66-77.
158. Colgan, S.P. and C.T. Taylor, *Hypoxia: an alarm signal during intestinal inflammation.* Nat Rev Gastroenterol Hepatol, 2010. **7**(5): p. 281-7.
159. Al-Bawardy, B., R. Shivashankar, and D.D. Proctor, *Novel and Emerging Therapies for Inflammatory Bowel Disease.* Front Pharmacol, 2021. **12**: p. 651415.
160. Catalan-Serra, I. and O. Brenna, *Immunotherapy in inflammatory bowel disease: Novel and emerging treatments.* Hum Vaccin Immunother, 2018. **14**(11): p. 2597-2611.
161. Khor, T.O., et al., *Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis.* Cancer Res, 2006. **66**(24): p. 11580-4.
162. Khor, T.O., et al., *Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer.* Cancer Prev Res (Phila), 2008. **1**(3): p. 187-91.
163. Song, J., et al., *Inactivation of Interleukin-4 Receptor alpha Signaling in Myeloid Cells Protects Mice From Angiotensin II/High Salt-Induced Cardiovascular Dysfunction Through Suppression of Fibrotic Remodeling.* J Am Heart Assoc, 2021. **10**(13): p. e017329.
164. Song, J., et al., *Myeloid interleukin-4 receptor alpha is essential in postmyocardial infarction healing by regulating inflammation and fibrotic remodeling.* Am J Physiol Heart Circ Physiol, 2021. **320**(1): p. H323-H337.
165. Cordes, T. and C.M. Metallo, *Itaconate Alters Succinate and Coenzyme A Metabolism via Inhibition of Mitochondrial Complex II and Methylmalonyl-CoA Mutase.* Metabolites, 2021. **11**(2).
166. He, R., et al., *Itaconate inhibits ferroptosis of macrophage via Nrf2 pathways against sepsis-induced acute lung injury.* Cell Death Discov, 2022. **8**(1): p. 43.
167. Muri, J., et al., *Electrophilic Nrf2 activators and itaconate inhibit inflammation at low dose and promote IL-1beta production and inflammatory apoptosis at high dose.* Redox Biol, 2020. **36**: p. 101647.
168. Li, J., et al., *Ferroptosis: past, present and future.* Cell Death Dis, 2020. **11**(2): p. 88.
169. Conrad, M. and J.P. Friedmann Angeli, *Glutathione peroxidase 4 (Gpx4) and ferroptosis: what's so special about it?* Mol Cell Oncol, 2015. **2**(3): p. e995047.
170. Tao, N., K. Li, and J. Liu, *Molecular Mechanisms of Ferroptosis and Its Role in Pulmonary Disease.* Oxid Med Cell Longev, 2020. **2020**: p. 9547127.
171. Tuo, Q.Z., et al., *Tau-mediated iron export prevents ferroptotic damage after ischemic stroke.* Mol Psychiatry, 2017. **22**(11): p. 1520-1530.
172. Fang, X., et al., *Ferroptosis as a target for protection against cardiomyopathy.* Proc Natl Acad Sci U S A, 2019. **116**(7): p. 2672-2680.
173. Gao, M., et al., *Glutaminolysis and Transferrin Regulate Ferroptosis.* Mol Cell, 2015. **59**(2): p. 298-308.
174. Tu, H., et al., *Combination of ponatinib with deferoxamine synergistically mitigates ischemic heart injury via simultaneous prevention of necroptosis and ferroptosis.* Eur J Pharmacol, 2021. **898**: p. 173999.

175. Guan, X., et al., *Galangin attenuated cerebral ischemia-reperfusion injury by inhibition of ferroptosis through activating the SLC7A11/GPX4 axis in gerbils.* Life Sci, 2021. **264**: p. 118660.
176. Guan, X., et al., *The neuroprotective effects of carvacrol on ischemia/reperfusion-induced hippocampal neuronal impairment by ferroptosis mitigation.* Life Sci, 2019. **235**: p. 116795.
177. Bomprezzi, R., *Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview.* Ther Adv Neurol Disord, 2015. **8**(1): p. 20-30.
178. Fisher, M. and S.I. Savitz, *Pharmacological brain cytoprotection in acute ischaemic stroke - renewed hope in the reperfusion era.* Nat Rev Neurol, 2022. **18**(4): p. 193-202.
179. Soares, R.O.S., et al., *Ischemia/Reperfusion Injury Revisited: An Overview of the Latest Pharmacological Strategies.* Int J Mol Sci, 2019. **20**(20).