

**UNDERSTANDING THE ROLE OF IMMUNOMETABOLITE ITACONATE IN THE
REGULATION OF HEPATIC LIPID METABOLISM DURING SEPSIS**

BY

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Dedication

I dedicate this dissertation to my beloved family, whose love and support have been instrumental in my academic journey. My affection for you all runs deep and fills my heart with gratitude. To my significant other, I am grateful for your attentive listening and unwavering support throughout all of my pursuits. Thank you for being my constant source of motivation and encouragement.

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“To Be Called a Refugee Is the Opposite of an Insult; It’s a Badge of Strength, Courage, and Victory.” -Tennessee Office for Refugees

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ABSTRACT

Sepsis is a life-threatening clinical complication with increased uncontrolled host immune response to an infection, mortality, morbidity, and financial burden worldwide. There has been a significant focus on understanding the role of metabolic reprogramming in the immune compartment during sepsis. However, there is a notable gap in our knowledge regarding the involvement of other vital organs responsible for maintaining metabolic integrity. Thus, the purpose of this work is to first expand our current understanding of how critical organs such as the liver contribute to preserving metabolism in the context of sepsis. In Chapter II, employing multi-omic approaches, we first demonstrate that mitochondrial reprogramming underlies impairment in energy production, remodeling of the TCA cycle, and an increase in oxidative stress, leading to the onset of dyslipidemia in the context of sepsis. Furthermore, the restoration of the pyruvate dehydrogenase complex (PDC) using dichloroacetate (DCA) improves hepatic metabolic dysfunction by enhancing the conversion of pyruvate to acetyl-CoA, highlighting the consequences of hepatic metabolic reprogramming.

One major limitation of Chapter II was the lack of understanding regarding the molecular regulators driving the observed alterations in hepatic lipid metabolism. Therefore, our subsequent goal was to identify and investigate novel molecular interactions governing the dysregulation of lipid metabolism. Consequently, through analysis of the unbiased metabolomics dataset generated in Chapter II, we identified itaconate as the top accumulated hepatic metabolite produced in

response to sepsis. However, understanding of itaconate's anti-inflammatory and immunometabolic activities in response to infections is mostly limited to immune cells making it pertinent to explore its functional role in central organs critical to maintaining systemic metabolism. Hence, to gain further insight into its physiological role in the liver during sepsis, we subjected wild-type (WT) and global Irg1 knockout (KO) mice to septic conditions.

In conjunction with our previous findings, we observed that WT septic mice develop hepatosteatosis. Interestingly, Irg1 knockout mice also develop this phenotype but to a more severe extent compared to WT counterparts in response to sepsis. For the first time, our data revealed itaconate as an important negative regulator of hepatic lipid accumulation in the context of sepsis. However, the exact molecular mechanism by which itaconate interacts with regulators of hepatic lipid metabolism during sepsis is yet to be uncovered. Therefore, our next objective was to investigate the role of itaconate in modulating hepatic lipid metabolism during overt inflammation. In Chapter IV our data revealed that itaconate plays a significant role in enhancing lipid clearance in hepatocytes by stabilizing the mitochondrial fatty acid uptake enzyme known as CPT1a. Additionally, proteomic analysis of hepatic substrates of itaconate has identified novel substrates involved in protein ubiquitination, which we hypothesize contributes to its ability to promote lipid clearance. Furthermore, we observed that mice lacking Irg1 also display impaired systemic fatty acid utilization ability and thermogenesis in response to sepsis.

In conclusion, our study contributes to the expanding understanding of itaconate as a metabolic regulator in non-immune cells in response to inflammation. These findings shed light on the mechanisms underlying hepatic lipid metabolism during sepsis and highlight the potential therapeutic implications of targeting itaconate in the management of sepsis-related complications such as dyslipidemia.

ABBREVIATIONS

APOA-1: Apolipoprotein A-1

APRs: Acute plasma response proteins

BCL-2: B-cell lymphoma-2 family proteins

BHB: Beta-hydroxybutyrate

C3a: C3 activator fragment

C5a: C5 activator fragment

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CDC: Centers for Disease Control

CEC: Cholesterol efflux capacity

CETP: Cholesteryl ester transfer protein

CLRs: C-type lectin receptors

CREB2: Cyclic-AMP response element-binding protein 2

CRP: C-reactive protein

CRTC2: CREB-regulated transcription coactivator 2

CRTCs:

CRTCs: cAMP response-regulated transcriptional co-activators

DAMPs: Damage-associated molecular patterns

DCA: Dichloroacetate

DIC: Disseminated intravascular coagulation

FFA: free fatty acids

FOXO1: Forkhead box O

GSA: Global Sepsis Association

HDL: high-density lipoprotein

HL: Hepatic lipases

ICU: Intensive care units

IFN- γ : Interferon gamma

IL-1: Interleukin-1

IL-10: Interleukin-10

IL-12: Interleukin-12

IL-18: Interleukin-18

IL-1 α : Interleukin-1 alpha

IL-1 β : Interleukin-1 beta

IL-6: Interleukin-6

IL-7: Interleukin-7

LCAT: Lecithin-cholesterol acyltransferase

LDL: low-density lipoprotein

LMWHs: Low-molecular-weight heparin

LPS: Lipopolysaccharide

NAFLD: Nonalcoholic Fatty Liver Disease

NLRs: NOD-like receptors

OXPHOS: Oxidative phosphorylation

PAI-1: Plasminogen activator inhibitor type I

PAMPs: Pathogen-associated molecular pattern molecules

PGC-1 α : Peroxisome proliferator-activated receptor-gamma coactivator

PLA₂: Phospholipase A2

PLTPs: Phospholipid transfer protein

PON1: Paraoxonase 1

PPAR: Peroxisome-proliferator activated receptors

PPAR α : Peroxisome-proliferator activated receptor alpha

PRRs: Pattern recognition receptors

RIG: Retinoic acid-inducible gene-like receptors

ROS: Reactive-Oxygen-Species

SAA: serum amyloid A

TC: total cholesterol

TCA: Tricarboxylic acid cycle

TF: Tissue factor

TGs: Triglycerides

TLR: Toll-like receptors

TLR4: Toll like receptor 4

TNF- α : Tumor necrosis factor-alpha

TRAF6: TNF receptor-associated factor 6

VLDL: Very-low-density lipoprotein

WHO: World Health Organization

A20: Alpha-induced *protein*

AIM2: Interferon inducible protein

AKT: Serine/threonine protein kinase	IRF1: Interferon regulatory factor 1
ALDOA: Aldolase	IRF3: Interferon regulatory factor 3
ALT: Alanine aminotransferases	Irg1: Immunoresponsive gene 1
ASC: Apoptosis-associated speck-like <i>protein</i> containing a CARD	IRI: Ischemia Reperfusion Injury
ATF3: Activating transcription factor 3	ITalk: Itaconate alkyne
ATP: Adenosine triphosphate	JUN: Transcription factor Jun
BCAA: Branched-chain amino acid	KEAP1: Kelch-like ECH-associated protein 1
BMDMs: Bone marrow-derived macrophages	LDHA: Lactate dehydrogenase:
CLYB: Citramalyl-CoA lyase	GAPDH: glyceraldehyde-3-phosphate dehydrogenase
CoA: Coenzyme A	LDL: Low-density lipoprotein
Cul3: Cullin 3	LDLR: Low-density lipoprotein receptor 1
DAMPs: Danger-associated molecular patterns	LPS: Lipopolysaccharide
DMI: Dimethyl Itaconate	M-CoA: Methylmalonyl-CoA
ECAR: Extracellular acidification rate	MCT4: Monocarboxylate transporter 4
ETC: Electron transport chain	MYD88: Myeloid differentiation primary response 88
eWAT: Epididymal white adipose tissue	NADH: Reduced nicotinamide adenine dinucleotide
FADH: Reduced flavin adenine dinucleotide	NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
FFA: Free fatty acid	NLRP: NLR family pyrin domain containing 3
AMPK: Adenosine monophosphate kinase	Nqo1: NADPH quinone dehydrogenase
FGF: Fibroblast growth factor	NRF2: Nuclear factor erythroid 2-related factor 2
FXR: Farnesoid X receptor	OXPHOS: Oxidative phosphorylation
GASMD: Gasdermin D	4-OI: 4-Octyl Itaconate
GLP-1: Glucagon-like peptide 1	PBMCs: Peripheral blood monocytes
GLUT: Glucose transporter	P-CoA: Propionyl coenzyme A
GSH: Glutathione	PFK: Phosphofructokinase
HFD: High-fat diet	PPAR: Peroxisome proliferator-activated receptor
HIF-1α: Hypoxia-inducible factor-1	ROS: Reactive oxygen species
HK: Hexokinase	SAM: S-adenosyl methionine
HMGB1: High mobility group box 1 protein	S-CoA: Succinate coenzyme A
Hmox-1: Heme oxygenase	SDH: Succinate dehydrogenase
I-CoA: Itaconyl coenzyme A	MUT: Methylmalonyl-CoA mutase
IDH: Isocitrate dehydrogenase	SREBF1: Sterol regulatory element binding transcription factor 1
IFNR: Interferon production regulator	
IFN-γ: Interferon gamma	
IκBζ: NF-kappa-B inhibitor zeta	
IL: Interleukin	
iNOS: Inducible nitric oxide synthase	

STAT: signal transducer and activator of transcription	COX7A2L: Cytochrome C Oxidase Subunit 7A2 Like
STING: Stimulator of interferon genes	ATPAF1: ATP Synthase Mitochondrial F1 Complex Assembly Factor 1
TCA: Tricarboxylic acid	NDUFAB1: NADH: Ubiquinone Oxidoreductase Subunit AB1
TF: Transcription factor	ACSL: Acyl-CoA Synthetase Long-Chain Family Member
TG: Triglyceride	CPT: Carnitine palmitoyl transferase
THR-β: Thyroid hormone receptor beta	ACAA2: Acetyl-CoA Acyltransferase 2
TLR4: Toll-like receptor 4	SLC25a20: Solute Carrier Family 25 Member 20
TNF-α: Tumor necrosis factor-alpha	FAO: Fatty acid oxidation
UPS: Ubiquitin proteasome system	ITalk: Itaconate alkyne
WD: Western diet	ILK: Integrin-linked kinase
TG: Triglyceride	UCP1: Uncoupling protein 1
FFA: Free fatty acids	RER: Respiratory exchange ration
FA: Fatty acids	LPS: Lipopolysaccharide
TCA: Citric acid cycle	BAT: Brown adipose tissue
Acod1: Aconitase decarboxylase 1	WAT: White adipose tissue
SDH: Succinate dehydrogenase	PPARα: Peroxisome proliferator-activated receptor alpha
ATF3: Activating transcription factor 3	SAM: S-adenosyl-methionine
NRF2: Nuclear factor erythroid 2-related factor 2	SAH: S-adenosyl-L-homocysteine hydrolase
KEAP1: Kelch-like ECH-associated protein	SREBF1: Sterol Regulatory Element-Binding Protein 1
GAPDH: Glyceraldehyde-3-phosphate	LDLR: Low-density lipoprotein receptor
4-OI: 4-Octyl itaconate	HRD1: ERAD-associated E3 ubiquitin-protein ligase HRD1
DMI: Dimethyl itaconate	
NAFLD: Non-alcoholic fatty liver disease	
OXPHOS: Oxidative phosphorylation	

CHAPTER I

1. Clinical context

i. Sepsis definition

The Third International Consensus (Sepsis-3) currently defines sepsis as a life-threatening organ dysfunction caused by a dysregulated host response to infection ¹. This serious condition is reported to affect over a million people in the U.S. and around 48.9 million people globally each year ². Depending on sepsis severity, septic patient mortality rates are estimated to be between 20% to 50% ³. Furthermore, the economic burden of inpatient hospital admissions and treatments for sepsis is immense making it one of the most expensive conditions treated across numerous healthcare systems globally ⁴. Just in the U.S. alone, inpatient hospitalization costs over \$45.5 billion, averaging about \$16,000 to \$18,000 per patient annually ^{3,5-7}. While anyone can get sepsis, children and older adults along with those with comorbidities and immunosuppression are at high risk ². Additionally, socioeconomic variables are correlated with significant disparities in sepsis-attributable morbidity and mortality ^{2,8,9}. Given this, numerous organizations like the CDC, GSA, and WHO are working towards raising awareness and improving treatments for sepsis globally ¹⁰.

i. Current sepsis therapy

Currently, targeted molecular therapeutics that significantly improve patient outcomes do not exist ⁹. This is partly because sepsis is a complex condition as pathophysiological dysfunctions are widespread making treatment a challenge.

Additionally, numerous preclinical and clinical trials have failed due to poor design and heterogeneous patient response. Given this, sepsis treatment currently relies on surveillance for early recognition, management of infection, hemodynamic changes and inflammation, and provision of supportive therapies as needed ¹¹.

ii. Innate immune activation and inflammation

Sepsis is distinct from localized infections in that it disrupts the body's normal inflammatory balance, resulting in inflammation throughout the body that can ultimately damage tissues and organs (**Fig 1**). The first step in defense against pathogens is the activation of innate immune cells such as macrophages, neutrophils, natural killer cells, and monocytes^{12,13}. These immune cells present endogenous PRRs that recognize PAMPs that are associated with pathogens or DAMPs molecules that are released from damaged host cells^{14,15}. These danger-associated molecules bind onto PRRs such as TLR, NLRs, RIG, and CLRs^{14,15}. This leads to the rapid activation of pathways associated with pro-inflammatory interleukins (IL-1, IL-12, and IL-18), TNF- α , and interferons¹⁴. In response to this increase in pro-inflammatory signaling, subsequent expression of other chemokines (IL-6, IL-1 β , IL-18, IFN- γ) proliferation of leukocytes, activation of complement and coagulation system, and acute phase hepatic response occurs^{14,16,17}. However, during sepsis, this innate immune response is exaggerated and consequently leads to the dysregulation of numerous downstream signaling transduction pathways resulting in physiological changes such as hemostatic and

metabolic perturbations and immunosuppression which worsens sepsis severity and recovery (**Fig1**).

iii. Sepsis and coagulopathy

One complication of sepsis that is associated with mortality is coagulopathy by DIC¹⁸⁻²⁰. DIC is the widespread activation of the blood coagulation system that leads to the abnormal formation of blood clots inside blood vessels throughout the bloodstream in response to inflammation^{21,22}. During sepsis, dysregulation occurs not just within the coagulation system but also in cells that regulate it and given the complication of this system, multiple pathways are found to be at play²². Activated monocytes, parenchymal cells, and damaged endothelium increase the expression and exposure of sub-endothelial TF during innate immune activation²³. This activates clotting factors and leads to platelet aggregation and adhesion, stimulation of coagulation cascades, and formation of microvascular thrombosis to limit the spread of pathogens²⁴. However, this response is exaggerated during sepsis, hence the hyper-coagulation and subsequently observed hypoxia and tissue damage^{25,26}. Furthermore, TFs also induce proinflammatory response via the activation of protease-activated receptors²⁷. Apart from increased TF-dependent pro-coagulation, reduction in fibrinolysis is also seen in septic patients^{12,23,27}. It is reported that early inhibition of plasmin, a fibrinolytic protease, due to increases in PAI-1 activity causes a decrease in the breakdown of fibrin in blood clots in an enzymatic process called fibrinolysis²⁷. Furthermore, increased PAI-1 is used to predict poor sepsis outcomes as it leads to elevated risks of microvascular thrombosis and coagulopathy, promoting organ failure and mortality^{12,23}.

Lastly, overt activation of the complement system is also reported to contribute to the onset of coagulopathy by DIC during sepsis²⁸. Acute activation of the complement system during the early stages of sepsis releases potent inflammatory mediators C3a and C5a^{29,30}. These fragments trigger complement-dependent oxidative bursts and ROS release from inflamed monocytes and granulocytes which promotes an increase in platelet function. Given the already underlying inhibition of fibrinolysis, this further increases thrombosis and exacerbates DIC observed during sepsis. Lastly, a robust increase in acute inflammatory CRP which is also used as a biomarker for sepsis is observed³¹⁻³³. CRP binds onto endotoxins and damaged cells and activates the complement pathways to increase their uptake by phagocytosis cells³⁴. However, CRPs can also promote monocyte-endothelin cell adhesion which activates PAI-1 and TF synthesis and secretion and increases fibrinolytic resistance and coagulation³⁵⁻³⁷. Furthermore, several clinical studies have attempted to treat septic patients with DIC with antithrombin, recombinant thrombomodulin, recombinant activated protein C, recombinant tissue factor inhibitor, and unfractionated heparin and LMWHs^{38,39}. However, these studies don't reveal concrete evidence to support the administration of these anticoagulants to septic DIC patients. Furthermore, a high risk of bleeding was observed in patients participating in these clinical trials. The complexity of the coagulation pathway along with poor discrimination between patient subgroups with septic-associated coagulopathy and sepsis-induced DIC is thought to be the reasons behind the failure of these anticoagulants clinically^{38,39}. Given that there are no approved therapeutics for sepsis induced DIC, currently

treatment focuses on early recognition of coagulation disorder and rapid treatment of underlying infection and inflammation.

iv. Sepsis-induced immunosuppression

The second line of defense against pathogens is achieved due to the adaptive immune response also referred to as acquired immunity. The adaptive immune response differs from the innate system as it is specific to a pathogen and the response is much slower⁴⁰. However, the effect of acquired immunity is a long-lasting defense and protection against recurrent infections that could last from weeks to months to a whole lifetime. Adaptive immune responses are carried out by white blood cells (lymphocytes) B cells and T cells. B lymphocytes can produce antibodies and are responsible for antibody-mediated immunity. On the other hand, T lymphocytes are involved in cell-mediated immunity which includes apoptosis of foreign antigen-presenting cells, stimulation of cytokines to mediate recruitment of phagocytic cells, inflammation, and regulation of other immune cell functions⁴⁰.

Following the resolution of an infection, our immune system begins to return to homeostasis as dampening of inflammatory activation and inflammation itself occurs. However, in the case of sepsis, the immune system fails to resolve completely and leads to impairment in the host's innate and adaptive immunity resulting in the onset of sepsis-induced immunosuppression^{40,41}. Due to this, patients who succumb to secondary infections post-recovery from sepsis have higher mortality rates as they no longer have a functioning immune system needed to survive this second hit⁴². One hallmark clinical feature underlying this

immunosuppression is lymphopenia, lymphocyte dysfunction, and in various cell subtypes⁴³. Septic patients with persistently low peripheral lymphocyte counts are found to be more susceptible to secondary infections and have high mortality rates⁴⁴⁻⁴⁷. Furthermore, sepsis survivors who display improvements in lymphocyte counts have better survival outcomes than patients whose counts do not improve⁴⁶. One mechanism underlying lymphopenia is leukocyte apoptosis which drives the loss of B cells, T cells, dendritic cells, macrophages, and C5a receptor-mediated neutrophil activation which consequently affects leukocyte function^{43,48}. However, the underlying molecular mechanisms governing immune cell apoptosis during sepsis are not well understood. Interestingly, administration of caspase inhibitors and anti-apoptotic proteins such as BCL-2 involved in regulation of immune cell apoptosis have been demonstrated to improve immune cell and host survival in both *in vitro* and *in vivo* models of sepsis⁴⁹. This highlights the potential of anti-apoptosis therapy for modulation of lymphopenia. Recent studies have shown promising results as an increase in CD4 and CD8 T cell counts was observed upon administration of anti-apoptotic cytokine recombinant IL-7 to septic patients^{50,51}. IL-7 is important for lymphocyte cell activation and proliferation⁵² and preliminary studies are now demonstrating the promising potential of such immunoadjuvant therapy for the treatment of sepsis-induced immunosuppression^{50,51}.

Lastly, repeated exposure to LPS following an initial encounter during sepsis can lead to endotoxin tolerance which is also associated with immunosuppression⁵³ observed during later phases of sepsis. It is reported that monocytes from septic

patients have a reduced ability to produce and secrete pro-inflammatory cytokines such as IL-1, IL-6, and TNF in response to the secondary endotoxin exposure ⁵³. On the contrary, altered changes favoring the increase in the production of anti-inflammatory mediators IL-10 in monocytes and a decrease in MHC class II cell surface receptor (HLA-DR) were observed in septic patients^{54,55}. Furthermore, inhibition of IL-10 in experimental models of sepsis has been shown to improve survival, and the ratio of IL-10 to lymphocyte counts is associated with mortality in severe septic patients⁵⁵. Overall, sepsis-induced monocyte metabolic reprogramming independently of lymphopenia also contributes to immunosuppression.

2. The liver during sepsis

Metabolic reprogramming during sepsis

It is now established that metabolic regulation and immune response during sepsis are intertwined. However, it is crucial to understand that this syndrome also leads to the dysfunction of multiple vital organs ⁵⁶ such as the heart, kidneys, lungs, central nervous system, hematopoietic system, and liver. Furthermore, multiple organ failure is considered to be a hallmark feature of sepsis and determines patient outcomes⁵⁷. While the concept of metabolic dysregulation contributing to host demise during sepsis is well-studied in the immune compartment, the role of the crucial organs in regulating the host response and recovery from sepsis is underappreciated. One organ that plays a crucial role in meeting the demands of infections is the liver (**Fig 2**). This organ can mediate an acute immune response as it functions as a lymphoid organ in response to hepatotropic and non-

hepatotropic pathogens. Elicited by upstream inflammatory signals like cytokines and glucocorticoids, the liver orchestrates the synthesis of positive APRs like CRP, complement factors, SAA, ferritin and haptoglobin. Production of these APRs is crucial as they serve physiological functions for the regulation of the innate immune system such as complement activation, phagocytosis and bacterial clearance, coagulation, and recruitment of immune cells to sites of inflammation. Additionally, since the liver is connected to the hepatic artery and portal vein, it is also able to directly filter and detoxify blood from pathogens and other inflammatory agents. However, in response to endotoxin-induced sepsis, the liver is also susceptible to injury which can cause local inflammation, immunosuppression, and organ damage with disease progression.

In addition to its immunogenic function, the liver also plays a crucial role in the regulation of systemic metabolism by supporting the metabolism of macromolecules and micronutrients, drug detoxification, digestion, and filtration. The liver's numerous physiological roles make it a crucial organ in promoting host survival during this period of intense inflammation ⁵⁸. However, as a result of sepsis-induced damage, the liver is subjected to metabolic dysfunctions that can promote organ damage and jeopardize host survival (**Fig 3**).

i. Glucose metabolism during sepsis

The occurrence of disturbances in carbohydrate metabolism is one of the most prominent characteristics of sepsis-driven metabolic disorders. Dysglycemia (both hyperglycemia and hypoglycemia), is a commonly observed clinical symptom in septic patients. Numerous observational and prospective randomized clinical trial

studies have established an adverse association between hyperglycemia and multiple organ dysfunction and a 30-day mortality rate in septic patients⁵⁹⁻⁶². Furthermore, the mortality risk for patients with a greater acute glycemic variability during sepsis is significantly higher compared to patients with low acute glycemic variability highlighting the consequences of metabolic instability on host survival^{63,64}. One explanation for the development of hyperglycemia during sepsis is the impaired glucose utilization by peripheral tissues alongside insulin resistance^{62,65} which is also associated with an increase in 30-day mortality^{62,65}. Given this, glycemic control with intravenous insulin therapy to improve septic patient outcomes is currently recommended by Surviving Guidelines for adults with sepsis or septic shock if their glucose levels are persistently ≥ 180 mg/dL until it ranges between 140-180 mg/dL⁶⁶⁻⁶⁸. It is important to note that goal of attaining normoglycemia in septic patients doesn't always benefit them and can rather be harmful. A meta-analysis that included 18,098 patients from 35 studies found that a low target concentration of <110 and 110-144 mg/dL was associated with a significant increase in risk of hypoglycemia and adverse outcomes in comparison to 144-180 and >180 mg/dL⁶⁹. No differences between target concentrations of 144-180 and >180 mg/dL were observed. Furthermore, the onset of hypoglycemia, independent of insulin therapy, is also a physiological response seen in subsets of septic patients and is associated with an increase in adverse events like mortality⁷⁰. This could be a result of depletion of nutrients due to infection-induced anorexia⁷¹, depletion of glycogen reserve and or impaired gluconeogenesis⁷², and an increase in peripheral glucose uptake and utilization⁷³. Given that both high and

low glucose levels are of concern, continuous monitoring of its concentration during the course of sepsis to minimize glycemic variability to avoid negative outcomes in critically-ill patients is crucial.

The liver and its primary cells, hepatocytes, play an important role in the regulation of systemic blood glucose homeostasis via hormone-signaling pathways triggered by insulin and glucagon. During the fasting state, glucagon stimulates hepatic glucose output by stimulating gluconeogenic gene transcription such as PGC-1 α via CREB2^{74,75}. On the contrary, during feeding, insulin diminishes hepatic PGC-1 α transcription and expression⁷⁵ via Akt-mediated phosphorylation of FOXO1 and suppression of nuclear translocation and target gene expression and gluconeogenesis⁷⁶. However, in response to critical illness like sepsis, these mechanisms that allow the liver to regulate glucose metabolism are altered which can ultimately drive the onset of dysglycemia as discussed earlier. One mechanism by which this is thought to occur is via the suppression of CRTCs that mediate CREB2 activation following its dephosphorylation and nuclear translocation. In response to pro-inflammatory cytokines like interleukin-1 β , an E3 ubiquitin ligase TRAF6, part of the TLR4 signaling pathway, is activated and associated with CRTCs⁷⁷. This leads to the ubiquitination of CRTCs (Lys628) which was found to inhibit binding of calcineurin and dephosphorylation of CRTC2, leading to glucagon resistance and impairment in hepatic gluconeogenesis.

ii. Lipid metabolism during sepsis

Another metabolic manifestation of sepsis is the derangement of lipid profiles. Septic patients display elevated levels of TGs and VLDL, and decreased

levels of TC, HDL, and LDL. Elevated triglycerides or hypertriglyceridemia observed in patients may be a result of increased synthesis of hepatic TG and VLDL due to heightened lipolysis and FFA release by white adipose tissues upon stimulation by cytokines (TNF- α , IL-1, IL-1 α , IL-1 β), catecholamines and bacterial endotoxins. Inflammatory cytokines can also induce the synthesis of fatty acids in the liver. Furthermore, in response to gram-negative infection the activity of lipolytic enzyme lipase that hydrolyzes TGs from circulating plasma lipoproteins is significantly diminished which eventually means a reduction in substrate availability necessary for tissues to undergo beta-oxidation for energy production. Additionally, hepatic PPAR- α levels are also downregulated during sepsis which compromises the hydrolysis of fatty acids through β -oxidation which can further drive the accumulation of FFAs, lipotoxicity, and inflammation of the liver as well as other organs ⁷⁸.

HDL functions to efflux cholesterol from peripheral tissues/bloodstream back to the liver where it can be metabolized. Besides this, HDL has anti-inflammatory properties as it promotes LPS binding and neutralization, leukocyte recruitment, and modulates corticosterone production ⁷⁹. However, during sepsis, there is a significant decrease in circulating HDL and remodeling of its composition and metabolism that is associated with an increase in adverse clinical outcomes and mortality ⁸⁰. HDL particles are found to be larger in size ⁸¹ and enriched in free cholesterols, TG, and free FA's, and depleted in cholesterol ester. Furthermore, rather than apoA-1, SAA is found to be the major apolipoprotein during inflammation. Studies have shown that this reduces the anti-oxidative and anti-

inflammatory properties of HDL particles. Additionally, the expression of important enzymes like LCAT, HL, CETP, PLTPs, and PLA₂ that are involved in HDL metabolism are altered during inflammation⁸². Furthermore, these alterations can lead to function changes such as reduction in CEC and arylesterase activity of HDL-associated PON1 which was found to be an independent predictor of 28-day ICU mortality^{82,83}. While the precise mechanism by which inflammation leads to a decrease in circulating HDL and its functionality is not fully understood, multiple mechanisms might be at play.

Additionally alteration in the expression of PPAR that are broadly involved in the regulation of numerous important genes including glucose and lipid metabolism also underlies the derangements in lipid metabolism observed in septic patients. PPAR α , more specifically, is regulated by FFA which downstream regulates the transcription of genes involved in both peroxisomal and mitochondrial β -oxidation of fatty acids. A decrease in hepatic expression is associated with increased sepsis severity in clinical and experimental studies⁸⁴⁻⁸⁶. Furthermore, *in vivo* studies have demonstrated that hepatic PPAR α deficiency results in metabolic shifts as it impairs lipid metabolism response and causes FFA accumulation in the liver, and aggravates mortality during bacterial sepsis^{78,85}. In conclusion, the alterations in lipid metabolism and regulatory mechanism in response to sepsis are part of a complex milieu that needs to be investigated further.

iii. Amino acid metabolism during sepsis

The liver also plays a central role in amino acid metabolism. Under physiological conditions, it is involved in the synthesis of plasma proteins, amino

acid interconversion, deamination of amino acids, and urea synthesis. The liver significantly reduces the production of various negative plasma proteins such as albumin as part of the inflammatory response to support the synthesis of positive acute-phase proteins which are crucial to innate immune response and function⁸⁷. Additionally, sepsis is characterized by the catabolism of energy substrates like carbohydrates, fats, and proteins where a high percentage of patients display muscle loss due to increased protein degradation⁸⁸. Amino acids such as alanine released into the bloodstream due to proteolysis can be used by the liver for the production of acute phase proteins and gluconeogenesis⁸⁹. However, studies have demonstrated a reduction in gluconeogenesis evident by a decrease in the utilization of precursor alanine for glucose production which doesn't help restore the energy balance dysregulation seen in sepsis^{72,90}. Accumulation of energy-rich substrates in circulation can further exacerbate the severity of sepsis⁸⁸ given that the liver is independently damaged due to inflammation and other underlying conditions during sepsis.

iv. Ketogenesis during sepsis

Sepsis induces a fasting response, as previously discussed, and is accompanied by elevated levels of plasma FFAs and ketone bodies. Ketogenesis, a metabolic pathway that predominantly occurs in hepatic mitochondria, generates ketone bodies such as BHB through the breakdown of fatty acids and amino acids when the supply of glucose is restricted. In sepsis, dysregulation of ketone body metabolism and a decrease in BHB levels are observed, which are linked to an increase in sepsis severity and mortality^{91,92}. Intriguingly, inhibition of ketogenesis

in endotoxin-induced septic mice has been demonstrated to be fatal, highlighting the protective role of ketone bodies, which becomes disrupted during sepsis⁹³. Moreover, ketone body administration in both human and mice have shown to aid with inflammation and fibrosis associated with NAFLD^{94,95}. Since the accumulation of free fatty acids is implicated in the development of sepsis, the use of ketone bodies could be viewed as a promising therapeutic approach for the management of hepatic steatosis.

Rationale and chapters

Chapter 1: Introduction

In sepsis, the balance of inflammatory response is closely linked to metabolic reprogramming as excessive hyperinflammatory response leads to metabolic shifts from OXPHOS to aerobic glycolysis. In immune cells, it is well established that excessive glycolysis can cause immunosuppression and that mitochondrial dysfunction and metabolic dysregulation play a critical role in the pathogenesis of sepsis. However, it is apparent that the effects of sepsis extend beyond the immune compartment and have a significant impact on the body as a whole. Therefore, to come up with novel therapeutics to improve sepsis survival it is essential to comprehensively study the metabolic relationship between hyperinflammation, immunosuppression, and metabolic status in metabolically active organs like the liver. Additionally, understanding the roles of hepatic mitochondria in metabolic reprogramming during sepsis could provide valuable insights into treatment and

prognosis. Numerous pieces of evidence in the literature have demonstrated the primary role of the liver in affording organismal adaptation to sepsis by promoting host survival. However, there is a lack of comprehensive knowledge of the hepatic metabolic manifestations of sepsis. ***Therefore, the overall goal of this thesis is to extend our current understanding of the liver in maintaining metabolic integrity as it is activated and responds to hyperinflammation observed in sepsis.***

Chapter 2: Itaconate and Regulation of signaling pathways

The concept of metabolic reprogramming through metabolites produced by different bioenergetic pathways to regulate the immune response during inflammation is widely recognized. One example of this is the regulation of immunomodulatory metabolic pathways via the TCA cycle metabolite itaconate in response to inflammation. While numerous studies have investigated its importance and biological function in immune cells, it is primarily through post-translational modification of regulatory cysteine residues of key proteins involved in inflammation and redox balance. However, emerging data suggest itaconate's expression and regulatory function in non-immune tissues, such as the heart, liver, lungs, and kidneys upon inflammation. As our lab focuses primarily on the liver, this chapter aims to summarize the classical pathways of itaconate as well as its synthetic derivatives in the regulation of hepatic metabolism by modulation of several key signaling pathways.

Chapter 3: Dichloroacetate reverses sepsis-induced hepatic metabolic dysfunction

Goal: Characterize the hepatic manifestations of sepsis and aim to identify identifying global metabolic pathways subjected to dysregulation. Additionally, explore the potential of DCA treatment in restoring bioenergetics and resolving sepsis.

To bridge the knowledge gap on how hepatic metabolic reprogramming supports host survival during sepsis, we conducted a study utilizing comprehensive integrative transcriptomics, metabolomics, and lipidomics profiling on liver tissue from a mouse model of chronic sepsis⁹⁶. Our analysis revealed significant dysregulation of hepatic mitochondrial energetics (OXPHOS & TCA), redox balance, and lipid metabolism in response to cecal ligation and puncture-induced sepsis. Additionally, we investigated the potential protective effects of restoring hepatic metabolic dysfunction observed during sepsis in our model. Our results show that administration of DCA to restore mitochondrial metabolic and bioenergetic processes restores multiple pathways involved in mitochondrial, lipid, and redox metabolism, ultimately leading to protective effects as previously demonstrated⁹⁷.

Chapter 4: Regulation of hepatic lipid metabolism by itaconate during sepsis

Goal: To gain a more detailed understanding of the specific effects of itaconate on non-immune cell metabolism during sepsis, with the ultimate goal of comprehensively evaluating its impact on hepatic lipid metabolism.

In our previous research, our lab observed an increase in the accumulation of itaconate, a TCA cycle metabolite, in septic hepatocytes during sepsis⁹⁶. However, the biological function of itaconate in non-immune cells and tissues during inflammation, such as sepsis, has not been fully understood. To address this knowledge gap, we conducted a study in which we exposed wild-type and *Irg1*-deficient mice, which cannot produce itaconate, to septic conditions. Interestingly, our findings indicate that itaconate plays a protective role against sepsis-induced steatosis, as the loss of *Irg1* led to the exacerbation of hepatic lipid droplets and triglyceride accumulation. Hence, the objective of this chapter is to investigate and reveal the potential mechanism responsible for this protective adaptation.

Chapter 5: Concluding remarks

Goal: Discuss the significance of the data generated in Chapter II and Chapter IV and future directions to address limitations.

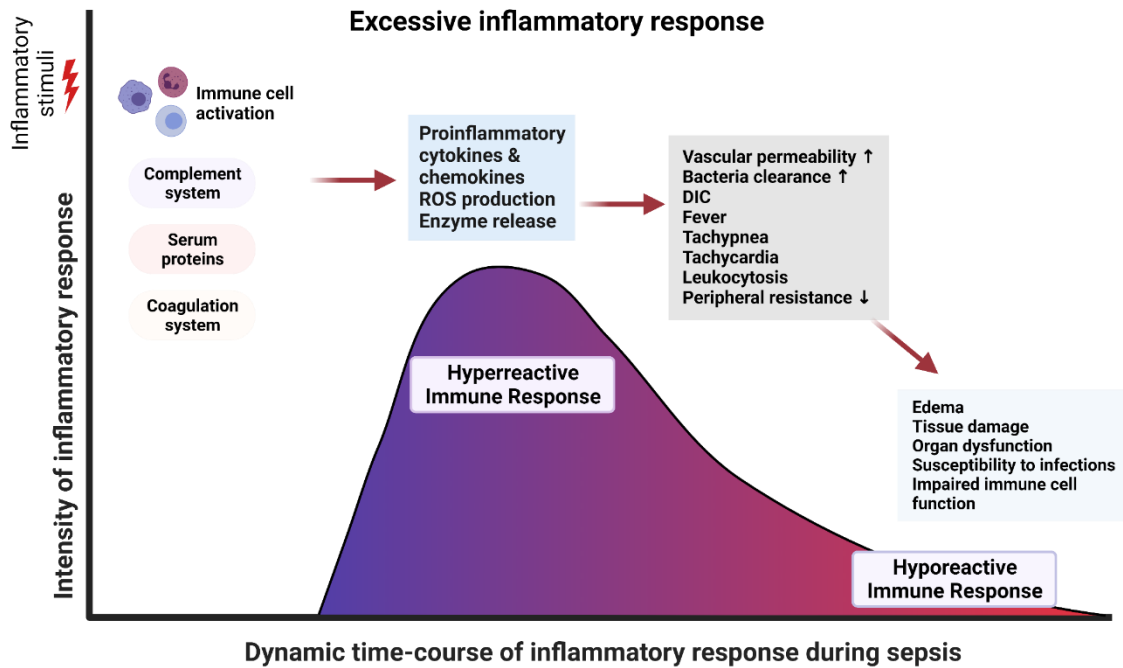
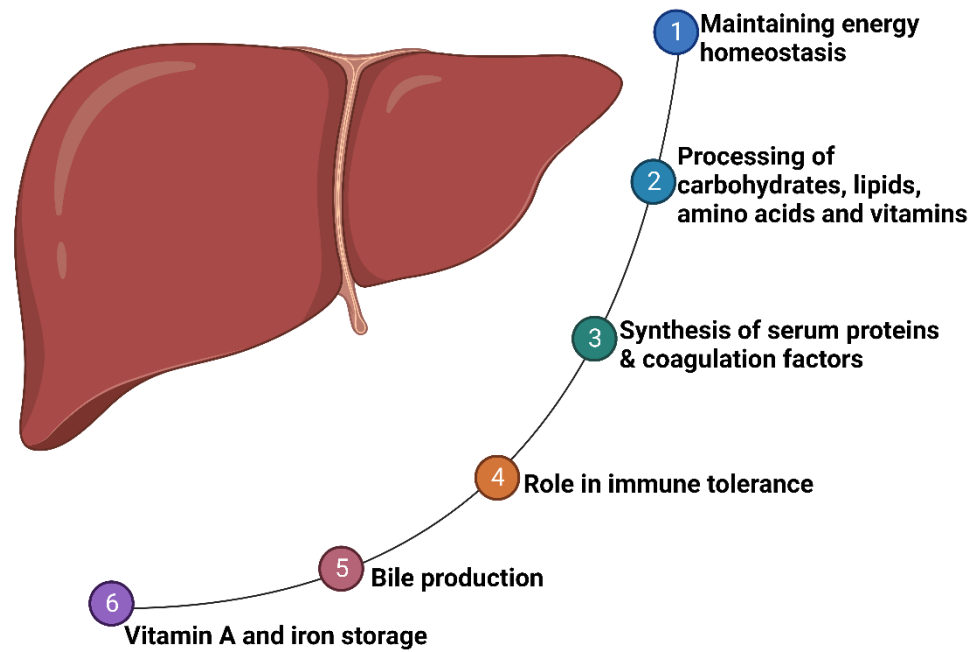


Figure 1: Dynamics of inflammatory response during sepsis. In response to inflammatory stimuli, the innate immune system is activated and produces proinflammatory cytokines, chemokines, and ROS. This excessive production of proinflammatory mediators leads to an increase in vascular permeability and tissue and organ damage as well as dysfunction of immune cells which can promote immunosuppression susceptibility to mortality due to secondary infections.

Figure 2: Role of the liver. Illustration representing the role in the liver in the maintenance of systemic energy metabolism as well as immune response.



CHAPTER II

Itaconate and Regulation of signaling pathways

I. Itaconate biosynthesis and metabolism

TCA located in the mitochondrial matrix of cells is a common metabolic pathway central to glucose, fatty acid, and amino acid metabolism. It is responsible for the production of the majority of reduced coenzymes like NADH and FADH needed for the generation of ATP in the electron transfer chain^{98,99}. The last decade of research has demonstrated the occurrence of distinct breaks in the TCA cycle in activated immune cells downstream of citrate to support the synthesis of several immunomodulatory metabolites^{100,101}. An example of this is the upregulation of mammalian *Irg1*, the gene encoding mitochondrial enzyme immune-responsive gene 1 protein in response to inflammatory stimuli in immune cells¹⁰²⁻¹⁰⁵. Mechanistically, immune receptors TLR4 and IFNR, adaptor protein MYD88-STING complex, ubiquitin ligase A20 and TF like, IRF1, IRF3-JUN, NF- κ B, and STATs have been demonstrated to regulate the expression of *Irg1* in stimulus-dependent manner^{106,107}.

Upon synthesis, IRG1 decarboxylates TCA cycle intermediate cis-aconitate into itaconate¹⁰⁴ (**Fig 1**). Itaconate can be cleared via C₅-dicarboxylate metabolism via acylation to itaconyl-coenzyme (I-CoA) by I-CoA transferase, then catabolized into citramalyl-CoA by citramalyl-CoA lyase (CLYB) and proposed to produce pyruvate and acetyl-CoA by¹⁰⁸ (**Fig 1**). Recently, a structurally similar metabolite mesaconate was found to be synthesized in LPS-

stimulated macrophages downstream of itaconate catabolism¹⁰⁹. Additionally, citraconate, an isomer of itaconate, was recently identified as a novel IRG1 inhibitor¹¹⁰.

2. Itaconate modulation of metabolism

To date, numerous studies have identified key nodes within central carbon metabolism that are impacted by itaconate after its upregulation in response to inflammation. Specifically, itaconate alters the enzymatic function of succinate dehydrogenase (SDH), several glycolytic enzymes, and methylmalonyl-CoA mutase (MUT) which collectively alters cellular metabolism and affects cell response.

2.1 SDH inhibition by itaconate

In the sixth step of the TCA cycle, SDH catalyzed the oxidation of succinate to fumarate coupled with the reduction of ubiquinone to ubiquinol in the ETC. During immune cell activation, oxidation of succinate by SDH drives the generation of reactive oxygen species in the ETC, which promotes HIF-1 α stability and subsequently transcription of pro-inflammatory targets like IL-1 β and IL-6 supporting a pro-inflammatory phenotype. Furthermore, upon classical activation of macrophages, nitric oxide mediated TCA cycle, and OXPHOS disruption alters metabolism and drives the accumulation of succinate which is observed in diverse cell types. In 2016, Cordes et al. and Lampropoulou et al. both demonstrated an

increase in IRG1/itaconate leads to the accumulation of intracellular succinate during immune cell activation^{111,112} (**Fig 2**). Additionally, owing to its structural similarity to succinate, itaconate was found to be a competitive and reversible SDH inhibitor that altered succinate levels in various cell types. Itaconate was also found to influence glutamine metabolism to support this buildup. Furthermore, inhibition of SDH by itaconate is observed to play a role in regulating immune response as it significantly reduces inflammation-induced cytokine production and ROS generation in macrophages. Interestingly this ability of itaconate to inhibit SDH is also linked with the development of immune tolerance after the LPS challenge as itaconate dampens TCA cycle activity and induces accumulation of succinate¹¹³. Furthermore, blocking LPS-induced *Irg1* expression with β -glucan counteracted the immunosuppressive effects of itaconate via restoration of the expression of succinate in tolerant monocytes, highlighting its role in induction of tolerance and regulation of trained immunity.

2.2 Glycolysis Inhibition by Itaconate

To support their activity, immune cells undergo dramatic metabolic reprogramming in response to the changes in their environment. Collectively, both innate and adaptive immune cells undergo a metabolic switch between OXPHOS and glycolysis to support immune cell response during an inflammatory event. The Warburg effect, initially only thought to occur in tumor cells, is also observed in activated macrophages. While there are two types of macrophages: M1 and M2, only M1 phenotype macrophages that are activated by LPS or IFN- γ switch from

OXPPOS to glycolysis to support a pro-inflammatory response. Increased levels of PFK1/2 together with glucose transporters (GLUT1/6), hexokinases (HK1/2), and monocarboxylate transporter 4 (MCT4) are indicative of the Warburg effect. Furthermore, this is associated with an increase in HIF-1 α stability due to the generation of ROS from ETC and OXPPOS and the accumulation of succinate in TCA cycle which promotes transcription of glycolytic genes and pro-inflammatory cytokines like IL-1 β .

Derivatives of TCA cycle like fumarate have been previously found to down-regulate glycolysis and mediate anti-inflammatory effects via succinylation and inactivation of a catalytic cysteine of glyceraldehyde-3-phosphate GAPDH and LDHA both clinically and experimentally. Given the electrophilic and structural similarities between the two metabolites, itaconate was also discovered to regulate aerobic glycolysis and exert anti-inflammatory effects (**Fig 3**). Utilizing a novel thiol-reactive probe 3,4,6-OAc₃ManNAz, Quin et.al identified 260 itaconate-modified cysteines in LPS-stimulated RAW 264.7 cells¹¹⁴. They further found itaconate covalently modified Cys73 and Cys339 of ALDOA, Cys245 of GAPDH, and Cys84 of LDHA and modulated their enzymatic activities¹¹⁴. Additionally, ALDOA was found to be a more relevant target of itaconate in regulating glycolysis as macrophages expressing the double mutated Cys73 and Cys339 ALDOA exhibited decreased glucose consumption and production of IL-1 β upon LPS stimulation. Following this discovery, Liao et al. demonstrated modification of Cys22 of GAPDH upon cell-permeable derivative 4-octyl itaconate (4-OI) treatment in LPS-induced macrophages¹¹⁵. Given that GAPDH is a rate-limiting enzyme regulating aerobic

glycolysis they also investigated if this alkylation at Cys22 influenced its function. Indeed they find this alkylation by itaconate inhibited aerobic glycolysis and alleviated inflammation as they observed decreased IL-1 β , TNF α , inducible nitric oxide synthase (iNOS), and inhibition of NF-kB indicating an inhibition in macrophage activation and inflammation. They further explored the effect of endogenous itaconate by utilizing *Irg1*^{-/-} BMDMs and find heightened GAPDH activity, lactate production, and ECAR indicating an increase in aerobic glycolysis. These cysteine modifications of glycolytic enzymes have been further verified by Qin et al. newest bioorthogonal probe ITalk which allows for analyzing targets of itaconation directly in living macrophages¹¹⁶. Additionally, itaconate inhibits glycolysis by inhibiting PFK-2 activity, lowering the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate in the liver¹¹⁷. However, the mechanism by which this occurs is not known. In conclusion, these studies so far describe itaconate's role in immunomodulation of the glycolytic burst that is initially needed to support the pro-inflammatory phase of macrophages which over time is necessary to regulate. Furthermore, new studies have now revealed the upregulation of *Irg1* in non-immune cells upon inflammation, making it crucial to investigate glycolysis regulation by itaconate and its function in other tissues important in the maintenance of systemic metabolism in the face of inflammation.

Apart from its anti-inflammatory effects on mammalian cells, itaconate is also considered an anti-microbial metabolite as it inhibits bacterial growth via inhibition of IDH via covalent adduct formation through its catalytic cysteine residue. This inhibition blocks the glyoxylate shunt that catalyzes the cleavage of isocitrate to

succinate and glyoxylate required for many different bacteria for optimal growth during infections when glucose isn't available.

2.3 MUT inhibition and impact on propionate metabolism by itaconyl-CoA

The carbon produced from catabolism of BCAA and odd chain β -oxidation and cholesterol generates propionyl-CoA (p-CoA) which can be converted into anaplerotic product succinyl-CoA by methylmalonyl-CoA mutase (MUT). Succinyl-CoA will then enter the TCA cycle as an intermediate metabolite. Recently a novel intersection between itaconate and vitamin B₁₂/cobalamin-dependent metabolism has been revealed^{111,118,119} (**Fig 4**). Coenzyme B₁₂ is a source of 5'-deoxyadenosyl radical and cob(II)alamin (Vitamin B₁₂). During the catalytic cycle, binding of the substrate methylmalonyl-CoA (M-CoA) triggers the homolysis of the cobalt-carbon bond of the bound CoA-B₁₂, however, it was reported that I-CoA can behave like the substrate-analog and bind to the same site³⁰. This irreversible binding promoted the addition of 5'-deoxyadenosyl radical of the CoA-B₁₂ to its double bond forming a biradical adduct that consisted of a tertiary carbon radical coupled to cob(II)alamin radical leading to. This further lead to the impairment of MUT activity as well as recycling by the endogenous CoA-B₁₂ machinery. Furthermore, inhibition of MUT leads to the accumulation of its substrate propionyl-CoA and M-CoA which will influence MUT-dependent anaplerosis and decrease succinyl-CoA for SDH reaction and carbon flux into the TCA cycle. This would result in the accumulation of odd chain FA as p-CoA is the end product of β -oxidation of odd-numbered FA's which can affect FA metabolism downstream¹⁰.

Inactivation of methionine synthase, another B₁₂-dependent enzyme, by I-CoA dysregulates the regeneration of methionine from homocysteine which leads to alterations in abundances of S-adenosylmethionine (SAM), a methyl donor in numerous biological and biochemical processes. Recently Aso et al. demonstrated a reduction in methionine adenosyltransferase (MAT) activity in T cell subsets upon itaconate exposure which led to a reduction in S-adenosyl-L-methionine/-adenosyl-homocysteine ratio and altered chromatin accessibility of essential transcription factors regulating cell differentiation and autoimmunity¹²⁰. Furthermore, deficiency in B₁₂ has been found to induce cholesterol biosynthesis by limiting SAM and modulating the methylation of SREBF1 and LDLR genes¹²¹.

While the consequences of I-CoA on B₁₂-dependent pathways in mammalian cells are only now being discovered, it does explain its anti-microbial effects. Inhibition of MUT by I-CoA blocks propionyl-CoA-dependent *Mycobacterium tuberculosis* growth³⁰ as it can no longer oxidize propionate as a source of carbon to support biomass increase.

3 Itaconate modulation of KEAP1

Regulation of transcription and gene expression is dependent on structural properties like how DNA is packaged as well as its interactions with regulatory proteins called TF. TFs bind onto specific upstream DNA-binding domains/promoter regions to stimulate gene expression. Furthermore, the activity of TFs can be regulated in different ways. One of the most common ways for alternating the regulatory function of TFs is via covalent modification such as post-translation

modification such as phosphorylation and ubiquitination. Studies investigating the regulatory effect of itaconate have demonstrated its ability to alter transcriptional response in various cell types. One of the first to be studied was the regulation of NRF2 expression by itaconate (**Fig 5**). NRF2 is a TF central to the regulation of oxidative and electrophilic stress. Under basal conditions, NRF2 is bound to a repressor protein (KEAP1), a substrate adaptor protein for Cul3-containing E3-ligase complex, which targets NRF2 for ubiquitination and degradation via the ubiquitin-proteasome system (UPS). Upon exposure to oxidative/electrophilic stress observed during inflammation, KEAP1 dissociates from NRF2 and allows for its translocation into the nucleus driving induction of anti-oxidative target genes like *Nqo1* and *Hmox-1*.

Given itaconate's electrophile properties and the fact that it had demonstrated anti-inflammatory effects via SDH inhibition, but not to the same extent as malonate (classic SDH inhibitor), Mills et al. investigated KEAP1/NRF2 as potential targets of itaconate¹²². Utilizing 4-OI, itaconate's ability to alkylate cysteine residue 151, 257, 288, 273, and 297 on KEAP 1 was revealed. 4-OI induced a decrease in ROS levels and IL-10 and IL-1B in activated macrophages which was diminished in response to NRF2 silencing/deletion demonstrating itaconate's anti-oxidative and anti-inflammatory properties through Nrf2. Furthermore, they reported 4-OI limited inflammation and mortality in an *in vivo* model of LPS-induced sepsis is dependent on NRF-2 activation. Recently, utilizing underivatized itaconate and itaconate-specific probes has resulted in a similar

finding of alkylation of KEAP1 by itaconate, further supporting NRF2 activation via itaconate^{114,116,123}.

Another TF that is reported to be regulated by itaconate derivative dimethyl itaconate (DI) is activating transcription factor 3 (ATF3). ATF3 is rapidly induced upon cellular stressors by inflammatory cytokines such as TNF- α and IL-1 β . DI and natural itaconate are reported to induce electrophilic stress due to covalent conjugation with GSH, reducing overall cellular GSH concentration which consequently leads to an increase in the generation of ROS¹²⁴. The electrophilic stress induced by DI was further reported to elevate protein expression of ATF3 and cause inactivation of a subunit of eIF2 α and subsequently inhibited I κ B ζ translation and reduced secondary inflammatory response. I κ B ζ is a member of the nuclear I κ B family of proteins that are associated with subunits of NF- κ B and regulates its transcriptional activity to modulate inflammation. Furthermore, this increase in ROS by itaconate has also been demonstrated to promote endotoxin tolerance as it induces A20 deubiquitinase expression to suppress NF- κ B and subsequent expression of inflammatory genes upon a second LPS challenge¹²⁵.

4. Itaconate modification of NLRP3

Inflammasomes are large multi-protein complexes found in cells of innate immune systems like macrophages that play crucial roles in shaping immune responses during host inflammatory diseases. So far four types of inflammasomes have been identified: NLRP1, NLRP3, NLRP4, and AIM2. Of these, one of the well-studied is NLRP3 inflammasome which is activated in a canonical or non-canonical

manner in cells of myeloid lineages like macrophages. Canonical NLRP3 inflammasome activation first requires initial priming by TLR ligand like LPS and is mediated by TLR4/MyD88/TRIF dependent pathway which induces pro-IL-1 β , IFN, and NLRP3 transcription via NF- κ B activation (signal 1). The second part of the activation results in NLRP3 self-oligomerization and recruitment of ASC and caspase-1 leading to proteolytic cleavage of pro-IL-1 β and pro-IL-18 along with its release and induction of pyroptosis via GASMD activation (signal 2). Extracellular DAMPs like ATP and ROS can also promote NLRP3 oligomerization and activation. Alternatively, during non-canonical NLRP3 activation, IFN produced during the priming step can also provide a feedback loop to activate caspase-11 (mouse 4/5) expression which promotes NLRP3 oligomerization to induce pyroptosis and release of mature IL-1 β .

Given the anti-inflammatory effects and decrease in mature IL-1 β and IL-18 release upon itaconate treatment, its role in the regulation of NLRP3 inflammasome was investigated previously. Initially, Lampropoulou et al. reported suppression of NLRP3 inflammasome as *Irg1*^{-/-} macrophages displayed elevated release of mature IL-1 β and IL-18 upon LPS and ATP stimulation¹¹². Following this, Swain et al. demonstrated that non-derivatized itaconate inhibited the maturation and release of IL-1 β in LPS-treated BMDMs indicating the inactivation of the NLRP3 inflammasome. However, this was independent of pro-IL-1 β expression indicating inhibition of NLRP3 at the level of signal 2 or the activation step¹²⁶. In contrast, 4-OI and DI which are stronger electrophilic derivatives were able to alter the

induction of pro-IL-1 β indicating the use of derivatives might not be reflective of mechanisms regulated by endogenous or natural itaconate. Furthermore, Hooftman et al. also confirmed that endogenous itaconate does regulate NLRP3 activation as LPS stimulated *Irg1*^{-/-} KO BMDMs exhibited increased NLRP3 activation in their hands as well¹²⁷. However, the mechanisms by which itaconate regulated NLRP3/caspase-1 dependent cleavage and release of IL-1 β was still not understood. To this end, they mechanistically demonstrated 4-OI blocked NLRP3 inflammasome activity via itaconation of Cys548 on NLRP3 which inhibited its interaction with serine/threonine-protein kinase Nek7 (NEK7)¹²⁷, an essential mediator of NLRP3 assembly and activation (**Fig 6**). Furthermore, 4-OI decreased the release of IL-1 β in an *in vivo* model of peritonitis and in PMBCs isolated from human patients with a cryopyrin-associated periodic disease which is a result of mutations in the *Nlrp3* gene highlighting its therapeutic potential in inflammatory diseases.

More recently, the accumulation of itaconate in synergy with iNOS in activated macrophages has been associated with immunotolerance via its regulation of NLRP3 activation¹²⁸. Bambouskova et al. described a novel mechanism to support this utilizing a model of tolerization via LPS priming (24 hours, signal 1) followed by a washout and rest phase for an hour before restimulation with LPS for another 3 hours (tolerizing phase) and ATP for forty-five minutes (signal 2 activation)¹²⁸. They find that restimulation of activated WT macrophages (24hr with LPS) after washout with a secondary challenge (3hr LPS,

45min ATP) resulted in downregulation of IL-1 β secretion while activated *Irg1*^{-/-} KO macrophages secreted IL-1 β to a similar extent as activated non-tolerized WT macrophages. This was independent of differences in pro-IL-1 β expression while ASC specking was greater in response to itaconate in tolerized macrophages demonstrating intrusion at signal 2 levels. Furthermore, they find tolerized *Irg1*^{-/-} KO macrophages exhibited GASMD processing/ cleavage and pyroptosis upon late NLRP3 activation which was abrogated in WT counterparts. To understand the mechanisms behind this, they performed a proteomic screening and identified Cys77 of GASMD as a target of itaconation, however, the functional consequence of this has yet to be understood. Furthermore, they identified iNOS as an important co-regulator of NLRP3 inflammasome tolerance that synergizes with itaconate as *Irg1*^{-/-} KO macrophages treated with iNOS inhibitor exhibited increased NLRP3 inflammasome activation. Additionally, treatment of *Nos2*^{-/-} and *Ifnar1*^{-/-} lacking iNOS activity with itaconate did not rescue inflammasome tolerance, further highlighting the synergism needed to establish late inflammasome tolerance. Overall these findings highlight the physiological consequence of endogenous itaconate isn't just purely immunosuppressive as some demonstrated, but rather immunomodulatory as prolonged activation of the inflammasome can result in pyroptotic cell death and tissue damage. Lastly, itaconate's role in the regulation of other types of inflammasomes in the face of inflammation needs to be further investigated.

5. Itaconate in liver injury/disease

Given itaconate's immunomodulatory properties in the immune compartment, researchers have begun to question this metabolite's functional properties in the non-immune compartment and therapeutic potential in the context of inflammatory diseases. Numerous pre-clinical studies have already demonstrated the therapeutic potential of itaconate and its derivatives in the resolution of inflammatory and oxidative stress-induced diseases such as autoimmune diseases, virus-induced inflammation, sepsis-induced inflammation, and ischemia-reperfusion injury. One organ that is of interest is the liver as it plays a central role in sensing and responding to systemic inflammation. In addition to hepatocytes that are crucial to the regulation of energy metabolism, the liver also harbors resident myeloid immune cells that are involved in maintaining local and systemic immunological homeostasis. Furthermore, many liver diseases demonstrate a perturbation of redox homeostasis and inflammation that could potentially be resolved with the administration of exogenous itaconate and or its derivatives.

5.1 Ischemia-reperfusion injury

Hypoxic hepatitis, also known as ischemic hepatitis, is a pathological condition that typically results due to an inadequate supply of blood and oxygen to the liver which can lead to hypoxia and cell damage. This condition occurs in numerous clinical settings such as infection-induced sepsis, liver transplant, dehydration, severe bleeding, or cardiac failure. Furthermore, due to alterations in

underlying mechanisms regulating immune, vascular, endothelial, and mitochondrial homeostasis, reperfusion of the liver post-ischemia exacerbates cellular dysfunction and leads to ischemia-reperfusion injury. IRI can be divided into two phases. The early phase of hepatic injury is characterized by robust activation of KCs, liver resident macrophage, upon reperfusion which has been shown to release significant levels of ROS that causes oxidative stress in the liver parenchyma and promote tissue damage¹²⁹. To maintain redox balance in the face of injury, the liver can up-regulate Nrf2 antioxidant pathways which are key for the regulation of oxidative stress.

Given itaconate's ability to activate Nrf2 signaling, Yi et al. investigated its protective role against oxidative injury upon reperfusion of ischemic liver via upregulation of Nrf2. They became the first to demonstrate increased expression of *Irg1* in non-parenchymal cells as well as hepatocytes in response to IR injury¹³⁰. Furthermore, they reported the loss of *Irg1* in either hematopoietic or non-hematopoietic compartments leads to exacerbation of hepatic oxidative injury and inflammation during reperfusion in an Nrf2- dependent manner. This response was even more severe in mice with loss of *Irg1* in both the hematopoietic and non-hematopoietic compartments, revealing an unappreciated regulatory role of itaconate in non-immune cells like hepatocytes. Exogenous administration of 4-OI to both WT and *Irg1* KO mice that underwent IR exhibited amelioration of IR injury demonstrated by the decrease in ALT and liver necrosis upon reoxygenation. Following this study, Ma et al. also confirmed that itaconate does reduce the degree of hepatic impairment and inflammation as they observed a decrease in serum ALT,

AST, TNF1- α and IL-1 β and improvement in liver morphology upon exogenous treatment with DI before reperfusion¹³¹. Moreover, exposure of isolated peritoneal macrophages to DI before reoxygenation reduced inflammatory response via reduction of p65 nuclear translocation, one of five subunits required for assembly and activation of NF-kB transcription complex, compared to the control group which presented with a significant increase in p65 upon reoxygenation. Additionally, DI also attenuated the expression of mediators of NLRP3 inflammasome activation like NLRP3, GASMD, caspase-1, and IL-1 β upon reoxygenation indicating inhibition of NF-kB pathway activity during IR, which in turn can reduce liver inflammation and injury (**Fig 7**).

Recently another interesting role of itaconate in preventing inflammatory injury in exercised mice before hepatic IR was revealed¹³². Mechanistically Zhang et al. reported 4 weeks of exercise before IR-induced HMGB1 release via TLR4 activation led to KC metabolic reprogramming favoring itaconate production that affected KCs in Nrf2 dependent manner. They demonstrated a primary challenge with pre-operative exercise therapy induces anti-inflammatory trained immunity in KCs via regulation of itaconate metabolism. This was contradictory to a previous study by Andres et.al where itaconate was found to inhibit the induction of trained immunity in human monocytes¹¹³. Furthermore, itaconate was found to be protective and mitigated liver injury upon secondary challenges like hepatic I/R and even LPS stimulation as loss of *Irg1* worsened hepatic injury and ameliorated the benefits of exercise. Interestingly other studies have also demonstrated modulation of the hepatic immune response or macrophage polarization during IR can

ameliorate hepatic injury¹³³⁻¹³⁵. Taken together, metabolites like itaconate that can influence immune cells to modulate their inflammatory response could be potentially exploited to develop novel pre-operative therapy especially when IR injury is anticipated as a complication to procedures like liver transplant.

Additionally, itaconate and its derivatives have been demonstrated to have an overall protective effect in renal¹³⁶, heart^{112 137} and brain¹³⁸ ischemia-reperfusion via regulation of inflammatory response and oxidative stress highlighting its regulatory role in various tissue types. Future studies using cell-specific *Irg1* deficient models (both immune and non-immune) are warranted to fully understand the global expression and functional relevance of itaconate and its therapeutic potential in the resolution of IR injury.

5.2 Itaconate and Hepatic Lipid Metabolism

The liver is known to play an important role in the regulation of lipid metabolism and homeostasis. It does so primarily through 1) acquisition of lipids via uptake of lipids and FA, 2) *De novo* lipogenesis, 3) TG synthesis and formation of lipid droplets (LD), 4) β -oxidation, and 5) LDL secretion for cholesterol transport¹³⁹.

The liver serves as an intermediate organ as it supplies energy to extrahepatic organs and alterations of hepatic metabolism can drive the onset of metabolic conditions like non-alcoholic fatty liver disease (NAFLD). NAFLD represents a variety of liver conditions ranging in severity from injury to the development of fibrosis. The most common and highly relevant risk factors for

NAFLD include a sedentary lifestyle, obesity, type II diabetes, and hypertension. Non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) are two types of NAFLD, with the latter being a malignant form. Both conditions demonstrate accumulation of intra-hepatic fat, metabolic stress, oxidative stress, and inflammation. Additionally, the liver's ability to regulate lipid metabolism is also influenced by hormones, metabolites, and transcriptional factors that are activated by various environmental cues. Since there are no defined pharmacological drugs for treatment, management of NAFLD relies on lifestyle changes along with anti-obesity, anti-diabetes, anti-fibrotic, anti-oxidant, and anti-inflammatory drugs. There are multiple ongoing clinical trials featuring new classes of drugs like therapeutics targeting FXR agonists (NCT05415722, NCT04408937), FGF stimulants (NCT03486899, NCT00968747, NCT05655221), GLP-1 agonists (NCT05203237, NCT05140694, NCT03648554), THR- β agonists (NCT05526144, NCT04951219), and PPAR regulators (NCT04849728, NCT03639623).

Early papers had initially demonstrated itaconate regulated β -oxidation of FAs to promote mitochondrial ROS generation and OXPHOS of macrophages^{140,141}. In the last few years, an unappreciated role of itaconate and its derivatives in the regulation of hepatic lipid metabolism in metabolic disorders has been identified. In early 2022 Chu et al. reported 4-OI attenuated oxidative stress and lipid accumulation in an *in vitro* FFA-induced NAFLD in various hepatocyte cell lines¹⁴². Mechanistically, they demonstrated this positive effect of itaconate via activation of the AMPK pathway via Nrf2-mediated inhibition of mitochondrial ROS production. Activation of AMPK signaling increased its phosphorylation which

inhibited downstream lipid droplet accumulation and triglyceride levels and alleviated FFA-induced lipid droplet accumulation. While the data in this paper demonstrated 4-OI's ability to regulate lipid metabolism, the role of endogenous itaconate and *in vivo* and clinical relevance wasn't investigated. The onset and progression of NAFLD is a complicated condition that doesn't just include hepatocytes but also a variety of immune cells (residential and recruited), endothelial cells as well as non-hepatic cells like adipose cells. Therefore, it is crucial to characterize the contributions of itaconate production in other cell types in the regulation of lipid metabolism during NAFLD. Additionally, few other studies have reported the induction of *Irg1* in non-immune cells in response to inflammation, further highlighting this need to investigate its regulatory role in those cell types.

More recently, Frieler et al. reported an alteration in overall energy metabolism in response to *Irg1* deletion in normal mice as there was an increase in glucose oxidation and a decrease in fat oxidation in *Irg1* KO mice compared to WT controls¹⁴³. Interestingly, as these mice aged, they spontaneously developed obesity and glucose intolerance. On the contrary, itaconate-expressing mice were leaner and protected from age-induced obesity. Furthermore, these knockout mice exhibited increased expression of genes involved in lipid and fatty acid biosynthesis and increased inflammatory gene expression in eWAT in comparison to wild-type obese controls. It was hypothesized that these effects of itaconate in counteracting lipid accumulation were due to its regulation of adipose lipid metabolism, however, the role of the liver was discounted even though induction of *Irg1* was not completely lost in the liver despite LysM-Cre KO of *Irg1* in myeloid cells upon LPS

stimulation. This suggests expression of *Irg1* in the liver is independent of expression in myeloid cells, however, based on the data it cannot be concluded what cell types are contributing to this observation.

Contrary to this, recently Weiss et al. reported hepatic macrophages are the source of itaconate accumulation in the liver during inflammation as macrophage-specific deletion of *Irg1* diminished itaconate abundance in hepatocytes in response to WD¹⁴⁴. However, liver-specific deletion of *Irg1* had no effects on itaconate abundance even though *Irg1* was no longer being expressed. Interestingly, differing from the findings of Frieler et al, they didn't observe expression of *Irg1* in adipose macrophages which do play an important role in promoting insulin resistance and hepatic lipid accumulation. Furthermore, they find WD fed *Irg1* KO mice had heightened hepatic steatosis that was associated with lipid accumulation in macrophages and not hepatocytes. Interestingly, they also observed upregulation of *Irg1* and itaconate levels in human NASH biopsy samples and a reduction of lipid accumulation in human hepatocytes upon itaconate treatment. Mechanistically, by utilizing isolated F4/80⁺ macrophages, they find itaconate produced by macrophages promotes fatty acid oxidation in hepatocytes which they proposed was to compensate for the reduction in ATP levels driven by the loss of mitochondrial substrate level phosphorylation and subsequent consumption of ATP during activation of itaconate to itaconate CoA. Lastly, administration of 4-OI reversed the hyperlipidemia and adiposity in WD-fed *Irg1* KO mice providing evidence for its therapeutic potential. Overall, their data identified a novel crosstalk between macrophage metabolism and hepatocytes in the regulation of lipid

metabolism that could be potentially exploited for the treatment of metabolic diseases like NAFDL.

Zhang et.al, have also confirmed this role of itaconate as deletion of *Irg1* in their HFD mice model also demonstrated aggravated NAFDL¹⁴⁵. They further reported decreased expression of *Irg1* in the steatotic liver and AML12 cells in response to HFD and palmitic acid/ oleic acid exposure as the underlying factor promoting the development of NAFLD. Mechanistically, *Irg1*/itaconate activated the Nrf2 pathway which lead to the inhibition of oxidative stress-induced AKT suppression. Exogenous administration of 4-OI was protective as it attenuated oxidative stress and hepatic steatosis via modulation of ATK. This was interesting as suppression of AKT signaling has been previously associated with metabolic dysfunction and its activation or restoration alleviates liver injury and steatosis during NAFDL¹⁴⁶. Additionally, DI and 4-OI have also been reported to have anti-melanogenic effects and improve osteoarthritis via regulation of AKT signaling^{147,148}. However, it is not known if the itaconation of cysteine residues of ATK independent of ROS regulation also accounts for some of the effects observed. Taken together, these data suggest that itaconate has the potential to modulate different pathways underlying the development of NAFLD. Future studies are however warranted to explore its full pharmacology potential and cell-specific effects concerning pathways critical to the regulation of energy and more specifically lipid metabolism.

6. Sepsis and itaconate

As discussed in Chapter I, sepsis due to hyperinflammation that occurs post-infection or traumatic events leads to tissue/organ damage and death if the host's response to the inflammation is uncontrolled. Additionally, in chapter II, in conjunction with what others have reported, we observed a break in the TCA cycle leading to alterations in multiple TCA intermediary and TCA cycle-derived metabolites such as itaconate. Furthermore, we identified itaconate as the most highly accumulated hepatic metabolite in response to sepsis. And given itaconate's immunomodulatory properties and association of sepsis with immune dysregulation, studies have already begun to untangle its biological function during sepsis. In LPS induced mice model of sepsis, administration of itaconate derivative 4-OI and DI have been reported to reduce oxidative stress, cytokine storm, and organ damage and improve survival ^{122,149-151}. While the exact mechanism by which itaconate exerts its effects on sepsis is not known, it is reported that Nrf2 accumulation and activation, inhibition of aldolase and other glycolytic mediators, SDH inhibition by itaconate, and inhibition of STING phosphorylation in activated macrophages all contribute towards dampening of the inflammatory response (**Fig 8**). As previously discussed, the development of immunosuppression is commonly observed with sepsis progression and after resolution. Due to the underlying immunosuppression, mortality rates due to secondary infections are high among sepsis survivors. During this phase, immune cells such as macrophages become tolerant to LPS and can no longer respond to the new inflammatory stimulus. PBMCs from septic patients and macrophages from LPS tolerized mice were found

to significantly upregulate *Irg1* expression¹²⁵. Furthermore, *Irg1* was found to increase A20 expression through ROS-mediated trimethylation of the 4th lysine residue of histone on the A20 promoter. This led to a significant reduction in cytokines levels of inflammation in LPS-tolerized macrophages associating itaconate with late-phase immunosuppression¹²⁵. Moreover, knockdown¹²⁵ and inhibition of *Irg1* expression by β -glucan¹¹³ reverted itaconate-induced endotoxin tolerance in stimulated macrophages. Taken together these results indicate itaconate's role in both anti-inflammation during the acute phase of sepsis as well as modulation of immunosuppression during the chronic phase of sepsis. However, more studies on mechanisms linking the two and how they can be exploited for sepsis treatment are warranted.

7. Conclusions

Some studies have reported the therapeutic value of itaconate and its derivatives in sepsis resolution, however, the role of non-immune mechanistic pathways tissues like the liver in haven't been well investigated. Furthermore, as discussed earlier, few studies have elucidated itaconate's role in the regulation of hepatic lipid metabolism in metabolic disorders like NAFLD (**Table I**). However, its role in the regulation of lipid metabolism that is robustly dysregulated during overt inflammation like sepsis is not known. Therefore, given the robust accumulation of itaconate in the liver, similar to mitochondrial reprogramming seen in macrophages, during sepsis in Chapter II, we wanted to further investigate its hepatic-specific biological function and association with lipid metabolism in Chapter IV. Molecular

regulations driving the dysregulation of lipid metabolism and their physiological consequences are not completely known given the complexity of this pathway. As of now, there are no specific therapeutic options available to treat sepsis and associated organ dysfunction. Therefore, it is imperative to delve deeper into the mechanisms underlying the onset of hepatic lipid metabolic reprogramming to identify potential therapeutic targets. Developing novel approaches for restoring and preserving liver function during sepsis and other inflammatory diseases depends on these new investigations.

Figure 2: Summarized illustration of SDH inhibition by itaconate.

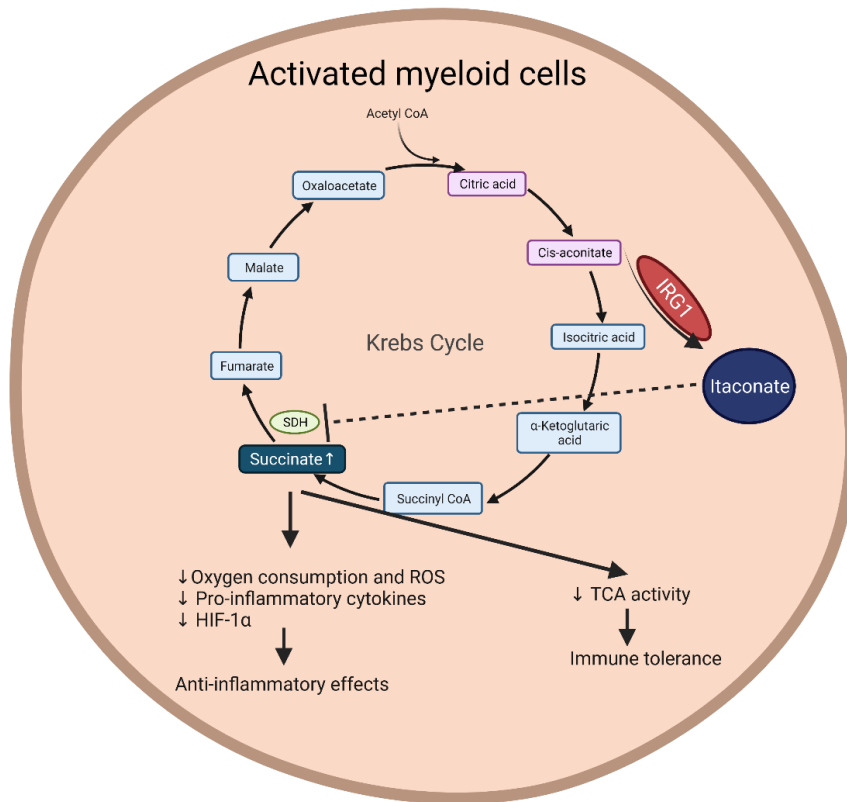
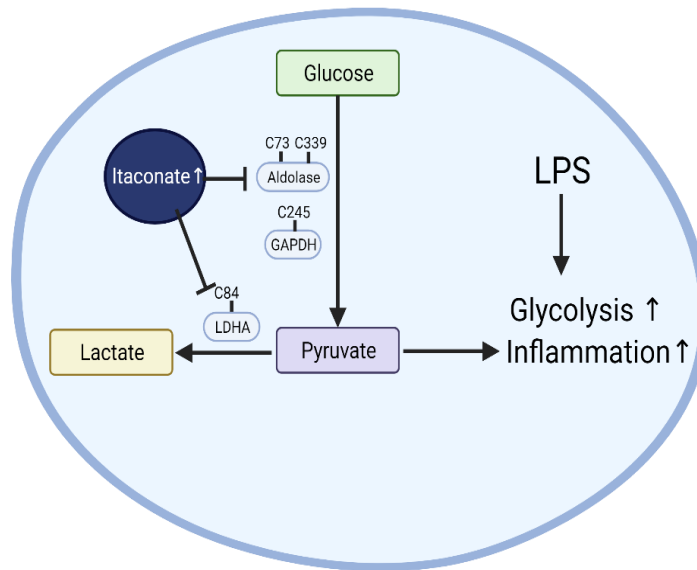


Figure 3: Cysteine residue modifications of glycolytic enzymes by itaconate.



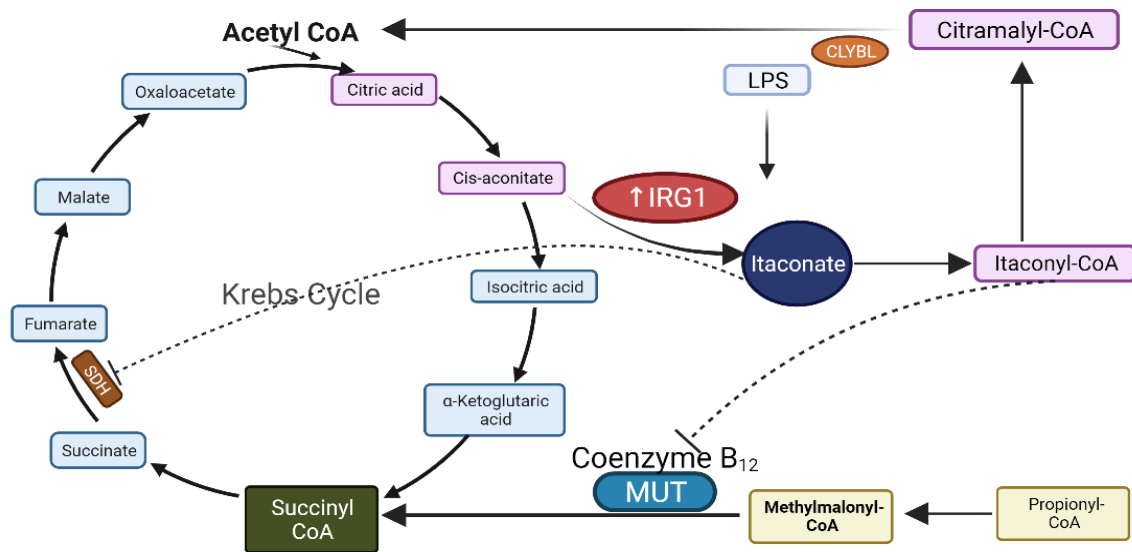
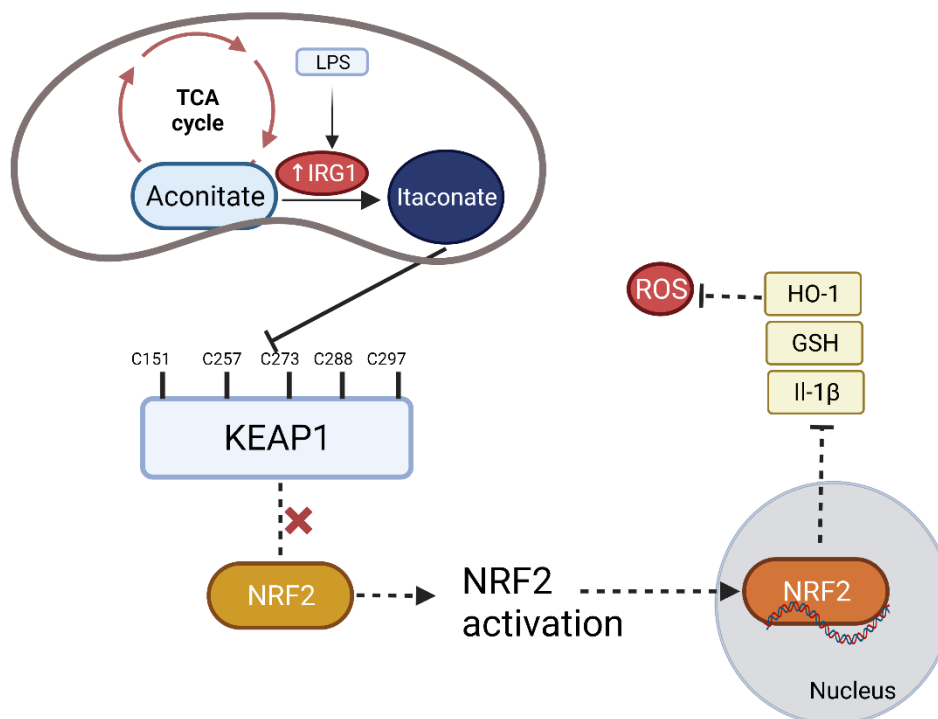


Figure 4: Graphical illustration summarizing itaconyl-CoA inactivation of coenzyme B₁₂. This leads to MUT inhibition and thus conversion of methylmalonyl- to succinyl-CoA is also inhibited.

Figure 5: Graphical illustration summarizing KEAP1 alkylation by itaconate leads to NRF2 activation.



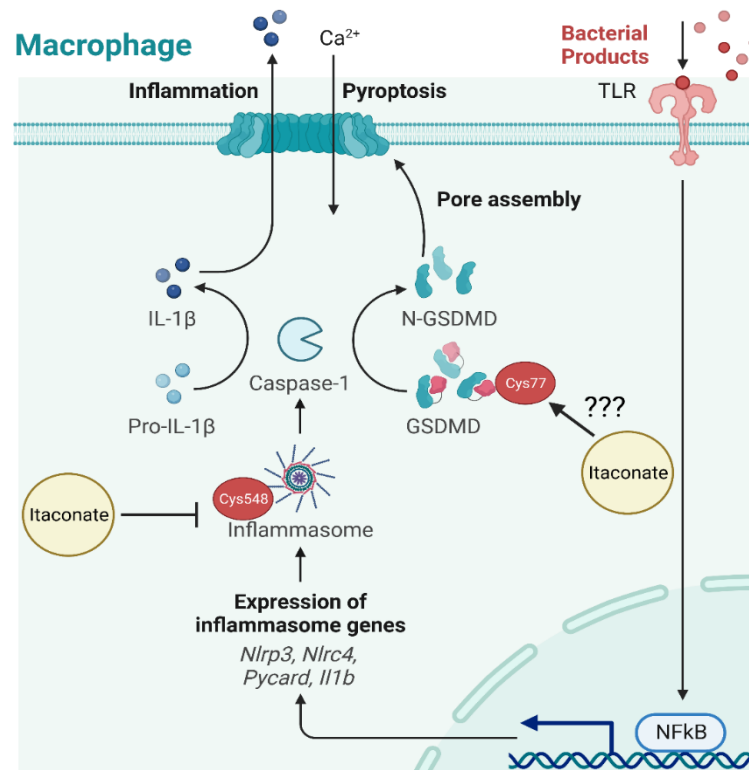


Figure 6: Graphical illustration summarizing NLRP3 inflammasome regulation by itaconate. Modification of Cys548 of NLRP3 by itaconate inhibits NLRP3-NEK7 interaction and activation which will prevent the processing of IL-1 β , IL-18, and GSDMD. Cys77 of GASMD is also a target of itaconation, however, the functional consequence is not understood.

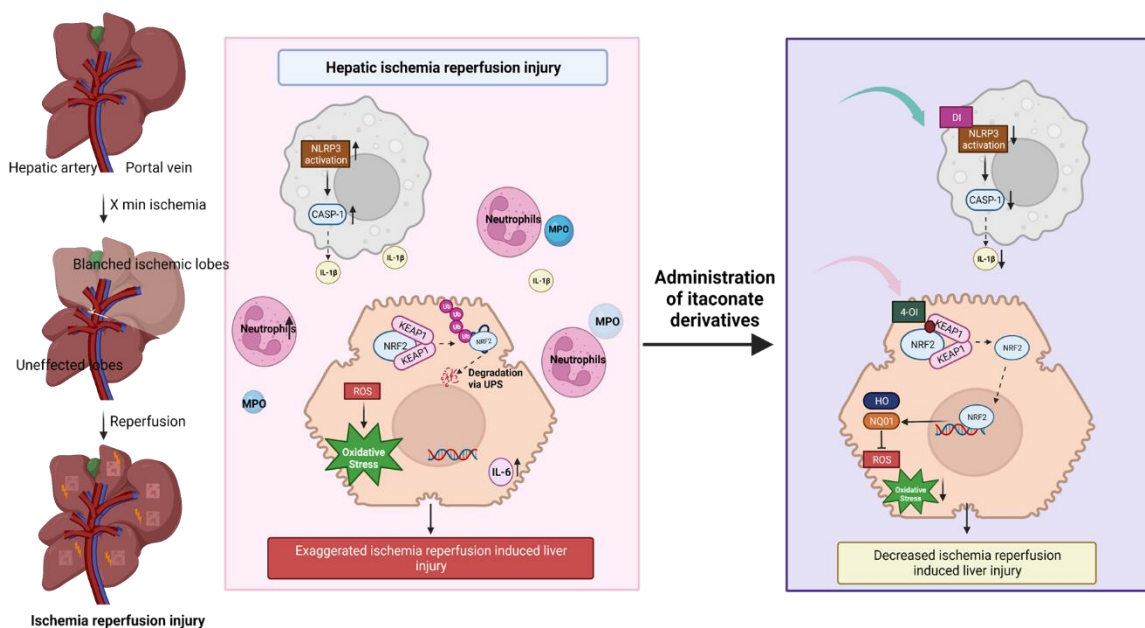


Figure 7 : Summarized graphical abstract of related pathways involved in itaconate regulation of ischemia reperfusion-induced liver injury. Administration of itaconate derivatives can exert anti-inflammatory and anti-oxidative effects by suppression of Keap1 and NLRP3 inflammasome in hepatic macrophages.

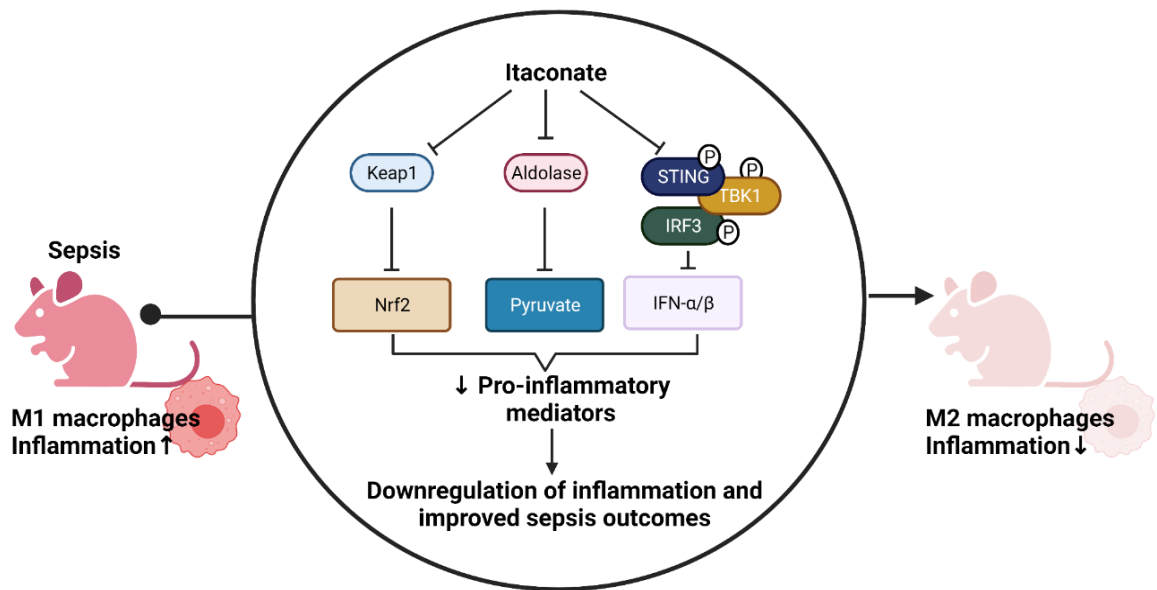


Figure 8: Summarized graphical abstract of itaconate relation of pathways involved in the improvement of sepsis. Itaconate can exert anti-inflammatory and anti-oxidative effects via activation of Nrf2 and suppression of aerobic glycolysis and STING phosphorylation in activated macrophages.

Table I: Therapeutic evidence of itaconate and its derivatives in the hepatic inflammatory disease models.

Model	Experimental condition	Outcome
Juvenile gibel carp ⁵⁴ -metabolism	0.1% 4-OI containing diet	- Inhibition of LPS-induced glycolysis in rat cells (NCTC1469) 4-OI inhibited glycolysis to reduce liver injury induced by enteritis
Juvenile gibel carp-metabolism ⁵⁵	0.1% 4-OI with soybean diet	Supplementation of 4-OI decreased hepatic inflammation and changes in glucose and lipid metabolism induced by soybean consumption via regulation of Nrf2-regulated PPARγ pathway
Male Sprague-Dawley rats-lipid metabolism ¹⁹	20g/L ITA solution/water	- Decreased visceral fat, triglycerides, and free fatty acids in rats supplemented with ITA - Inhibition of glycolysis by ITA due to reduction in fructose-2,6-bisphosphate
Mouse-Liver ischemia/reperfusion ³²	IP injection of 4-OI (25mg/kg) 2 hours pre I/R	- Irg1 KO demonstrated suppression of Nrf2 pathway and an increase in oxidative stress - Decreased hepatic injury with 4-OI administration
Mouse- CLP-induced sepsis ⁵⁶	IP injection of 4-OI (25 & 50mg/kg)	OI improved liver damage caused in response to sepsis
Mouse- Hepatocarcinogenesis ⁵⁷	Diethylnitrosamine administration to Irg1 ^{+/+} and Irg1 ^{hep-/-}	- Increased expression of Irg1 in response to DEN - Irg1 promoted apoptosis of hepatocytes independent of itaconate due to trapping of anti-apoptotic Mcl-1 which downstream leads to the release of cytochrome c initiating apoptosis and subsequent hepatocarcinogenesis. - DI inhibited NF-kB/NLRP3 pathway activation and promoted the resolution of inflammation and IR injury.
Mouse-Liver ischemia/reperfusion ³³	IP injection with dimethyl itaconate (DI)	DI inhibited NF-kB/NLRP3 pathway activation and promoted the resolution of inflammation and IR injury.
Mouse- HFD-induced NAFLD ⁴⁸	Oral gavage of 50mg/kg 4-OI for 6 weeks of the 14-week HFD feeding period	- Deletion of Irg1 worsens HFD-induced NAFLD and modulates oxidative stress and AKT signaling in fatty liver 4-OI improves HFD-induced steatosis via activation of P13K/AKT.
Infection- Zika virus ⁵⁸	In vitro 150uM 4-OI in Huh7 cells infected with Zika	Hih-7 cells pretreated with 4-OI reduced viral replication.
In vitro-metabolism ¹⁴	2mM Itaconic acid	- Exogenous ITA leads to an increase in endogenous ITA - Itaconate increases succinate accumulation in Huh7 cells but isn't metabolized to further fuel carbons into TCA cycle - ITA is a competitive and reversible SDH inhibitor, drives cells to accumulate glutamine-derived succinate, alters BCAA and nitrogen metabolism, modulates propionate metabolism by influencing vitamin B12 and MUT activity and increases odd chain FA abundances while decreasing acetyl-CoA levels.
Mouse-Concanavalin A (Con A) induced autoimmune hepatitis ⁵⁹	IP injection of 4-OI (50mg/kg) 2 hours before Con A administration	4-OI attenuates Con A-induced liver injury by upregulating Nrf2/HO-1 pathway and inhibiting ROS-mediated apoptosis of hepatocytes and pyroptosis of recruited macrophages
Mouse –Xenobiotic induced hepatic lipid disorder ⁶⁰	Oral gavage of 30ug/kg BW Tetrachlorodibenzo-p-dioxin (TCDD)	- TCDD causes an increase in hepatic itaconate which correlated with a decrease in vitamin B12 and inhibition of MUT. Hypothesized that conversion of itaconate into itaconyl-CoA inhibits MUT activity and represses genes associated with vitamin B12 dependent carboxylation pathway in liver in response to xenobiotics like TCDD - Decrease in MUT activity due to decrease in vitamin B12 following TCDD redirects propionyl-CoA metabolism to alternate vitamin B12 independent b-oxidation like pathway driving the accumulation of acrylyl-CoA which can contribute to hepatotoxicity and possibly the progression of steatosis to steatohepatitis and fibrosis.
In vitro (hepatocytes)-dyslipidemia ⁴⁵	25,50 & 100 uM 4-OI for 4 hours before FFA loading	4-OI alleviated FFA-induced lipid accumulation in hepatocytes 4-OI enhances Nrf2 which regulated ROS production - Decrease in ROS levels induced an increase in AMPK and ACC1 phosphorylation and alleviated lipid deposition in FFA-stimulated hepatocytes.
Mouse- HFD-induced obesity ⁴⁶	WT vs KO exposed to HFD	- Increase in hepatic Irg1 expression and itaconate abundance in response to LPS (Global & myeloid Irg1 KO) - Basal level decrease in fat oxidation and increase in glucose oxidation in Irg1 KO mice.
Mouse-WD induced NAFLD ⁴⁷	WT and KO exposed to western diet (WD)	- WD feeding increases hepatic Irg1 expression but was dependent on myeloid compartment Irg1 activation since macrophage-specific Irg1 KO lead to abrogation of Irg1 expression in hepatocytes but hepatocyte specific KO of Irg1 still have preservation of Irg1 expression. - Increased lipid accumulation in the liver of Irg1 KO on WD compared to WT on WD. - Increase insulin insensitivity and glucose intolerance in Irg1 KO fed a WD. - Loss of hepatic macrophage in Irg1 KO fed a WD. - Increase in b-oxidation and OXPHOS in response to Irg1 deficiency in WD fed mice. - Increase itaconate in human livers from NASH patients - Itaconate suppresses substrate level phosphorylation which reduces ATP level and as a compensatory mechanism causes b-oxidation and glycolysis increase to partially restore ATP levels and also regulates lipid levels.

Chapter III

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Dichloroacetate reverses sepsis-induced hepatic metabolic dysfunction

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Abstract

Metabolic reprogramming between resistance and tolerance occurs within the immune system in response to sepsis. While metabolic tissues such as the liver are subject to damage during sepsis, how their metabolic and energy reprogramming ensures survival is unclear. Employing comprehensive metabolomic, lipidomic, and transcriptional profiling in a mouse model of sepsis, we show that hepatocyte lipid metabolism, mitochondrial TCA energetics, and redox balance are significantly reprogramed after cecal ligation and puncture (CLP). We identify increases in TCA cycle metabolites citrate, cis-aconitate, and itaconate with reduced fumarate and triglyceride accumulation in septic hepatocytes. Transcriptomic analysis of liver tissue supports and extends the hepatocyte findings. Strikingly, the administration of the pyruvate dehydrogenase kinase (PDK) inhibitor dichloroacetate (DCA) reverses dysregulated hepatocyte metabolism and mitochondrial dysfunction. In summary, our data indicate sepsis promotes hepatic metabolic dysfunction and that targeting the mitochondrial PDC/PDK energy homeostat rebalances transcriptional and metabolic manifestations of sepsis within the liver.

Sepsis is a potentially life-threatening condition that occurs due to the body's overwhelming response to an infection. This reaction leads to aberrant immune responses and if not diagnosed and treated early after its onset, may limit survival by inducing coagulopathy, altered microvasculature, and dysregulation of the host's metabolism and organ function ¹⁵²⁻¹⁵⁸. Sepsis accounts for one in three hospital deaths in the U.S. and millions of deaths each year globally, highlighting its hazard to public health ^{7,159}. The high mortality rate associated with sepsis reflects the lack of a clinically viable molecular-based therapeutic target. Therefore, understanding the pathogenesis of sepsis at both the molecular and organismal level is of utmost importance to address major gaps in knowledge regarding sepsis.

The response to sepsis has classically been characterized as a biphasic phenomenon, where the acute phase is characterized by high energy consumption and hyper-inflammation associated with increased oxidation, followed by cellular reprogramming to a low energy, anti-inflammatory state of immunometabolic paralysis, with accompanying organ failure ¹⁵⁹⁻¹⁶². Additionally, during the early phase of sepsis, increased catabolism of fats, proteins, and carbohydrates, associated with high rates of oxygen consumption and ATP synthesis is observed ¹⁶³⁻¹⁶⁶. Following the acute phase is a hypometabolic state where ATP production and mitochondrial respiration decreases ¹⁶³. It is postulated that this is a protective mechanism to overall lower the metabolic demands of the cell and help with its recovery ^{152,167,168}.

Of particular interest, during the hyper-inflammatory anabolic phase of sepsis, an increase in the expression and activity of pyruvate dehydrogenase

kinase 1 (PDK1) consistently occurs ^{97,169}. This enzyme is one of four PDK isoforms that reversibly phosphorylates serine residues on pyruvate dehydrogenase complex (PDC) E1a subunit, inhibiting the conversion of pyruvate to acetyl coenzyme A (acetyl CoA) ¹⁷⁰. Inhibition of this important enzyme central to glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), and the lipogenic pathway is thought to be an important mechanism driving the dysfunction of mitochondrial respiration and cell bioenergetics observed during sepsis ^{97,171}. Therefore, PDK serves as a potential therapeutic target as its inhibition would allow for the downstream oxidation of glucose to continue, restoring OXPHOS and mitochondrial function known to be altered in both immune and non-immune cells during sepsis ^{97,166,172,173}.

The liver is an important metabolic and immune organ due to its role in nutrient metabolism and production of acute phase proteins. However, our understanding of transcriptional alterations and subsequent metabolic phenotypes elicited by sepsis still remain limited ^{166,174-177}. Furthermore, the mechanisms by which DCA elicits hepatic metabolic changes in response to sepsis are completely unknown. Hence, we set out to characterize the hepatic manifestations of sepsis, with the overall goal of identifying global metabolic pathways subject to dysregulation and whether these pathways are restored by DCA treatment.

Results

Sepsis Impairs Hepatic Mitochondrial Metabolism

To test the long-term hepatic transcriptional changes elicited by sepsis, we performed RNA-seq in whole livers thirty-hours post cecal ligation puncture (CLP). At this time point, septic mice exhibit tolerance, in which immunometabolic paralysis and end organ dysfunctions decrease survival. Ingenuity pathway analysis (IPA) of the top physiological pathways subject to alteration in response to sepsis revealed a significant increase in the acute phase response pathway, highlighting a persistent inflammatory state in the liver into the chronic phase of sepsis (Fig. 1a). Of particular interest, OXPHOS and mitochondrial dysfunction were the top two enriched pathways in the liver of septic mice (Fig. 1a). To gain further insight into the effects of sepsis on the transcriptional regulation of mitochondrial function, we performed gene set enrichment analysis (GSEA) of the OXPHOS pathway (GO:0006119). Septic mice at 30 h decreased the transcriptional output of OXPHOS components as evidenced by a negative GSEA enrichment score (Fig. 1b). Given the connection between OXPHOS and the tricarboxylic acid (TCA) cycle, we next asked if polymicrobial infection would elicit similar alterations in this pathway. In accordance with the changes observed in the OXPHOS pathway, we found a negative enrichment score for TCA cycle enzymes (GO:0006099) in the liver of CLP mice (Fig. 1c). Thus, our transcriptome data indicate that sepsis impairs mitochondrial metabolism in the liver.

Next, we wanted to assess if the transcriptional changes elicited by sepsis would manifest in altered hepatic TCA cycle metabolism. Therefore, we performed global unbiased metabolomic screening in isolated hepatocytes from control and septic mice by Ultrahigh Performance Liquid Chromatography-Tandem Mass

Spectroscopy (UPLC-MS/MS). In line with altered transcriptional regulation of the TCA cycle, we found that sepsis altered the relative abundance of multiple metabolites involved in the TCA cycle (Fig. 1d). In particular, significant elevation of citrate and cis-aconitate was observed in septic hepatocytes at 30h (Fig. 1e&f). Unlike macrophages, which shift their ratio of succinate and α -ketoglutarate to favor succinate accumulation over α -ketoglutarate¹⁷⁸⁻¹⁸⁰, we found no changes in the levels of these metabolites in hepatocytes (Fig. 1G&H). Furthermore, unlike macrophages¹⁸¹, levels of fumarate decreased, but malate levels were unchanged in hepatocytes from septic animals (Fig. 1I&J). Monocytes, macrophages and dendritic cells reprogram the TCA cycle during acute inflammation to a catabolic tolerance phenotype by shunting cis-aconitate to itaconate through the enzymatic action of aconitase decarboxylase (ACOD1; also known as immune responsive gene 1 [IRG1])¹⁸¹⁻¹⁸⁴. Accordingly, we also found that sepsis significantly induces *ACOD1* and elevates itaconate in isolate hepatocyte preparations (Fig. 1K&L).

Sepsis Shifts the Hepatic Redox Balance towards Oxidative Stress

Perturbations in OXPHOS have been shown to promote the production of reactive oxygen species (ROS)¹⁸⁵. Furthermore, itaconate has been shown to induce activation of the master KEAP1/NRF2 antioxidant pathway leading to increased expression of redox regulatory enzymes in macrophages¹⁸³. Therefore, we examined if sepsis invokes a similar gene expression profile in hepatocytes. GSEA of ROS metabolism (GO:0072593) revealed a positive enrichment, highlighting the transcriptional induction of genes involved in the regulation of redox

state in livers of septic mice (Fig. 2a). Given the induction of these genes, we wanted to determine if sepsis alters key metabolites involved in the maintenance of cellular redox balance, focusing on the transsulfuration pathway leading to glutathione biosynthesis. Sepsis did not alter intracellular homocysteine levels in isolated hepatocytes (Fig. 2-figure supplement 1), however, cystathionine decreased significantly (Fig. 2b). Furthermore, we found a trend for decreased cysteine levels in the liver ($p=0.0534$) (Fig. 2c) and significantly decreased glycine (Fig. 2d). Most dramatically, sepsis depleted hepatocyte glutathione (Fig. 2e) and led to decreased oxidized glutathione (GSSG) (Fig. 2f). Ophthalamate has recently been shown to be a biomarker of oxidative stress and signals consumption of hepatic GSH ¹⁸⁶. Functionally, we observe a significant accumulation of hepatic ophthalamate levels in response to sepsis, signifying a state of oxidative stress (Fig. 2g).

Sepsis promotes hepatic steatosis

Thus far, our data has revealed that sepsis induces significant impairments in hepatocyte OXPHOS, alterations in the TCA cycle, and induction of oxidative stress, findings that are also metabolic hallmarks of fatty liver disease ¹⁸⁷. Because perturbations in global lipid profiles occur in septic patients ¹⁶⁵, we evaluated the effects of CLP on the hepatic lipid metabolism pathway. In support of human data, our transcriptome data indicate significant repression of lipid metabolism in livers of septic mice (Fig. 3a). Sepsis also decreased transcriptional output of fatty acid metabolic process components in the liver, as evidenced by a negative GSEA

enrichment score (Fig. 3b). In particular, we find alterations in genes involved in both fatty acid oxidation as well as fatty acid biosynthesis (Fig. 3-figure supplement 1).

Since transcriptional regulation of hepatic lipid metabolism is significantly altered during sepsis, we further investigated the functional manifestation of the observed gene expression profile. Consistent with our transcriptional profiling studies, our unbiased metabolomic screening in hepatocytes also identified lipid metabolism as the top metabolic pathway altered in sepsis (Fig.3c). We observed increases in virtually all fatty acids and acylcarnitine derivatives assayed (Fig. 3d). The dysregulation in hepatic lipid metabolism in response to sepsis ultimately culminates in the development of steatosis, as evidenced by increased free fatty acids, mono-, di- and triglycerides (Fig. 3e). Intriguingly, genes involved in fatty acid elongation and desaturation show impaired expression in response to CLP at the time point surveyed (Fig. 3-figure supplement 1), suggesting the metabolic changes observed at our time point potentially arise from earlier transcriptional changes and the observed repression is compensatory in nature.

Sepsis remodels the hepatic lipidome

Given the severe dysregulation in hepatic lipid metabolism triggered by sepsis, we next sought to characterize the global consequences on hepatic lipid profiles. This was achieved by targeted lipidomic screening. Parallel to our unbiased metabolomic screening, we find sepsis leads to profound accumulation of almost all lipid species surveyed (Fig. 4a). We next evaluated the hepatic lipid composition

to discern which lipid species are most vulnerable to sepsis-induced alterations. We find very little alterations to the ceramide pool in septic hepatocytes (Fig. 4b), with only modest changes in dihydroceramide (Fig 4-figure supplement 1). However, the hepatic fatty acid fractions displayed significant remodelling in response to sepsis (Fig. 4b). Specifically, we find increases in free fatty acid species such as 16:0, 18:1 and 18:2 in septic hepatocytes (Fig 4-figure supplement 2). Moreover, hepatic phospholipid composition showed robust alterations following CLP (Fig. 4b). Of the phospholipids (PLs) most significantly affected, we find accumulation of phosphatidylcholines (PCs), phosphatidylethanolamine (PE) and sphingomyelin (SM) (Fig. 4c). Of the hepatic PC pool, we find the most abundant species (16:0/18:2 & 16:0/18:1) to show increased accumulation in response to sepsis (Fig 4-figure supplement 2). We also observed significant increases in the most abundant PE species (18:0/20:4, 16:0/22:6 & 18:0/22:6) and SM species (22:0, 16:0 & 20:0) in septic hepatocytes (Fig. 4-figure supplement 2). One phospholipid species, lysophosphatidylcholine, displayed a decrease in concentration during sepsis, however, the hepatic concentration of this phospholipid species is significantly lower than other PL species assayed (Fig. 4c). Finally, we detected no differences in other PL species such as phosphatidylinositol (PI) and lysophosphatidylethanolamine (LPE) and no differences in hepatic cholesterol ester levels (Fig 4-figure supplement 3). Collectively, our data detail the impact of sepsis on hepatic lipid composition and robustly show a global rise in nearly all lipid species.

PDK inhibition attenuates sepsis-induced transcriptional and metabolic changes in the liver

We have previously reported that PDK inhibition by the pyruvate analog and pan-PDK inhibitor dichloroacetate (DCA) promotes PDC-dependent immunometabolic adaptations to sepsis and increases survival ¹⁸⁸. Given the establishment of hepatocyte mitochondrial dysfunction and steatosis during sepsis, we asked whether pharmacological targeting of the hepatic PDK/PDC axis would mitigate the disruption of key metabolic and bioenergetic processes induced by sepsis. To test this postulate, we administered DCA to septic mice 24 h after CLP onset and assessed the global transcription profile of liver 6 h later via RNA-seq. DCA treatment of septic mice reversed the majority of sepsis-regulated gene networks (Fig. 5a). Most notably, pathways involved in redox metabolism, lipid metabolism and mitochondrial dysfunction are returned to basal levels with the administration of DCA in septic mice (Fig. 5b).

Given our previous results indicating the transcriptional changes associated with sepsis in the liver underlie functional alterations in metabolism, we next sought to determine if the DCA reversal of transcriptional changes leads to reprogramming of metabolism to a pre-sepsis state. Our RNA-seq data showed a positive enrichment score for production of ROS along with negative enrichment for glutathione conjugation. Strikingly, the depletion of redox components cystathionine, cysteine, glycine, hypotaurine, taurine and glutathione elicited by sepsis was completely reversed by administration of DCA (Fig. 5c). More

importantly, we observed a significant decrease in hepatic ophthalmate levels, signifying a decrease in oxidative stress (Fig. 5c). The restoration of key redox metabolites in septic mice following DCA administration prompted us to investigate the mitochondrial consequences of this pharmacological intervention. With the exception of fumarate, DCA completely restores TCA metabolite levels to that of control levels (Fig. 5d). Finally, we wished to define the effects of DCA treatment on the hepatic lipidome. Lipidomic analysis shows a protective effect of DCA in ameliorating sepsis-induced steatosis. In summary, these data reveal pharmacological inhibition of the PDK pathway restores transcriptional and metabolic changes associated with sepsis in the liver.

Discussion

The failure of over 50 trials of anti-inflammatory and anti-cytokine agents for treatment of severe sepsis has led to a reevaluation of the concept of sepsis as causing death by unbridled inflammation ¹⁸⁹. Instead, it has become increasingly clear that mitochondrial bioenergetics and metabolic reprogramming play vital roles in organ and immune cell dysfunction during sepsis that may lead to sustained organ failure and immunometabolic paralysis ^{190,191}. Consequently, much attention has been focused recently on the immunometabolic consequences of inflammation in immune cells ¹⁹². However, significant gaps exist in our understanding of the metabolic adaptations induced by systemic inflammation from sepsis in vital organs. Addressing this limitation is critical because sepsis survival depends on restoring both organ and immune cell homeostasis following dysregulated and

disseminated inflammation ¹⁹³. In the present study, we reveal severe disruption of several key hepatic metabolic pathways in septic mice, including TCA cycle activity, OXPHOS, redox and lipid metabolism during the prolonged tolerant phase of sepsis. Importantly, DCA administration restored TCA cycle flux, ameliorated oxidative stress and reversed sepsis-induced steatosis.

Remodeling of TCA cycle flux is well appreciated to support immune cell effector function in the face of inflammatory challenges ^{154,180,194,195}. For example, succinate primes inflammation through succinate dehydrogenase (SDH)-mediated ROS generation and ATP synthesis ^{180,194}. SDH-derived ROS enhances IL-1 β production by activating HIF-1 α and activating the NLRP3 inflammasome ²⁰. Inhibition of SDH activity increases IL-10 production and skews inflamed immune cells towards an anti-inflammatory response ¹⁸⁰. Fumarate, on the other hand, possesses anti-inflammatory properties as it inhibits pro-inflammatory cytokine production either by activating the KEAP1/NRF2 pathway or via inhibiting pathways like NF- κ B and MAPK that lie downstream of TLR signaling in immune cells ^{196,197}. Our findings extend the concept that the TCA cycle is subject to reprogramming in response to sepsis in non-immune cells such as hepatocytes. Furthermore, we found similarities as well as differences in terms of TCA cycle remodeling in hepatocytes compared to immune cell effects, clearly indicating this pathway is regulated in a cell-type and context-dependent manner during sepsis.

In response to LPS stimulation, macrophages significantly upregulate IRG1 and accumulate itaconate from the decarboxylation of cis-aconitate ^{181,183}. One of

the primary functions itaconate serves in immune cells is to limit IL-1 β production through direct inhibition of SDH and activation of the KEAP1/NRF2 and I κ B ζ -ATF3 pathways ^{181,183,198}. In line with what occurs in macrophages, we observed an increase in IRG1 and itaconate in septic hepatocytes at 30 h. While we did not fully characterize the hepatic function of itaconate, a recent paper demonstrates its anti-inflammatory effects during Ischemia-reperfusion (IR) injury in the liver ¹⁹⁹. The deletion of IRG1 heightens inflammation and liver damage and renders hepatocytes susceptible to oxidative injury after I/R injury. Further, itaconate administration reduces liver damage and inflammation associated with I/R in IRG1 KO mice, also emphasizing its anti-inflammatory and hepatoprotective effects ¹⁹⁹. Given the immunomodulatory effects of itaconate and its accumulation in the liver in our study, we hypothesize that itaconate directs hepatocyte shifts in both TCA cycle metabolism and mitochondrial energetics. Moreover, immune cells have been shown to depend on itaconate accumulation to confer tolerance ²⁰⁰. Our similar findings within the liver during the prolonged phase of sepsis suggests this organ may utilize similar mechanisms to promote tissue tolerance during chronic sepsis. Intriguingly, DCA reduces itaconate levels, which we also find in a human monocyte model of sepsis ²⁰¹.

Similar to immune cells, liver cells increase ROS production during sepsis ^{202,203}. We offer a potential mechanism whereby sepsis leads to significant depletion of redox metabolites within the glutathione pathway ultimately culminating in loss of cellular glutathione levels resulting in ophthalmate accumulation in hepatocytes.

Sepsis profoundly alters lipid metabolism, resulting in significant increases in plasma fatty acid and glycerol concentrations, changes reported to predict prognosis in septic patients ²⁰⁴. Furthermore, patients who died from sepsis had evidence of hepatic steatosis affecting 5%-80% of liver parenchyma ²⁰⁵. Human data supports a robust hepatic steatosis phenotype subsequent to sepsis. One vital lipid metabolic pathway which can contribute to steatosis is *de novo* lipogenesis. While we did not directly measure hepatic lipogenesis, our metabolomics screening did show an increase in hepatic citrate and free fatty acid levels suggesting a increased lipogenesis during sepsis. Functionally, immune cells exploit the lipogenic pathway for the generation of malonyl-CoA and subsequent malonylation of glycolytic enzymes, which ultimately contributes to the metabolic adaptation of macrophages to inflammatory cues ²⁰⁶. While this work did not investigate the mechanisms and factors driving alterations in hepatic lipid metabolism during sepsis, we hypothesize altered regulation of transcription factor peroxisome proliferator-activated receptor alpha (PPAR α) to be play a critical role. PPAR α is considered to be one of the key regulators of fatty acid oxidation as it controls the expression of numerous genes in this pathway upon activation by unsaturated fatty acids ²⁰⁷. Multiple studies have reported altered expression of PPAR α during sepsis ²⁰⁸⁻²¹⁰. Of interest, recently Wyange et al. reported the biological activity of PPAR α to be significantly lowered in a CLP model of sepsis ²¹¹. This resulted in impaired fatty acid uptake and β -oxidation, driving the increase of fatty acids levels in circulation and accumulation of lipid droplets in the liver ²¹¹. Furthermore, Wyange et al. also demonstrated that activation of PPAR α improved hepatic lipid

metabolism and survival during sepsis, highlighting the protective nature of this pathway during sepsis ²¹¹.

In addition to accumulation of free fatty acids and triglycerides in the liver, our lipidomic analysis revealed significant increases in phospholipids. In the present study, we find hepatic levels of the phospholipids phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin to be heightened during sepsis, indicating abnormal metabolism of phospholipids. PC and PE are the most abundant phospholipids that make up cellular membranes and an abnormally high ratio of PC and PE has been reported to influence energy metabolism and linked to fatty liver disease and impairment in liver regeneration after injury ²¹²⁻²¹⁴. Altered PC:PE ratio has also been shown to influence the dynamics and regulation of lipid droplets contributing towards steatosis, a phenotype we also report in our study ^{212,213,215}. The dysregulation of hepatic phospholipid metabolism may be a contributing mechanism underlying steatosis, which is a topic of future investigation. Additionally, phospholipids serve as precursor molecules to bioactive lipids, which are involved in numerous signal transduction cascades ²¹⁶. Investigating whether signaling properties of bioactive lipids in the liver during sepsis is pathogenic or contributes to sepsis resolution is also needed in the future.

PDC is a master metabolic regulator controlling the conversion of pyruvate to acetyl-CoA in the mitochondria ²¹⁷ and its inactivation contributes to the metabolic reprogramming that occurs in immune cells in response to inflammatory signaling ^{171,217}. PDK is a negative regulator of the PDC, as it phosphorylates PDC and inhibits the conversion of pyruvate to acetyl-CoA ²¹⁷. During sepsis, the expression

and activity of PDK1 in immune cells is heightened, contributing to the dysfunction of mitochondrial metabolism. One mechanism driving this observed increase is through pro-inflammatory mediators such as LPS and interferon gamma (IFN- γ)²¹⁸. Another possible mechanism contributing to PDK activation in the context of sepsis is through glucocorticoid signaling²¹⁹. In fact, starvation is well established to activate the PDK pathway²²⁰⁻²²⁴. Based on our findings of elevated glucocorticoid levels in both the circulation and the liver, we hypothesize the stress hormone pathway as a contributor to PDK activation during sepsis.

We reported in a sepsis monocyte model that DCA reduced TCA cycle catabolic effects concurrent with increasing amino acid anaplerotic catabolism of branched-chain amino acids, leading to increased TCA-driven anabolic energetics¹⁷¹. In the present study, we find restoration of hepatic TCA metabolites, decreased triglyceride accumulation, lessening of lipid synthesis and amelioration of oxidative stress in septic mice after DCA administration. Overall, we demonstrate that hepatic transcriptional and metabolic dysfunction improves after targeting the PDK/PDC axis with DCA.

Notably, our interpretation of results is based on a single 30 h time point, which was selected based on our previous publication showing reversal of disease tolerance mechanisms and increased survival following DCA intervention at 24 h¹⁸⁸. Indeed, the metabolic changes at 30 h in this study align with repressed mitochondrial respiration and extracellular acidification reported in this earlier publication. Future time course studies will take advantage of this data to expand our knowledge of hepatic metabolic perturbations during the sepsis response and

provide higher granularity of the regulatory events underlying the DCA effects. Furthermore, while our current work provides a comprehensive atlas of hepatic metabolic and transcriptional pathways subject to dysregulation during prolonged sepsis, acute metabolic manifestations on a global scale remain unknown. Indeed, it is well documented that early metabolic changes are induced in the immune compartment in response to pathogen exposure which ultimately shape the host response to infection including induction of inflammation as well as establishment of immune tolerance ²²⁵. Previous work from our group demonstrates extreme metabolic flexibility that occurs in a coordinated fashion in myeloid cells ²²⁶, opening the possibility that the liver may behave in a similar dynamic fashion. Moreover, prior work has demonstrated rearrangements of certain hepatic metabolic pathways at the transcriptional level within hours of sepsis induction ²²⁷. For example, amino acid, fatty acid and redox pathways are downregulated transcriptionally in the liver within six hours of sepsis ²²⁷. Intriguingly, we find similar alterations at the metabolite level during prolonged sepsis, indicating early transcriptional changes elicited by sepsis may prime the liver for the later metabolic manifestations observed in our current study. Future work evaluating the contribution of these early transcriptional changes to acute metabolic reprogramming in hepatocytes during the resistance phase is needed. These studies will illuminate whether the findings reported here represent protective mechanisms associated specifically with tissue tolerance or are pathogenic in nature, a product of aberrant response to pathogenic exposure.

In terms of molecular mechanisms underlying the hepatic metabolic manifestations of sepsis, several potential candidates exist. Firstly, the mammalian target of rapamycin (mTOR) could be contributing to sepsis-induced metabolic disturbances in the liver. The foundation for this hypothesis is due to mTOR being demonstrated to be an essential component of the inflammatory response as well as underlying the glycolytic switch observed during acute sepsis ^{228,229}. The metabolic signature observed here during the prolonged phase of sepsis is characterized by decreased mitochondrial and glycolytic derived energy, consistent with a state of impaired mTOR activity. Evaluating the effects of sepsis on altering hepatic mTOR signaling will shed light on whether there is acute activation of this pathway during the resistance phase and whether it shifts towards inhibition during the tolerant phase. Opposite to the anabolic mTOR pathway is the AMP activated kinase (AMPK) pathway which promotes catabolism. AMPK is known to play a critical role in maintaining cellular energy homeostasis and its activation is important in the restoration of metabolic balance. Numerous studies utilizing animal models such as acute lung injury, ischemia-reperfusion, acute kidney injury and hemorrhagic shock have shown protective effects of AMPK, its activation decreasing organ injury and inflammation ²³⁰⁻²³². Of interest, activation of AMPK during sepsis minimizes liver and renal endothelial damage by decreasing inflammatory cytokine production ²³³. Similarly, using transgenic mouse models, others have shown that deletion of AMPK α in myeloid cells exacerbates polymicrobial sepsis by promoting the release of HMGB1 and endotoxin shock, while activation of AMPK had protective effects ²³⁴. Overall, given the evidence, it

is clear that activation of the AMPK signaling serves to limit inflammation and overall organ dysfunction. However, the exact mechanism by which AMPK signaling is dysregulated and the impact on metabolic remodeling in the liver during sepsis needs further investigation.

While we did not directly test here the physiological/pathophysiological contribution of individual metabolic pathways during the course of sepsis, we hypothesize the overwhelming hepatic metabolic response during prolonged sepsis is pathogenic in nature. Our interpretation is based on our DCA results. We have previously demonstrated that DCA treatment reverses immunoparalysis and promotes host survival¹⁸⁸. Our results indicate that the vast majority of metabolic changes observed during non-lethal sepsis are completely reversed in DCA treated mice. We surmise that if these metabolic pathways were contributing to host survival, the DCA treatment would have enhanced their activity (ie DCA would increase metabolites increased in response to sepsis). Furthermore, we postulate that the observed hepatic metabolic changes associated with prolonged sepsis may in fact be directly contributing to immunoparalysis and subsequent host demise. Further work dissecting the contribution of these hepatic metabolic changes during the course of non-lethal sepsis is needed to establish the physiological/pathophysiological contribution in the context of pathogen resistance and tissue tolerance.

Alternatively, it is possible that some of the metabolic changes associated with sepsis in the liver confer host protection and tissue resolution. For example, we observed a significant decrease in hepatic fumarate levels 30 h post-sepsis and

an even further reduction in the DCA treated septic mice. We speculate the decrease in endogenous hepatic fumarate may lend itself to organ protection and sepsis resolution. Fumarate derivatives however have been shown to possess anti-inflammatory, antioxidant and pro-survival properties in the liver during sepsis ²³⁵. Whether endogenous fumarate possesses differential effects than its derivatives (dimethyl fumarate) is unknown. Other TCA cycle derivatives such as dimethyl itaconate and 4-octyl itaconate have shown to exert differential effects than the endogenous form ²³⁶. Future work dissecting the role of endogenous fumarate and derivatives during lethal and non-lethal sepsis is warranted to illuminate if this metabolite promotes tissue damage and if the reduction is compensatory in nature.

In particular, further investigation specifically into the PDK pathway to determine which isoform underlies the hepatic manifestations of sepsis is needed. This is warranted given multiple isoforms of PDK expressed in the liver ²³⁷ and DCA inhibits multiple isoforms of PDK ^{238,239}. The results of this study fill a gap in understanding how sepsis at the molecular level alters liver metabolism and expand the current concepts on physiology and pathophysiology of resistance and tolerance of hepatocyte during sepsis ^{240,241}. It is still unclear from our studies however if the effects of DCA on reversing hepatic metabolism are due to its effects on ameliorating immunoparalysis and enhancing pathogen clearance.

Here, we have confirmed and extended our previous findings that stimulation of mitochondrial OXPHOS metabolism by DCA in murine sepsis may be therapeutic in ameliorating the sepsis-induced immunometabolic paralysis of liver and potentially other vital organs. While encouraging, we recognize the limitations of

extrapolating such positive findings in murine models to septic patients. Nevertheless, intravenous DCA has been used safely in critically ill patients ²⁴²⁻²⁴⁴, supporting the translational potential of the current and related investigations. Furthermore, our data demonstrate that the reversal of organ metabolic dysfunction may be a potential mechanism by which DCA confers protection in murine models of sepsis.

FIGURES

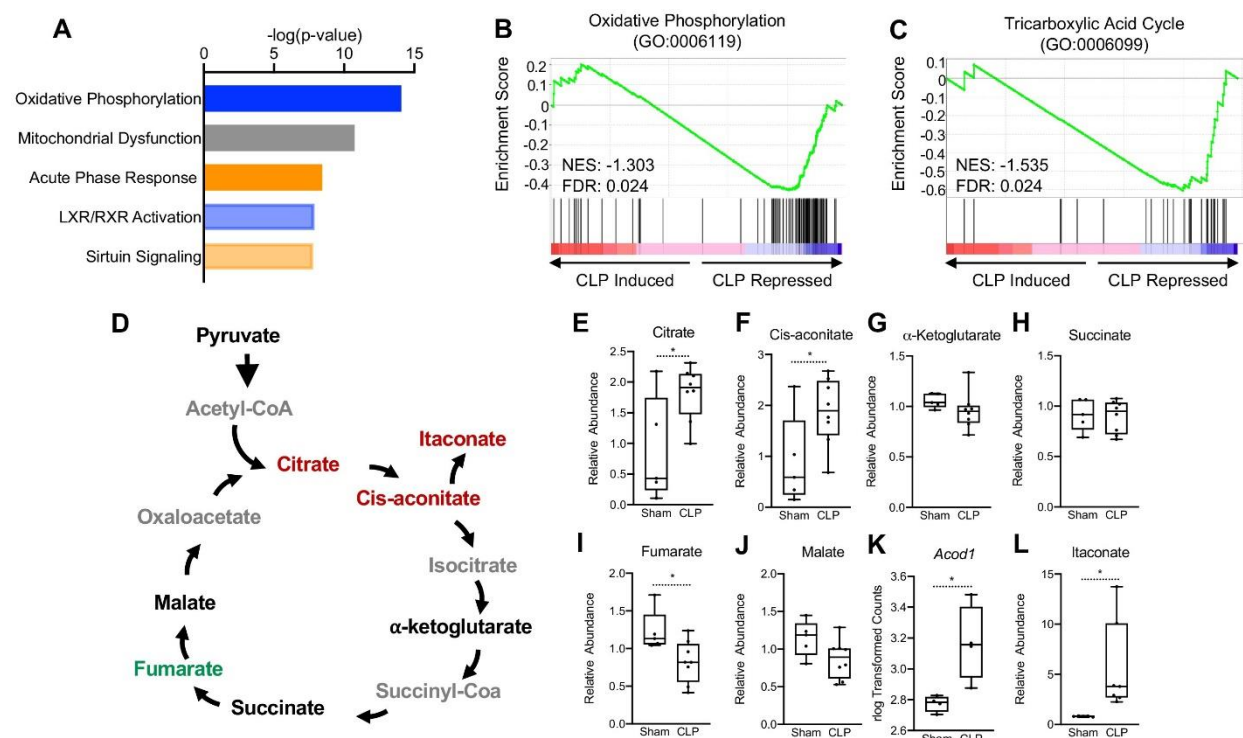


Figure 1: Sepsis Impairs hepatic mitochondrial metabolism. (A) Top five canonical pathways subject to transcriptional alterations in the liver identified by ingenuity pathway analysis of RNA-seq of sham versus CLP mice (n = 4 mice per group). Blue represents a negative z-score and orange represents a positive z-score. Shading indicates intensity of pathway activation/inhibition. (B) GSEA of the oxidative phosphorylation pathway (GO:0006119) indicating a negative normalized enrichment score (NES = -0.853). (C) GSEA of the TCA cycle pathway (GO:0006099) indicating a negative enrichment score (NES = -1.1956). (D) Schematic representation of hepatic TCA cycle metabolites altered during chronic sepsis. Red denotes a metabolite increased in response to sepsis; green indicates a metabolite decreased in response to sepsis; black indicates a metabolite unchanged in response to sepsis; gray indicates a metabolite not measured in our metabolomic screening. (E–J) Relative metabolite levels measured by ultrahigh-performance liquid chromatography–tandem mass spectroscopy (UPLC–MS/MS) from livers of sham and CLP mice 30 hr post-surgery (n = 5 sham; 8 CLP). (K) rlog transformed counts from RNA-seq of sham and CLP mice 30 hr post-surgery. (L) Relative itaconate levels in livers of sham and CLP mice measured by UPLC–MS/MS (n = 5 sham; 8 CLP). *p<0.05 as determined by Student’s T-test.

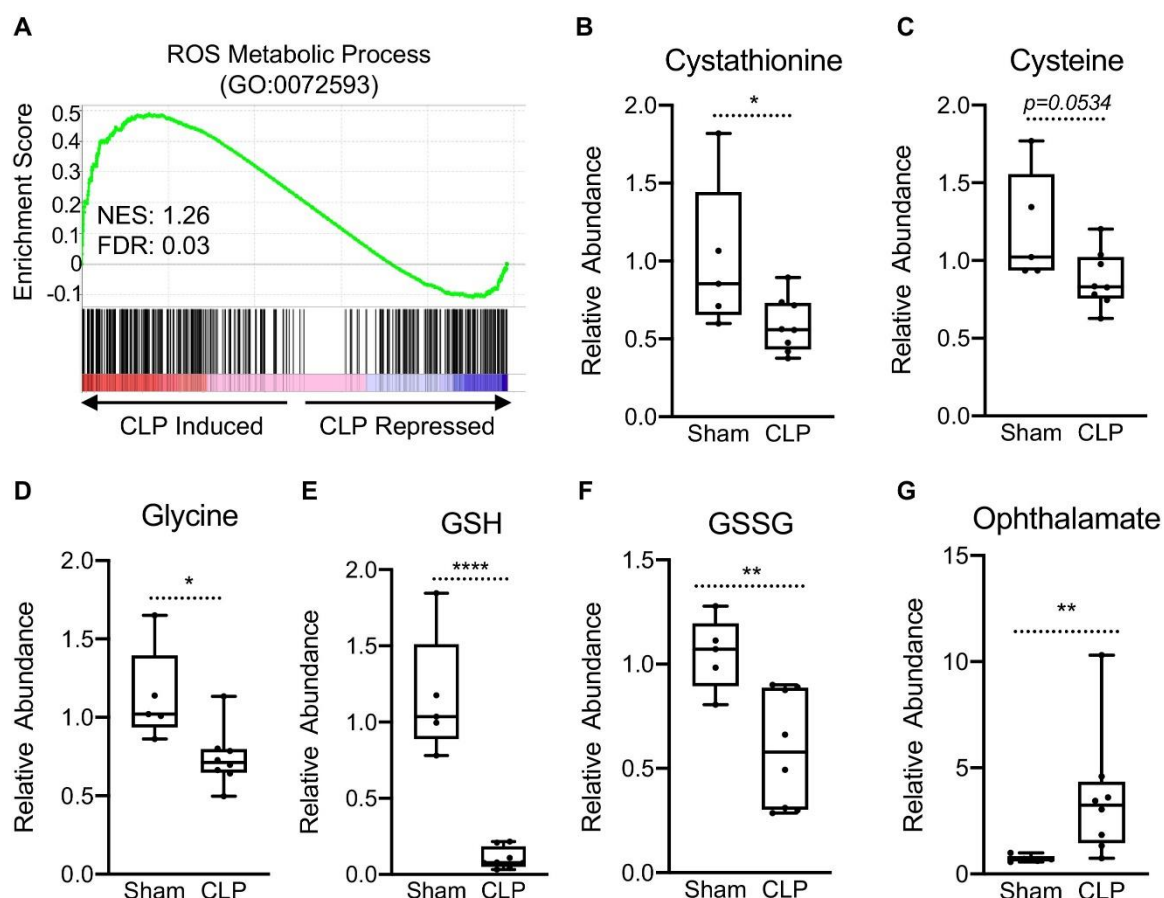


Figure 2: Impaired hepatic redox balance in septic mice. (A) GSEA of the ROS metabolic pathway (GO:0072593) from RNA-seq from livers comparing sham to CLP showing a positive enrichment score (NES = 1.117) (n = 4 mice per group). (B–F) Relative metabolite levels of metabolites involved in redox balance measured by UPLC–MS/MS (n = 5 sham; 8 CLP). (G) Relative levels of ophthalmate involved in redox balance measured by UPLC–MS/MS. *p<0.05, **p<0.01, ****p<0.0001 as determined by Student's T-test.

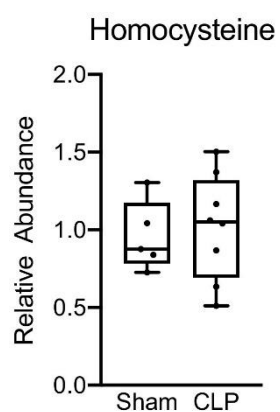


Figure 2 S1: Hepatic homocysteine levels in sham and CLP mice. Ultrahigh-performance liquid chromatography–tandem mass spectroscopy measurement of homocysteine from isolated hepatocytes 30 hr post-surgery. n = 5 sham, n = 8 CLP.

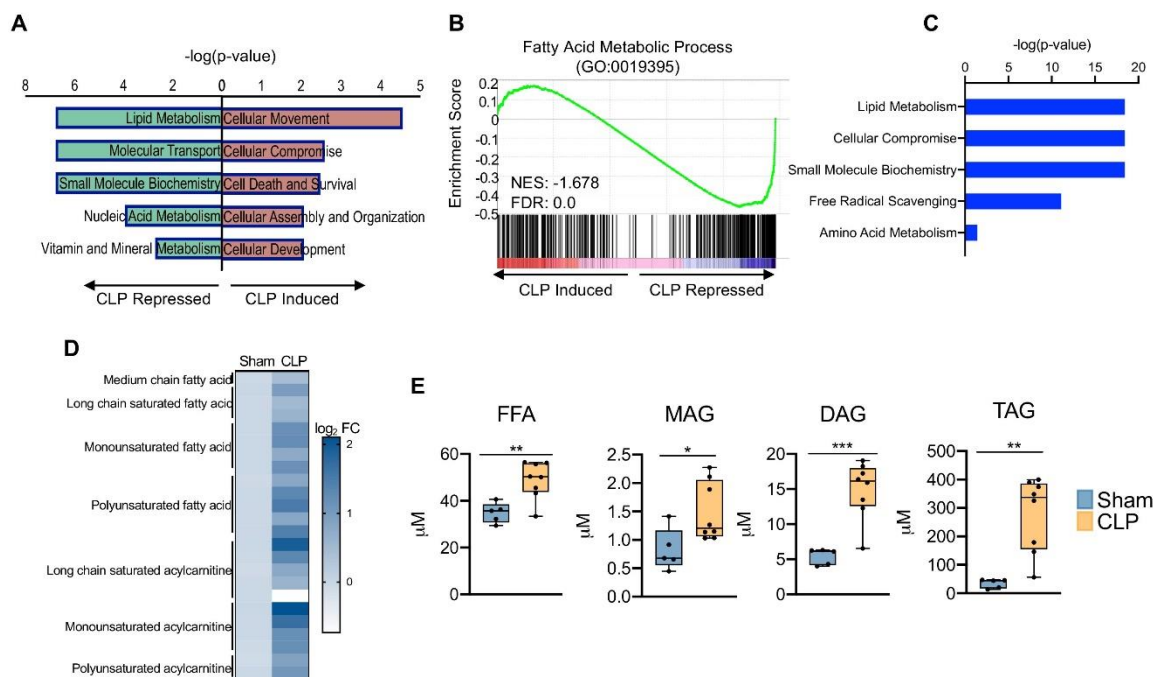


Figure 3: Sepsis promotes hepatic steatosis. (A) Top five induced and repressed physiological pathways in the liver of sham versus CLP mice identified by IPA of RNA-seq. (B) GSEA of the fatty acid metabolic pathway (GO:0019395) from RNA-seq from livers comparing sham to CLP showing a negative enrichment score (NES = -1.018) (n = 4 mice per group). (C) IPA of top five metabolic pathways significantly altered in the liver in response to sepsis identified by global metabolomic screening. (D) Heatmap representation of log₂ fold change of different lipid species in sham and CLP mice measured by UPLC-MS/MS. (E) Quantification of lipids by UPLC-MS/MS in hepatocytes isolated from sham and CLP mice 30 hr post-surgery (n = 5 sham; n = 8 CLP). *p<0.05, **p<0.01, ***p<0.001 as determined by Student's T-test.

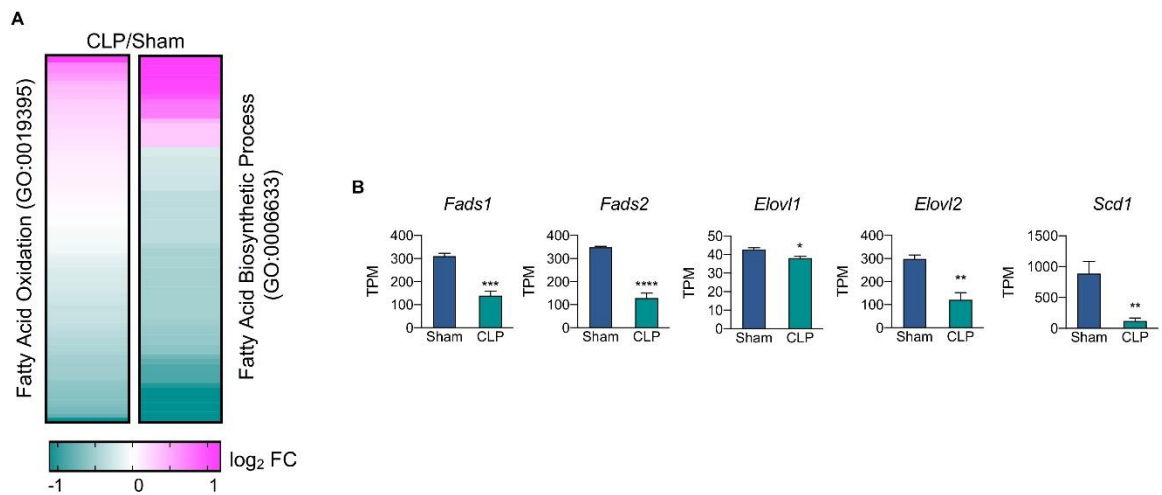


Figure 3S1: Fatty acid oxidation and biosynthetic pathways in septic livers. (A) Heatmap representation of average $\log_2$ fold change of genes involved in fatty acid oxidation (GO:0019395) and fatty acid biosynthetic process (GO:0006633) in CLP versus sham operated mice (n = 5 sham; n = 8 CLP). (B) Transcripts per million for lipid elongation and desaturation genes from RNA-seq. n = 4 samples per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ as determined by Student's T-test.

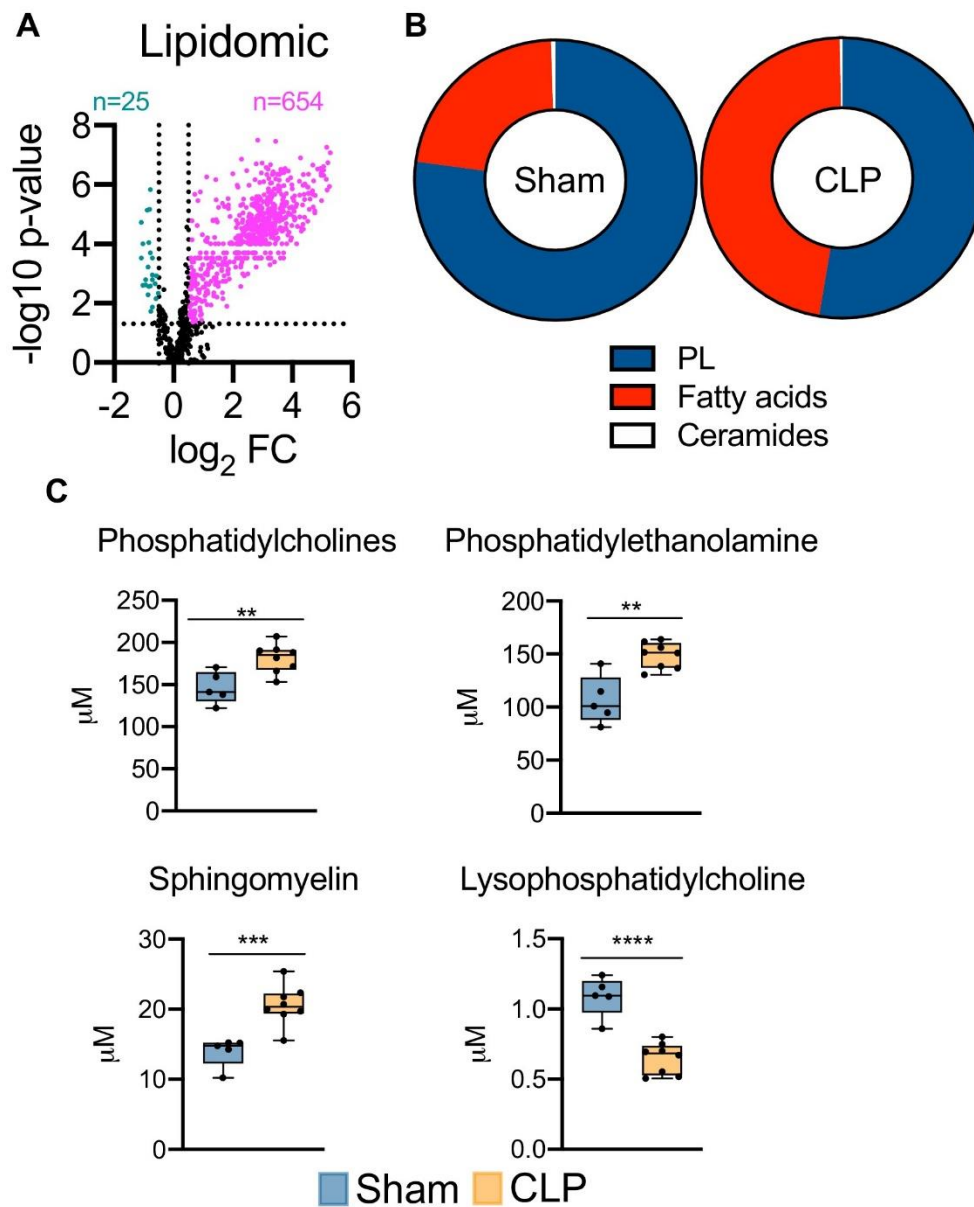


Figure 4: Sepsis reprograms the hepatic lipidome. (A) Volcano plot of significantly altered lipids from sham and CLP hepatocytes measured by UPLC–MS/MS ($n = 5$ sham; $n = 8$ CLP). (B) Pie chart of lipid compositions from sham and CLP mice. (C) Quantification of lipids by UPLC–MS/MS in hepatocytes isolated from sham and CLP mice 30 hr post-surgery ($n = 5$ sham; $n = 8$ CLP). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ as determined by Student's T-test.

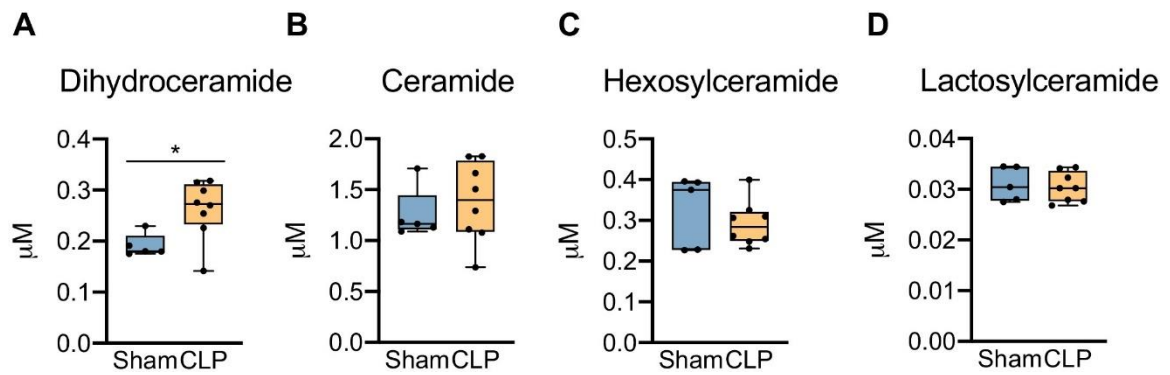


Figure 4 S1: Hepatic ceramide levels in sham and CLP mice. Ultrahigh-performance liquid chromatography–tandem mass spectroscopy measurement of (A) dihydroceramide, (B) ceramide, (C) hexosylceramide, and (D) lactosylceramide from isolated hepatocytes 30 hr post-surgery. n = 5 sham, n = 8 CLP. *p<0.05 determined by Student's T-test.

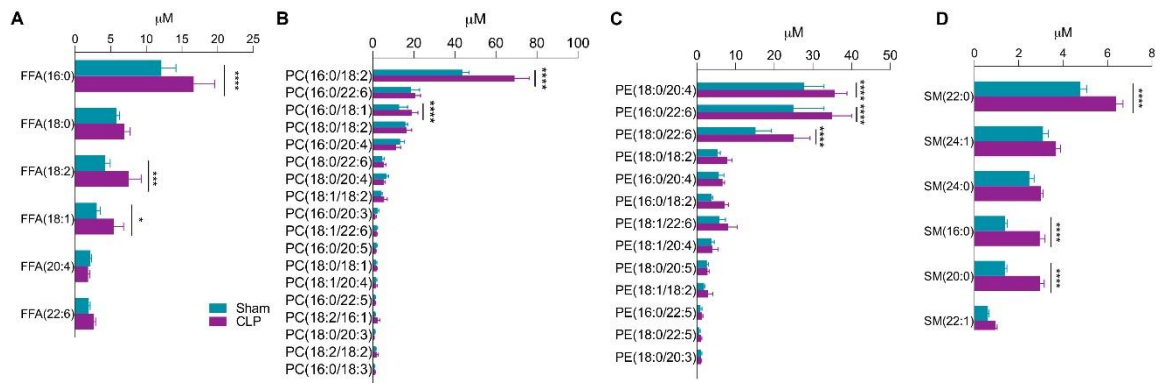


Figure 4 S2: Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin quantifications. Ultrahigh-performance liquid chromatography–tandem mass spectroscopy measurement of (A) free fatty acid species, (B) phosphatidylcholine species, (C) phosphatidylethanolamine species, and (D) sphingomyelin species from isolated hepatocytes 30 hr post-surgery. $n = 5$ sham, $n = 8$ CLP. **** $p < 0.0001$ determined by two-way ANOVA followed by multiple comparisons test.

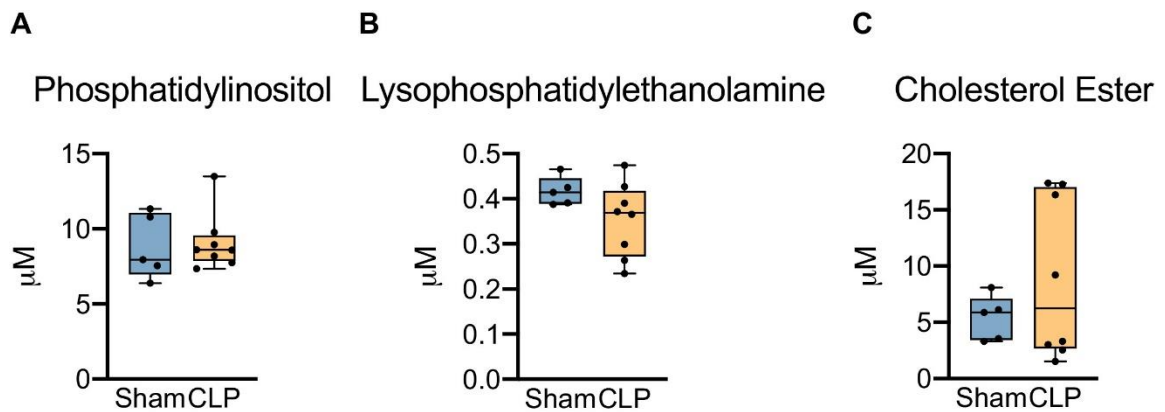


Figure 4 S3: PI, LPE, and CE levels in septic livers. Ultrahigh-performance liquid chromatography–tandem mass spectroscopy measurement of (A) phosphatidylinositol, (B) lysophosphatidylethanolamine species, and (C) cholesterol ester from isolated hepatocytes 30 hr post-surgery. $n = 5$ sham, $n = 8$ CLP. **** $p < 0.0001$ determined by two-way ANOVA followed by multiple comparisons test.

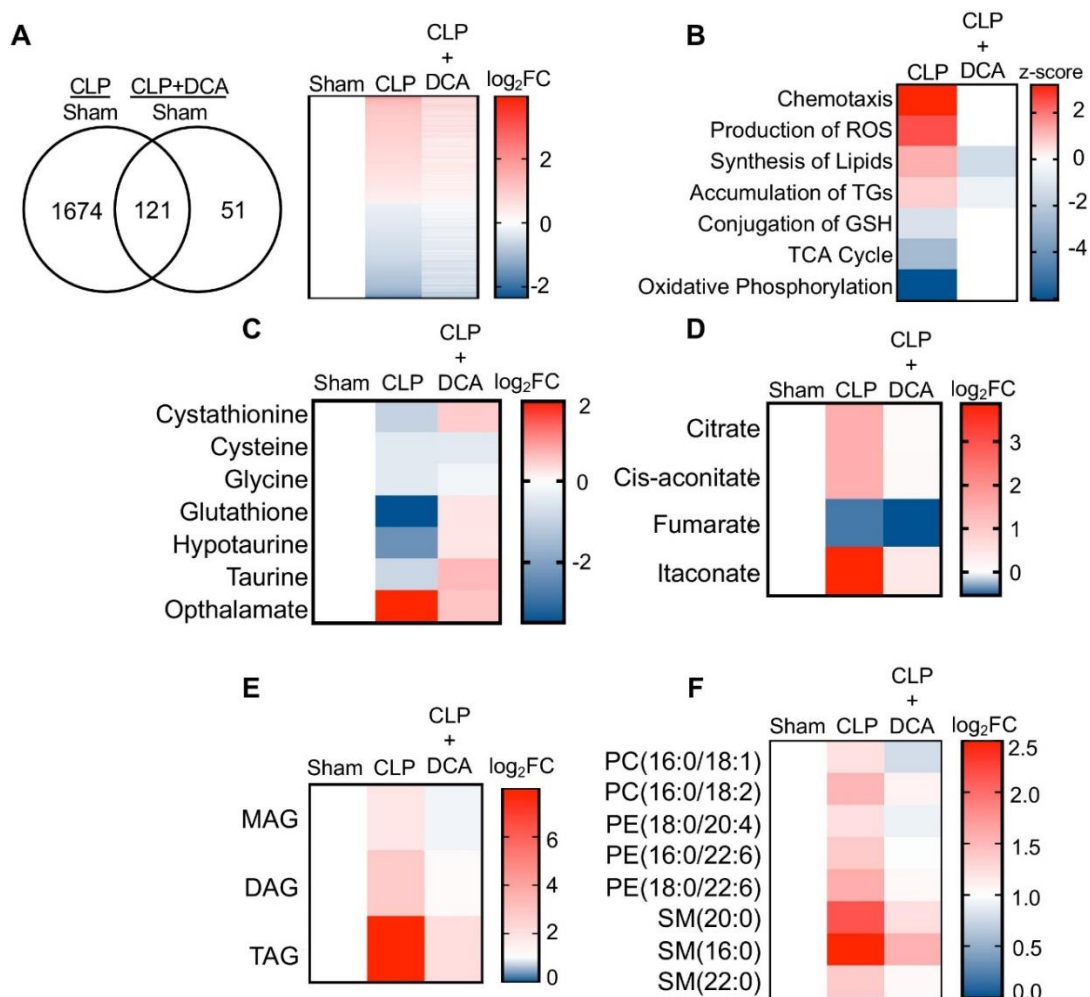


Figure 5: PDK inhibition restores hepatic metabolism in septic mice. (A) Venn diagram of differentially expressed genes (DEGs) assessed by RNA-seq in CLP versus sham compared to CLP+DCA versus sham 30 hr post-surgery (left). Heatmap of average log₂ fold change of DEGs in sham, CLP, and CLP+DCA (right) ($n = 4$ mice per group). (B) Heatmap depiction of z-scores of top canonical pathways identified by IPA in CLP versus sham and CLP+DCA versus sham. (C) Heatmap depiction of average log₂ fold change in metabolite levels involved in redox balance in sham, CLP, and CLP+DCA 24 hr post-surgery measured by UPLC–MS/MS ($n = 5$ sham; $n = 8$ CLP and CLP+DCA). (D) Heatmap depiction of average log₂ fold change in metabolite levels involved in TCA cycle in sham, CLP, and CLP+DCA 30 hr post-surgery measured by UPLC–MS/MS ($n = 5$ sham; $n = 8$ CLP and CLP+DCA). (E) Heatmap depiction of average log₂ fold change in MAG, DAG, and TAG levels in sham, CLP, and CLP+DCA 30 hr post-surgery measured by UPLC–MS/MS ($n = 5$ sham; $n = 8$ CLP and CLP+DCA). (F) Heatmap depiction of average log₂ fold change in phospholipids in sham, CLP and CLP+DCA 30 hr post-surgery measured by UPLC–MS/MS ($n = 5$ sham; $n = 8$ CLP and CLP+DCA).

Material and Methods

Animal Experiments

Male C57BL/6J mice aged 8-10 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were subject to a 12:12 hour dark/light cycle with *ad libitum* access to standard rodent chow and water. Cecal ligation puncture model was used to induce sepsis as previously described^{188,245}. Mice were randomly assigned to our experimental groups. Briefly, cecum was ligated and punctured two times with a 22-gauge needle. Contents were then returned and incision was closed in two layers (peritoneum and skin). Sham operation where abdominal incision was made but cecum not ligated or punctured was used as a control. Subcutaneous fluids (1ml normal saline) were given to each animal. Mice were euthanized 30 hours post-surgery for tissue collection. Dichloroacetate (DCA) (Sigma; MO, USA) was administered (25mg/kg) intraperitoneally at 24 hours post-surgery and tissues collected 6 hours post DCA administration (30 hours post-surgery). No explicit power analysis was used to calculate sample size. Our sample size was based on consultation with Metabolon for metabolomic screening.

Hepatocyte Isolation

Hepatocytes were isolated via portal vein perfusion and collagenase digestion as previously described²⁴⁶. Following perfusion, liver cells were liberated by gentle dissociation in DMEM (ThermoFisher; CA, USA). Cells were then filtered through nylon mesh to remove cellular debris and connective tissue and resulting cells

pelleted by centrifugation at 50g for 1 minute. After three washes with DMEM cells were counted and viability assessed via Trypan Blue exclusion.

RNA-sequencing

RNA was isolated from whole liver tissues using Trizol and the RNeasy RNA isolation kit (Qiagen; MD, USA) according to manufacturer's protocol. One microgram of high quality RNA (RIN>8) was used as a template for library generation using the Illumina TruSeq RNA Sample Prep Kit v2 (Illumina; CA, USA) according to the manufacturer's protocol. Generated libraries were then poly(A) enriched for mRNA prior to sequencing. Indexed samples were sequenced at 100bp-paired-end protocol with the NovaSeq 6000 (Illumina), generating approximately 20-30 million reads per sample. Sequenced reads were aligned to the University of California Santa Cruz (UCSC) mm10 reference genome using STAR v2.5 as previously described ²⁴⁷. The mapped read counts were quantified by Subread feature Counts v1.5.0-p1 ²⁴⁸. Differentially expressed genes (DEGs) were determined by DESeq2 v1.14.1 ²⁴⁸ using a false discovery rate of 0.05. Ingenuity Pathway Analysis (Qiagen) and Gene Set Enrichment Analysis v4.0.3 (GSEA) were further used as previously described ²⁴⁹. Sequencing was performed one time with four biological replicates per group.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy

Hepatocytes were isolated described above for metabolomic screening via Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy

(UPLC-MS/MS) (Metabolon; NC, USA). Briefly, 150-200 microliter cell pellets per animal were used as starting material. Samples were prepared using the automated MicroLab STAR system (Hamilton; NV, USA). Proteins were precipitated using methanol under vigorous shaking for 2 minutes followed by centrifugation. Prior to analysis organic solvents were removed with TurboVap (Zymark; MA, USA) and overnight storage under nitrogen. Dried samples were reconstituted with solvents compatible with the three following analytical methods: 1) reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), 2) RP/UPLC-MS/MS with negative ion mode ESI and 3) HILIC/UPLC-MS/MS with negative ion mode ESI. Resulting samples were analyzed with the ACQUITY UPLC (Waters; MA, USA) and a Q-Exactive high resolution/accurate mass spectrometer (ThermoScientific; MA, USA) interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. An acidic positive ion condition was used on an aliquot optimized to detect more hydrophilic compounds. Another acidic positive ion condition was run but chromatographically optimized for hydrophobic compounds. Basic negative ion optimized conditions was also used on a separate C18 column. Resulting raw data was extracted and peaks identified using Metabolon's hardware and software. Compounds were identified by comparing to known library entries of purified standards or recurrent unknown entities. A library of authenticated standards containing retention time/index (RI), mass to charge ratio (m/z) and chromatographic data (MS/MS spectral data) on all library compounds. Three criteria are used to identify chemicals: 1) RI within a narrow window of proposed

identification, 2) accurate mass match to the library ± 10 ppm and 3) MS/MS forward and reverse scores between the experimental data and authentic standards. Peaks were quantified using area under the curve (AUC). For complex lipid panel lipids were extracted with methanol:dichloromethane in the presence of internal standards. Extracts were concentrated under nitrogen and reconstituted in 250ml of 10mM ammonium acetate dichloromethane:methanol (50:50). Mass spec analysis was performed in a Shimadzu LC with nano PEEK tubing and the Sciex Slexlon-5500 QTRAP (Sciex; MA, USA). Both negative and positive mode electrospray was used. Individual lipid species were quantified by taking the peak area ratios of target compounds and their assigned internal standards then multiplying by the concentration of added internal standards. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of each class comprised by individual fatty acids. Mass spec analysis was performed one time.

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CHAPTER IV

Itaconate stabilizes CPT1a to enhance lipid utilization during inflammation

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Keywords: sepsis, liver, itaconate, fatty acid metabolism, thermogenesis

Abstract

Sepsis is a condition characterized by an inflammatory response to an infection, which can result in organ dysfunction as it progresses. The dysregulation of energy balance, caused by changes in mitochondrial bioenergetics and cellular signaling pathways, contributes to impaired lipid metabolism in the liver during sepsis. However, there is still limited knowledge regarding the specific regulators that govern the molecular interactions supporting lipid metabolism in the liver during sepsis. Therefore, this study aims to address this knowledge gap by investigating the role of itaconate as a modulator of hepatic lipid metabolism during overt inflammation. Our research findings reveal that itaconate plays a significant role in enhancing lipid clearance in hepatocytes by stabilizing the mitochondrial fatty acid uptake enzyme known as CPT1a. Additionally, we have discovered novel substrates of itaconate involved in protein ubiquitination, which may contribute to its ability to promote lipid clearance. Furthermore, we have observed that mice deficient in itaconate not only exhibit defects in hepatic lipid metabolism but also display potential dysregulated systemic fatty acid utilization and impaired thermogenesis following an endotoxin challenge. In conclusion, our study contributes to the expanding understanding of itaconate as a metabolic regulator in non-immune cells in response to inflammation. These findings shed light on the mechanisms underlying hepatic lipid metabolism during sepsis and highlight the

potential therapeutic implications of targeting itaconate in the management of sepsis-related complications such as dyslipidemia.

Introduction

Sepsis is described as a life-threatening organ dysfunction caused by a dysregulated host response to infections¹. Our understanding of sepsis has shifted to incorporate metabolic dysfunction as central component of its pathogenesis. Inflammation-driven metabolic reprogramming and its consequences in the immune compartment have been investigated extensively²⁵⁰⁻²⁵². However, our understanding of metabolic derangements in central organs like the liver is limited. We have previously shown the liver is a target for profound transcriptional and metabolic remodeling in response to polymicrobial sepsis. Specifically, we observe an alteration in mitochondrial function, TCA cycle remodeling, and hepatic lipid accumulation following sepsis²⁵³. Additionally, we have extended these findings to show that hepatic metabolic dysregulation contributes to altered systemic metabolism during sepsis²⁵⁴. Our previous work is consistent with human studies indicating hepatic steatosis is induced during sepsis and an independent predictor of 30-day mortality²⁵⁵⁻²⁵⁷. Furthermore, inhibition of the master lipid sensor peroxisome proliferator-activated receptor alpha (PPAR α) within hepatocytes exacerbates sepsis-induced pathology²⁰⁸. Collectively, these studies highlight the essential role of hepatic lipid metabolism in maintaining organismal adaptations to

sepsis. However, molecular regulators coordinating hepatic metabolic responses to sepsis remain largely unknown.

Immune response and metabolic alterations are intertwined consequences of inflammation. Of particular interest is the extensive reprogramming of mitochondrial metabolism in cells of myeloid lineage favoring the production of metabolites with immunomodulatory properties²⁵⁸. Itaconate is one such metabolite produced by the decarboxylation of a citric acid cycle (TCA) intermediate cis-aconitate via aconitase decarboxylase 1 (*Acod1*) also known as immune-responsive gene 1 (*Irg1*)^{259,260}. Numerous studies have shown itaconate exerts anti-inflammatory and anti-oxidative effects via multiple mechanisms including induction of Nrf2¹²² and ATF3¹²⁴, inhibition of succinate dehydrogenase (SDH)¹¹² and NLRP3 inflammasome¹²⁷, and inhibition of glycolysis^{115,261}. Additionally, the therapeutic potential of itaconate derivatives has shown promise in a variety of pre-clinical models of inflammatory diseases²⁶¹.

While studies have focused on itaconate's function in the immune cells, its role in metabolically active tissues such as the liver is less understood. We have previously demonstrated sepsis elicits significant accumulation of itaconate within hepatocytes²⁵³. However, the biological function of itaconate, particularly its metabolic effects, within hepatocytes during the course of sepsis remains unknown. Therefore, utilizing *Irg1* knockout mice deficient in itaconate synthesis we aimed to address the effects on hepatic metabolism in response to polymicrobial sepsis.

Results

***Irg1* deficiency exacerbates hepatic lipid accumulation during sepsis**

We have previously reported sepsis induces a state of hepatic steatosis²⁵³. Given itaconate accumulates within hepatocytes during sepsis and previous reports demonstrating the ability of itaconate to modulate lipid metabolism^{141-143,262}, we sought to determine its role, if any, in altering the course of steatosis during sepsis. To achieve this, we subjected 8-10 weeks old male C57BL/6NJ (WT) and C57BL/6NJ-*Acod1*^{em1(IMPC)J/J} (*Irg1* KO) to sepsis via cecal slurry injection for 24 hours as previously described²⁶³. To further investigate the role of itaconate in modulating hepatic lipid homeostasis during sepsis, we first evaluated the level of triglyceride accumulation. Consistent with our previous reports we find sepsis induced hepatic lipid accumulation as shown by increased oil red O staining and quantification (Fig. 1a-b). Remarkably, septic *Irg1* KO mice exhibited enhanced lipid droplet accumulation and significantly higher triglyceride levels compared to WT littermates (Fig. 1a-b). Given the aberrant steatosis observed in response to *Irg1* deficiency in male mice, we next evaluated if inflammation drives the development of hepato-steatosis in a sex-dependent manner. Parallel to male mice, female *Irg1* KO mice injected with endotoxin exhibited a higher degree of lipid droplet accumulation compared to endotoxin treated WT littermates as demonstrated by enhanced BODIPY staining in liver sections (Supplemental Fig. 1). Next, we determined if itaconate is directly modulating hepatocyte lipid metabolism or if this phenotype is driven by secondary effects such as enhanced inflammation as

previously reported¹⁸¹. To achieve this, we employed an *in vitro* model of steatosis via oleate loading in AML12 cells and primary hepatocytes. Utilizing the cell-permeable itaconate derivative, 4-octyl itaconate (4-OI), we find that both primary hepatocytes and AML12 cells pretreated with 4-OI (250uM) demonstrate lower oleate-induced lipid droplet formation (Fig. 1c). Taken together, our data demonstrate itaconate acts as an anti-steatotic metabolite within the liver both *in vivo* and *in vitro*.

4-OI promotes mitochondrial fatty acid uptake and clearance

While our data has revealed anti-steatotic properties of itaconate, mechanisms conferring these actions are not resolved. To gain mechanistic insight into the anti-steatotic effects of itaconate, we performed discovery-based untargeted proteomic analysis of AML12 cells treated with 4-OI for 24 hours. Analysis of our proteomic data indicated significant regulation of several different pathways related to lipid metabolism by 4-OI (Fig. 2a). Notably, we find enhanced expression of proteins involved with oxidative phosphorylation such as COX7A2L, ATPAF1, NDUFAB1, as well as the fatty acid β -oxidation enzymes ACSL3-5, CPT1a, CPT2, and ACAA2 following 4-OI stimulation (Fig. 2b). Enhanced expression of CPT1a, CPT2, and SLC25a20 were verified in independent experiments via western blot analysis (Fig. 2c). Given the stimulatory effect of 4-OI on carnitine shuttle enzyme expression, we hypothesized loss of endogenous itaconate during inflammatory settings would result in impaired expression. Assessing the protein expression of these enzymes in endotoxin-treated WT and

Irg1 KO mice revealed loss of *Irg1* significantly decreases the expression of CPT1a, CPT2, and SLC25a20 (Fig. 2d). These data reveal a stimulatory effect of 4-OI and endogenous itaconate on the expression of carnitine transport enzymes within hepatocytes. Lastly, we investigated whether the regulation of CPT1a by itaconate is linked to its anti-steatotic effects. We repeated the lipid loading experiment, however, this time we inhibited CPT1a via pharmacological inhibition with etomoxir. We find etomoxir treatment reverses the anti-steatotic effects of 4-OI resulting in lipid droplet formation similar to oleate-loaded control cells (Fig. 2e). Collectively, our data demonstrates upregulation of β -oxidation enzymes in response to itaconate, which affords, at least in part, its anti-steatotic effects. However,

4-OI stabilizes CPT1a protein expression

The upregulation of carnitine shuttle enzyme expression in response to itaconate stimulation, to our knowledge, has not been shown before. Therefore, we aimed to determine the mechanism underlying this upregulation. Initially, we assessed transcript levels of CPT1a, CPT2, and SLC25a20 in AML12 cells stimulated with 4-OI. We find very modest induction of these genes, however, not to the extent we are observing at the protein level (Supplemental Fig. 2a). Furthermore, gene expression of these enzymes in liver lysates of endotoxin stimulated WT and *Irg1* KO mice do not support the repression we observe *in vivo* (Supplemental Fig. 2b). These data indicate itaconate may upregulate the expression of these enzymes at the post-translational level. Increased protein expression can be conferred through enhancing protein stability. Therefore, we first tested the stability of CPT1a in

response to 4-OI stimulation in the presence of the protein synthesis inhibitor cycloheximide (CHX). CPT1a displayed a prolonged half-life of about 24 hours in vehicle treated cells (Fig. 3a). In contrast, stimulation with 4-OI significantly extended the half-life of CPT1a protein (Fig. 3a). The stability of proteins is regulated at the post-translational level through activation of the ubiquitin system. Activation of E1-E3 ubiquitin ligases leads to ubiquitination of target substrates and subsequent proteasomal degradation. Given the extended half-life of CPT1a in the presence of 4-OI, we sought to determine if this is mediated via alterations in its ubiquitination status. To achieve this, we immunoprecipitated CPT1a in vehicle and 4-OI stimulated cells in the presence of proteasome inhibitor MG132. Immunoprecipitation of CPT1a and subsequent western blotting of ubiquitin revealed little to no ubiquitination in both vehicle and 4-OI treated groups in the absence of MG132 (Fig. 3b). However, in the presence of MG132 we observe robust polyubiquitination of CPT1a in the vehicle treated group (Fig.3b). In contrast, 4-OI stimulation drastically reduced levels of CPT1a polyubiquitination (Fig. 3b). Taken together, our data indicate 4-OI interferes with CPT1a ubiquitination promoting its stabilization.

Alkylation of cysteine residues can lead to various post-translation modifications (PTM) that are crucial for the regulation of cellular processes. Since thiol sidechains are highly nucleophilic, they can participate in Michael's reaction and undergo conjugation addition with compounds like itaconate that have an electrophilic α,β -unsaturated carboxylic acid to form adducts. Given that itaconate

and its derivatives have been shown to modulate biological function via the alkylation of numerous proteins, we next tested the hypothesis that itaconate interacts with CPT1a and hinders ubiquitination. This is based on previous reports in macrophages in which CPT1a was shown to interact with itaconate via the bioorthogonal probe ITalk²⁶⁴. To determine initially if itaconate interacts with the hepatic proteome we labeled AML12 cells with ITalk for 4 hours followed by click reaction to a azide-rhodamine probe. In-gel fluorescence of both Raw Macrophages and AML12 cells treated with ITalk show a banding pattern compared to vehicle treated cells (Fig. 3c). Additionally, pre-treatment of the competitive inhibitor 4-OI blocks this banding pattern, indicating the specificity of the ITalk probe for itaconation (Fig. 3c). These data indicate that hepatic proteins are indeed subject to itaconation. Next, we performed ITalk labeling in AML12 cells followed by subsequent azide-agarose click reaction to allow for mass spectrometry identification of hepatic itaconated proteins. We identified 123 proteins that had ≥ 1.5 fold enrichment over vehicle treated cells. We initially surveyed the hepatic itaconated proteins to determine if indeed CPT1a was enriched. Contrary to our hypothesis and previous reports, we did not identify CPT1a as a hepatic itaconation substrate. Therefore, we next performed pathway analysis to gain insight into biological pathways that may afford itaconate's ability to stabilize CPT1a protein. Consistent with previous reports, we find enrichment in proteins involved in glutathionylation and NRF2 mediated oxidative signaling (Fig. 3d). Additionally, we found proteins involved in protein ubiquitination to show enrichment in the itaconation group (Fig. 3d). These proteins range from E1-E3 ubiquitin ligases as

well as various components of the proteasome and ubiquitin peptidases (Fig. 3d). Identification of these components involved in proteasomal turnover of proteins within the liver is the first to our knowledge to be demonstrated. We hypothesize itaconation of ubiquitin ligases and proteasome components may confer the stabilizing effects of itaconate on CPT1a. Very little is known about ubiquitin ligases that control the proteasomal turnover of CPT1a. Moreover, the ubiquitination components disrupted by itaconate responsible for mediating its stabilizing effects on CPT1a are completely unknown. To begin to investigate this gap in knowledge we focused on the E3 ubiquitin ligase NEDD4. This particular E3 ligase has been shown to regulate mitochondria proteins such as VDAC, indicating its capacity to interact with mitochondrial associated proteins²⁶⁵. We performed immunoprecipitation of CPT1a firstly to determine if there's an interaction with NEDD4 under basal conditions. We find Nedd4 interacts with CPT1a under basal conditions and that the addition of MG132 enhances that interaction (Fig. 3e). Intriguingly, addition of 4-OI reduces the interaction between Nedd4 and CPT1a, under both basal and MG132 stimulated conditions (Fig. 3e). These data indicate a novel interaction between the E3 ubiquitin ligase NEDD4 and CPT1a, which is disrupted by itaconate. These data collectively indicate itaconate may directly target ubiquitin ligases in order to stabilize CPT1a to confer its anti-steatotic properties.

Irg1 deficiency impairs the thermogenic program during sepsis

Apart from the liver, adipose tissues also play a central role in the maintenance of whole-body energy homeostasis. While white adipose tissues

function to store excess energy in the form of triglycerides, brown adipose tissue (BAT) are metabolically active adipose depots which contribute to non-shivering thermogenesis²⁶⁶. This is achieved, in part, through oxidation of fatty acids and activation of UCP1²⁶⁷. A decrease in body temperature during sepsis is an independent predictor of mortality^{268,269}. Therefore, given the importance of thermogenesis during sepsis and our findings that *Irg1* deficiency impairs hepatic β -oxidation, we endeavored to determine if this has effects on BAT function. We assessed core body temperature in response to an endotoxin challenge in WT and *Irg1* KO mice. WT display a significant drop in body temperature in response to LPS treatment, peaking at 12 hours and returning to near baseline by 24 hours (Fig. 4a). We find *Irg1* KO mice have a significantly more dramatic drop in body temperature following LPS treatment compared to their WT littermates (Fig. 4a). Next, we aimed to characterize potential mechanism underlying the hypothermic phenotype in *Irg1* KO mice. To this end, we assessed the protein expression of UCP1. We find impairment in UCP1 gene and protein levels in *Irg1* deficient mice following endotoxin treatment, independent of PGC1 α expression (Fig 4b-d). Furthermore, the BAT of endotoxin challenged *Irg1* KO mice was larger in weight compared to LPS-injected WT septic mice (Fig 4e). In summary, these data indicate an impairment in UCP1-driven thermogenesis in the BAT of *Irg1* deficient mice following an inflammatory challenge. Furthermore, our data indicate this could be mediated by impaired BAT fatty acid oxidation, as evidenced by the larger BAT depot in inflamed *Irg1* KO mice.

***Irg1* deficiency impairs systemic substrate utilization during sepsis**

We have previously reported a global shift in systemic fuel preference from glucose to fatty acid oxidation in response to CLP-induced sepsis²⁷⁰. Our data thus far indicate itaconate deficiency impairs lipid metabolism during inflammation at the organ level. However, it remains unknown the systemic effects of *Irg1* KO on sepsis-induced shifts in fuel preference. However, it is known that *Irg1* KO mice favor glucose oxidation over fatty acids under baseline conditions²⁷¹. Given this, we next investigated the effects of itaconate deficiency on inflammation-induced metabolic flexibility. We first assessed energy expenditure in LPS-injected WT and *Irg1* KO mice by using indirect calorimetry-enabled metabolic cages. We observed a decrease in energy expenditure in both groups in response to LPS, however, no significant differences between the groups was appreciated, under both baseline and post-LPS injection (Fig 5a-d). Next, we interrogated the respiratory exchange ratio (RER) as an indicator of systemic fuel preference. Consistent with our previous study²⁷⁰, WT septic mice demonstrated a shift in fuel preference indicated by a decrease in RER from about 1 to 0.7 (Fig 5e-h), signifying a higher reliance on fatty acid oxidation over glucose utilization. Interestingly, we found *Irg1* KO mice had a decrease in RER following LPS treatment, however, but not to the same extent as their WT-type counterparts (Fig. 5g&h), indicating itaconate deficiency leads to a subtle defect of systemic fat utilization.

Discussion

Our studies identify itaconate as a central modulator of lipid metabolism during the course of overt inflammation. Specifically, we show itaconate may

enhance lipid clearing in the liver through the stabilization of mitochondrial fatty acid uptake enzyme CPT1a. Additionally, we have uncovered novel itaconate substrates involved in protein ubiquitination which may underlie the lipid clearing effects of itaconate. Lastly, we demonstrate systemic defects in lipid metabolism and thermogenesis in itaconate deficient mice following endotoxin challenge. These studies extend our understanding of itaconate as a metabolic regulator in response to inflammation.

Mitochondrial bioenergetics and metabolic reprogramming play a crucial role in promoting both immune and non-immune changes in response to inflammation^{85,100,253,272,273}. Furthermore, metabolic dysregulation in the context of sepsis can profoundly contribute to impaired lipid metabolism, which can enhance sepsis severity and mortality^{96,270,274-276}. We previously demonstrated sepsis elicits a systemic increase in fatty acid mobilization and utilization²⁵³, which supports a systemic shift in fuel preference from glucose to fatty acid oxidation²⁷⁰. Additionally, we have shown sepsis induces dyslipidemia, which in turn promotes the development of hepatic steatosis^{96,270}. While the exact mechanisms driving alterations of hepatic lipid metabolism during inflammation are not fully understood, dysregulation of hepatic PPAR α signaling has been implicated^{78,85,277}. These pre-clinical findings are consistent with human septic patients, which show the presence of hepatic steatosis^{205,278}. Our data identifies itaconate as a novel pathway for the regulation of hepatic lipid metabolism during sepsis.

The role of itaconate in modulating fatty acid oxidation is becoming more appreciated. Several previous studies have demonstrated itaconate promotes β -oxidation^{141,140}. Our studies demonstrate itaconate protects against aberrant hepatic steatosis during sepsis. Furthermore, we identify the β -oxidation pathway as a target of itaconate. Interestingly, *Irg1* KO mice have been shown to have decreased fatty acid oxidation and enhanced glucose oxidation compared to wild-type mice¹⁴³. Our data sheds insight into the mechanism by which this may be afforded. Specifically, the modulation of CPT1a and other carnitine shuttle enzymes underlies, at least in part, the regulatory role of itaconate on the β -oxidation pathway.

Previously, it has been reported that itaconate plays a role in regulating the β -oxidation of fatty acids to fuel OXPHOS. This has been observed in various cell types, including hepatocytes from mouse models of NAFDL, tissue-resident macrophages in peritoneal tumors¹⁴⁰, macrophages from zebrafish¹⁴¹ as well as T cell subsets Th17- and Treg- polarizing T cells¹²⁰. This is in conjunction with our observations given significant upregulation in proteins governing the β -oxidation and OXPHOS pathway by itaconate. Interestingly, we also observed itaconate regulation of key steps within cholesterol biosynthesis in ALM12 cells when assessing pathways involved in cellular metabolism. We hypothesize this is due to the inactivation of vitamin B₁₂ by itaconyl-CoA, resulting from itaconate activation^{111,119}. This can cause impairment of methionine synthase activity, an enzyme dependent on vitamin B₁₂, leading to dysregulation in the conversion of

homocysteine to methionine, and ultimately, alterations in the abundance of S-adenosylmethionine (SAM), a methyl donor in numerous biological and biochemical processes²⁷⁹. Interestingly deficiency in B₁₂ has been found to induce cholesterol biosynthesis by limiting SAM and modulating the methylation of SREBF1 and LDLR genes¹²¹. Additionally, alteration in intracellular SAM abundance and reduction in SAM/SAH ratio by itaconate was found to influence TH17/ Treg cell differentiation as a result of tri-methylation of histone H3 protein induced epigenetic reprogramming¹²⁰. However, the exact mechanism by which itaconate regulates metabolic and epigenetic reprogramming to enhance hepatic cholesterol synthesis is not known and needs to be further investigated given the crucial role of cholesterol in maintaining cellular organization, steroid hormone, bile acids, and vitamin D synthesis²⁸⁰.

Frieler et al. recently uncovered a protective role of endogenous itaconate in regulating adipocyte metabolism through altering glucose homeostasis, lipolysis, and adipogenesis during HFD-induced obesity¹⁴³. Furthermore, they demonstrated endotoxin induces the expression of *Irg1* in brown adipose tissue (BAT). However, the biological function of inflammation induced *Irg1* in BAT was not studied. A previous study demonstrated improved body temperature and clinical scores in septic mice upon exogenous 4-OI administration¹²². However, the role of endogenous itaconate in modulating body temperature in response to inflammation has yet to be investigated. Our study begins to fill this gap by showing a pro-thermogenic effect of itaconate during endotoxin challenge. While the mechanism underlying these effects were not investigated in the current study, we show a

defect in the primary thermogenic mediator UCP1. Research has demonstrated that inter-organ crosstalk between BAT and the liver is essential to elicit non-shivering thermogenesis. Specifically, BAT utilizes hepatic derived acylcarnitines released in response to cold stress²⁸¹. Intriguingly, these studies demonstrated a reliance on hepatic CPT1 function to mediate cold stress induced acylcarnitine secretion²⁸¹. Our data demonstrates impaired CPT1a expression in the liver of endotoxin challenged *Irg1* KO mice. These findings in conjunction with previous literature may provide a potential physiological mechanism by which itaconate deficiency hinders hepatic acylcarnitine production due to impairment in CPT1a expression. Future studies targeting hepatic CPT1a expression in *Irg1* KO mice would uncover causal links between hepatic fatty acid oxidation and thermogenesis in response to endotoxin. This is important, as clinically and in murine models of sepsis, the onset of hypothermia is observed and considered a predictor of mortality^{277,282}.

Given its electrophilic properties and its ability to directly alkylate cysteine residues, we initially hypothesized regulation of FAO was driven by enhancement of CPT1a due to its itaconation. Contrary to our hypothesis, proteomic profiling of ITalk substrates in AML12 cells did not reveal CPT1a as a target of itaconation. However, proteomic analysis revealed enrichment of the protein ubiquitination pathway by ITalk. We found this of interest, as this may explain the stabilizing effects of itaconate on CPT1a protein expression. The regulation of protein ubiquitination by itaconate is a mechanism that has been implicated in numerous models of inflammation. The first is the classical upregulation of NRF2 due to the alkylation of cysteine residues of KEAP1¹⁴², which functions as an adaptor of the

Cul3-based ubiquitin E3 ligase complex. This covalent modification promotes the dissociation of KEAP1 from CUL3 to inhibit the conjugation of ubiquitin onto the N-terminal domain of NRF2^{122,283,284}. Additionally, 4-OI has been shown to negatively regulate osteoclastogenesis and inflammatory response by suppressing E3 ubiquitin-protein ligase HRD1 to activate Nrf2 signaling²⁸⁵. However, the mechanism underlying the inhibition of HRD1 by itaconate was not investigated or discussed. While the bona fide E3 ligases responsible for proteasomal turnover of CPT1a have yet to be discovered, our data indicate a potential interaction with the E3 ligase NEDD4. We hypothesize alkylation of reactive cysteine residues within the catalytic domains of NEDD4 by itaconate inhibits its interaction with CPT1a leading to hypoubiquitination and subsequent stabilization of protein. Future studies aimed at dissecting the regulatory role of NEDD4 in the proteasomal turnover of CPT1a are warranted and would reveal a potential regulator of CPT1a expression at the post-translational level within hepatocytes.

Accumulation of itaconate in the mouse liver during sepsis has highlighted the necessity to understand its functional role within this compartment. By endeavoring to uncover the biological role of itaconate in hepatocytes, we have uncovered a novel function of itaconate within the liver and systemically, to aid in fatty acid processing in the face of inflammation. Furthermore, our work identifies a potentially new mechanism of action via the stabilization of CPT1a. Finally, our work has uncovered systemic effects of endogenous itaconate on metabolic flexibility and thermogenesis in response to inflammation. Overall, these findings suggest that interventions aimed at regulating the *Irg1*/itaconate axis may hold potential

therapeutic advantages in regulating dyslipidemia at both the local and systemic levels observed during sepsis. Future work is necessary to understand cellular sources of itaconate, and the role of this immunometabolite in coordinating interorgan crosstalk during sepsis.

Figures

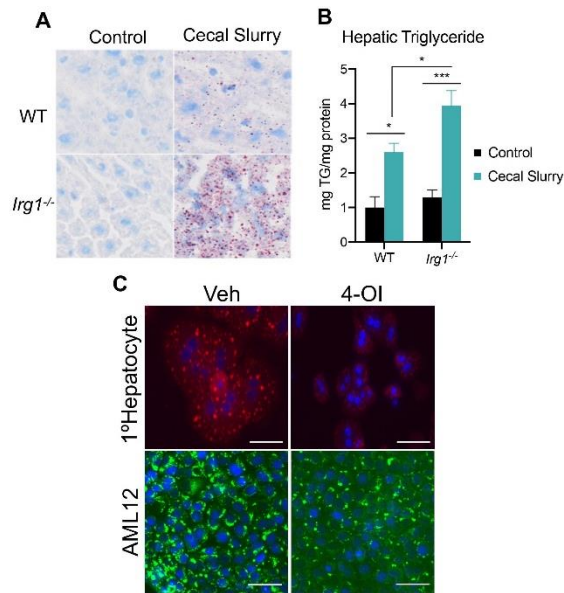
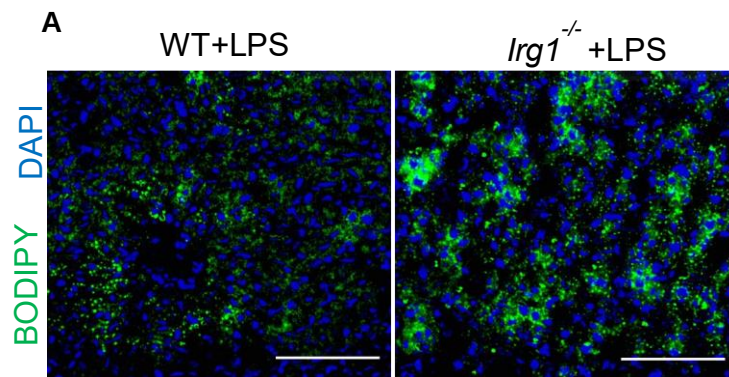


Figure 1: *Irg1* deficiency promotes exacerbation of hepatic lipid accumulation during sepsis. (A) Oil red O staining of liver sections of WT and *Irg1* KO control and septic mice. (B) Hepatic triglyceride measures. N=7 mice per group. (C) BODIPY staining of livers from WT and *Irg1* KO mice injected with LPS. (D) Oleate-loaded AML12 cells and primary hepatocytes treated +/- 4-OI (250μM) stained with either BODIPY (top panel) or Nile Red (bottom panel). Data are represented as mean ± SEM. * denotes $p < 0.05$, *** $p < 0.001$



Supplement Figure 1a: Overt hepatic lipid droplet accumulation in female septic *Irg1* KO compared to WT KO mice. (A) BODIPY staining of livers from WT and *Irg1* KO female mice injected with LPS (5mg/kg body weight).

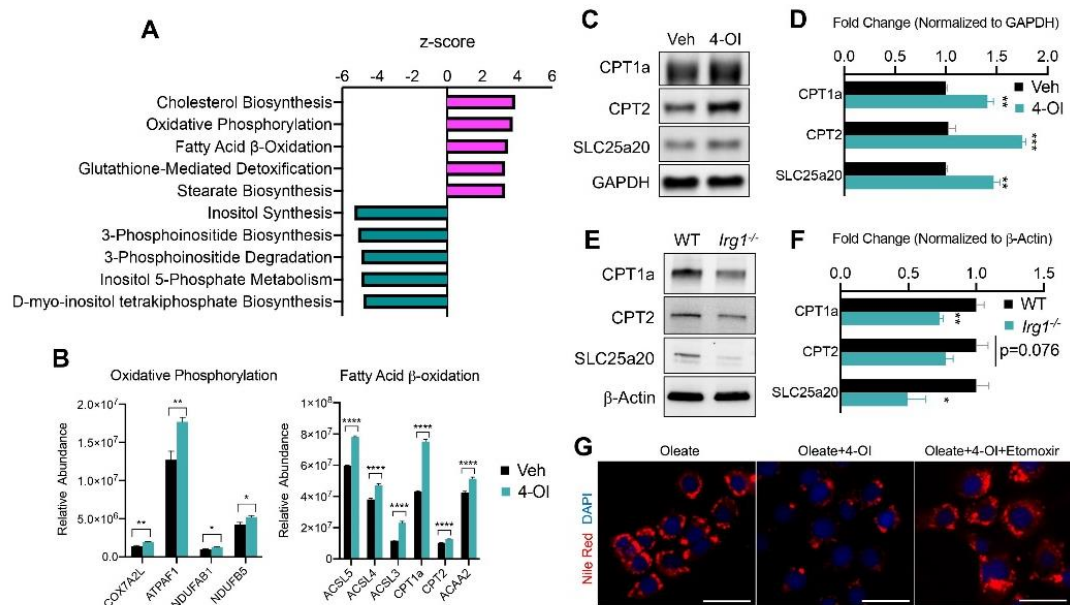
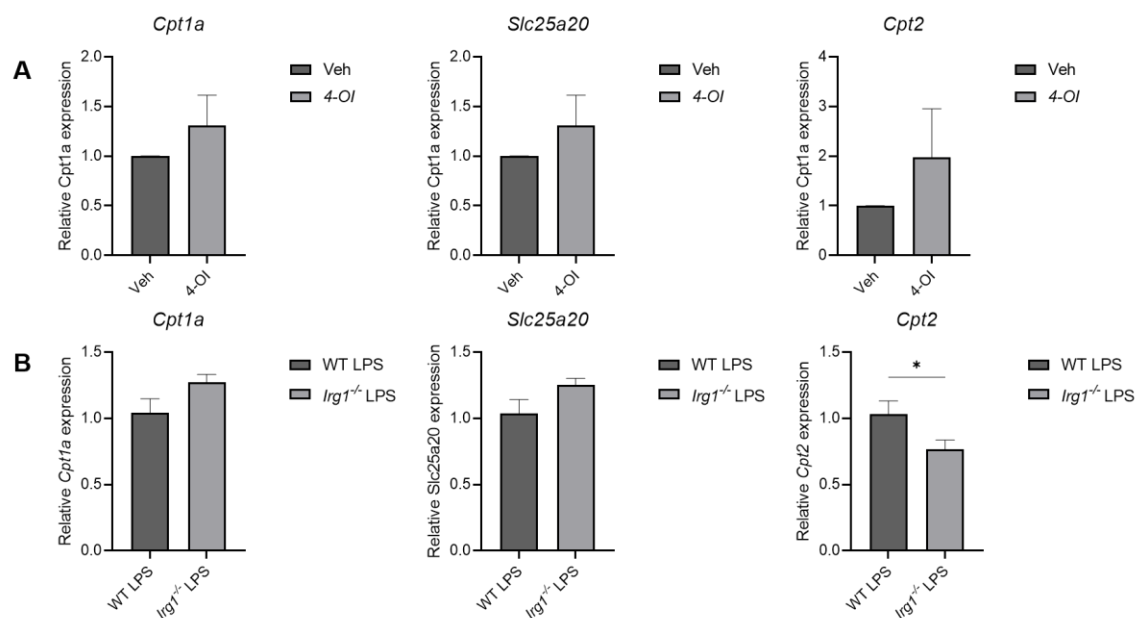


Figure 2: 4-OI promotes mitochondrial fatty acid uptake and clearance. (A) Pathway analysis of global proteomics of AML12 cells stimulated with 4-OI for 24 hours. n=5 biological replicates per group. (B) Quantification of CPT1a/CPT2 in proteomics analysis. (C) Western blot of CPT1a, CPT2 and SLC25a20 in AML12 stimulated with 4-OI for 24 hours. (D) Western blot of liver lysates of LPS injected WT and *Irg1* KO mice. (E) Nile Red images of lipid-loaded hepatocytes treated with 4-OI (250 μ M) +/- etomoxir (4 μ M). N=3 independent experiments. * * denotes $p < 0.05$, *** $p < 0.001$



Supplement Figure 2: Proteins of the carnitine shuttle pathway. Gene transcription levels of *Cpt1a*, *Slc25a20*, *Cpt2* were detected via RT-qPCR in (A) AML12 cells treated with 4-OI for 24 hours (n=3 independent) and (B) liver lysates from 24 hours WT and *Irg1* KO LPS (5mg/kg body weight) challenged mice. (n= 7 WT, n=9 KO).

* denotes p<0.05

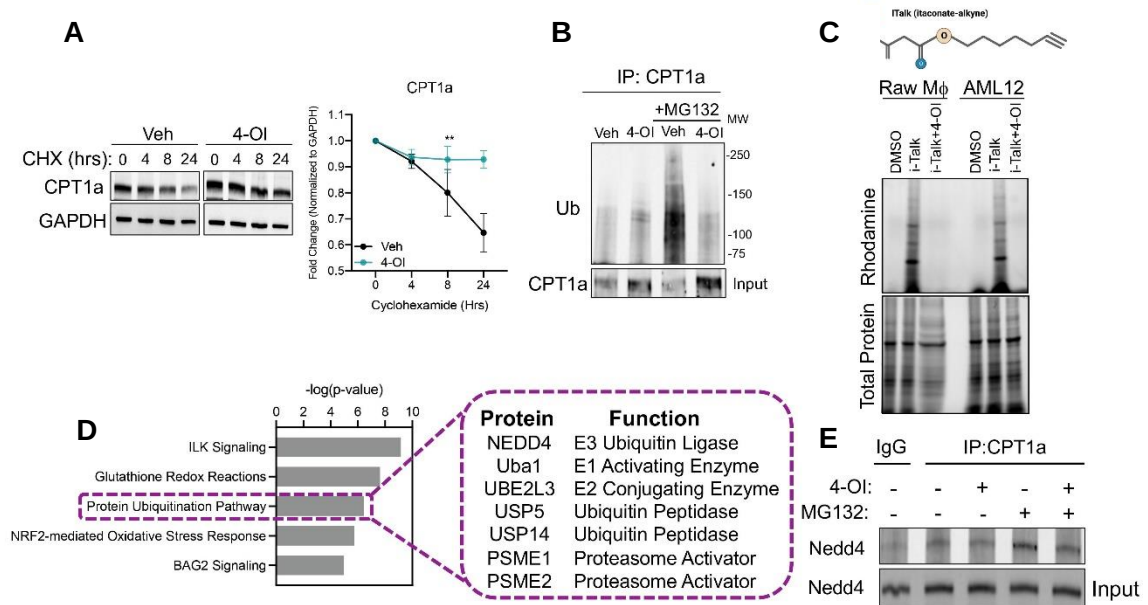


Figure 3: 4-OI stabilizes CPT1a protein expression. (A) Western blot and quantification of CPT1a in AML12 cells that were pretreated with vehicle or 4-OI for 24 hours, then stimulated with CHX. n=3. (B) Immunoprecipitation of CPT1a in AML12 cells pretreated with 4-OI for 24 hours followed by stimulation with MG132 for 6 hours. Equivalent amounts of proteins were immunoprecipitated with anti-CPT1a and subjected to Western blot analysis with ubiquitin antibody. (C) ITalk labeling of macrophages and hepatocytes in vitro and western blot image of rhodamine fluorescence (top) and total protein (bottom) in Raw macrophages and AML12 cells. (D) Pathway analysis of global proteomics of AML12 cells stimulated with ITalk for 12 hours. (D) Equivalent amounts of proteins were CPT1a was immunoprecipitated with anti-CPT1a and subjected to Western blot analysis of NeDD4 protein expression. N=3 independent experiments. ** denotes p<0.01

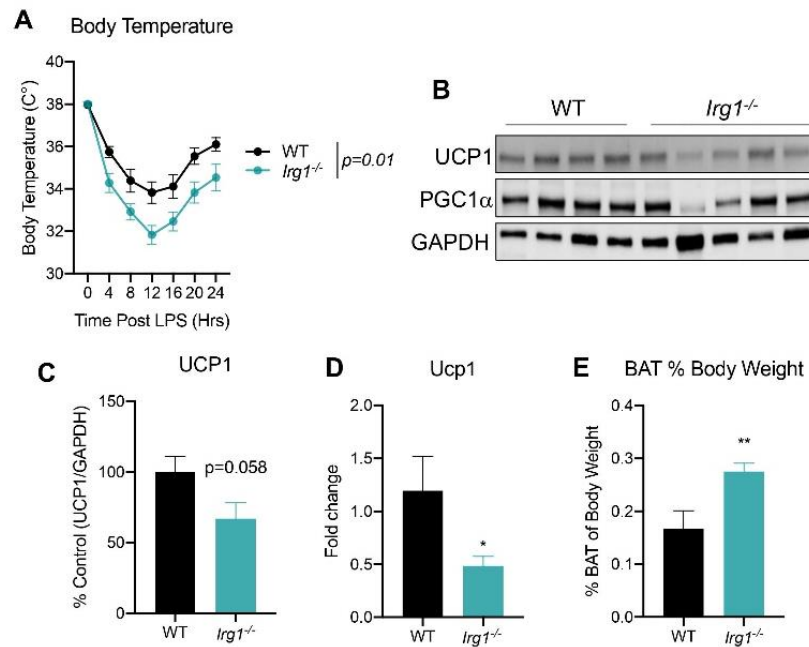


Figure 4: Irg1 deficiency worsens hypothermia response and promotes BAT dysfunction during sepsis. (A) Core body temperature in female WT and Irg1 KO mice following LPS injection. n=5-8 mice per group. (B) Western blot of UCP1, PGC-1a and GAPDH in BAT of LPS injected WT and Irg1 KO mice. Quantification on the right. n=5-8 mice per group. (C) Western blot (D) mRNA expression of UCP1 in BAT of LPS injected WT and Irg1 KO mice. n=5-8 mice per group. (E) BAT weight 24h post-LPS injection in WT and Irg1 KO mice. n=5-8 mice per group. * denotes $p<0.05$, ** $p<0.01$.

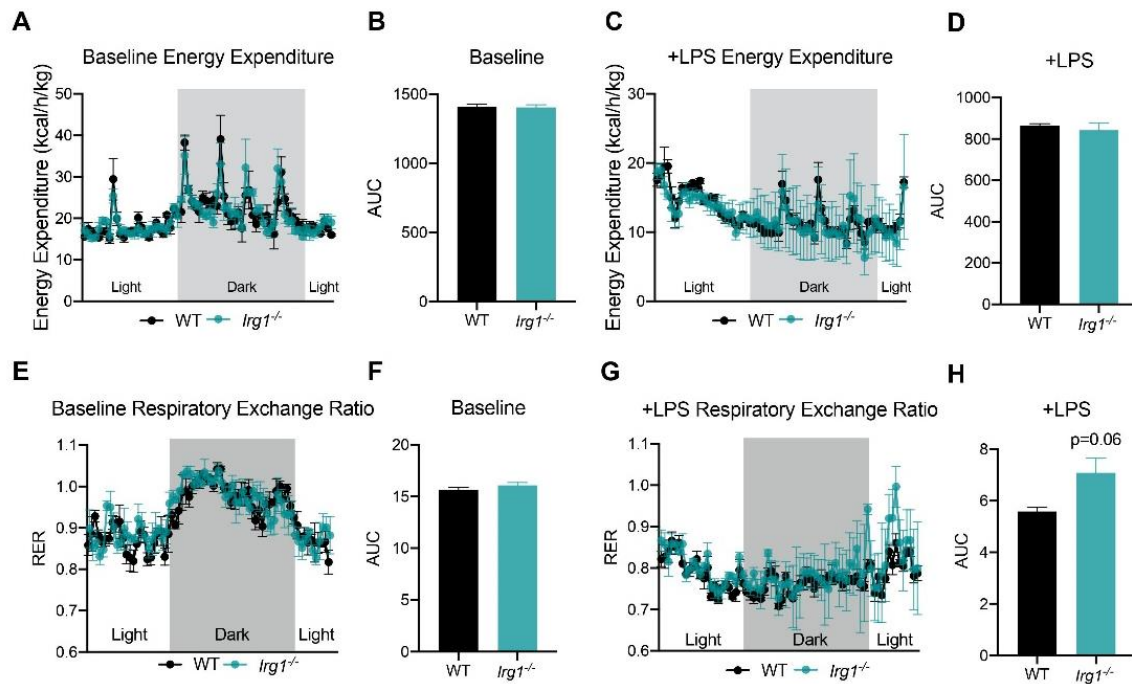


Figure 5: *Irg1* deficiency impairs systemic substrate utilization during sepsis.

(A) Energy Expenditure (kcal/hr/kg) during baseline and (C) post-LPS injection in female *Irg1* KO and WT controls. Plots represent 24- hour cycle. n=3 mice per group. (B,D) Area under the curve for energy expenditure values over 24-hour cycle from panel B and D. (E) Respiratory exchange ratio (RER) during baseline and (G) post-LPS injection in female *Irg1* KO and WT littermate controls. Plots represent 24- hour cycle. n=3 mice per group. (F,H) Area under the curve for RER values over 24-hour cycle from panel F and H. Statistical significance was calculated using an unpaired 2-tailed Student's t test.

Experimental procedures

Antibodies

Primary antibodies used in the study are as follows: mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology, SC-32233), rabbit polyclonal anti-ACSL1 (Protein Tech, 13989), mouse monoclonal anti-CPT1a (Protein Tech, 151841), rabbit polyclonal anti-CPT2 (Protein Tech, 2655), rabbit polyclonal anti-SLC25A20 (Protein Tech, 19363), mouse monoclonal anti-Ubiquitin (Cell Signaling Technology, 3936) and mouse monoclonal anti- β -actin (Cell Signaling Technology, 3700S).

Western Blot and co-immunoprecipitation

Cells were lysed in 1X Laemmli SDS sample buffer and boiled for 5 min. Lysates were resolved on SDS PAGE gels and blotted onto PVDF membranes using Trans-Blot Turbo RTA Midi 0.45 μ m LF PVDF Transfer Kit (Bio-Rad, 1704275). The transfer was run in Tran-Blot Turbo Transfer System (Bio-Rad, 1704150) using a Mixed MW (1.3A-25V-7M) preset. The membranes were then blocked with InterceptTM (TBS) Blocking Buffer (LI-COR, 927-60001) for 45 minutes at room temperature and incubated in primary antibody (1:1000) overnight at 4°C. On day 2 membranes were washed three times with TBS with 0.05% tween20 (Bio-Rad) and then incubated with IRDye[®] 800CW Goat anti-Mouse IgG (LI-COR, 926-32210) and IRDye[®] 680RD Goat anti-Rabbit IgG (LI-COR, 926-68071) secondary antibodies (1:10000) in blocking buffer for 1 h at room temperature.

Band images were visualized using ChemiDoc™ MP Imaging System (Bio-Rad, 12003154).

Co-immunoprecipitation experiments were performed utilizing Pierce™ MS Compatible Magnetic IP Kit (Thermo Fisher Scientific, 90409). 1mg of total protein was incubated with 5ug of anti-CPT1a overnight at 4°C, then incubated with pierce protein A/G magnetic beads for an hour at room temperature. Beads were washed and then boiled for 7 min in 1X Laemmli SDS sample buffer. Proteins were analyzed using western blotting with ubiquitin antibody and visualized using ChemiDoc™ MP Imaging System (Bio-Rad, 12003154).

Cell culture

Murine hepatocyte cell line AML12 (ATCC, #CRL-2254) was maintained on plastic cell culture plates in Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F-12) supplemented with 10% FBS, Gibco™ Insulin-Transferrin-Selenium supplement (Gibco), and penicillin and streptomycin in a humidified incubator (37°C, 5% CO₂). *In vitro* experiments were done 12 hours after plating cells on desired plates. Reagents added to cell culture media are as follows: 250 μM 4-octyl itaconate (Tocris Bioscience, 6662), 4mM Etomoxir (Sigma, E1905), Oleate (Sigma, O1008), Cyclohexamide (Sigma, C4859), 10uM MG132 (Cell Signaling 2194S).

RNA isolation and RT-qPCR.

Liver tissues were homogenized in 1mL TRIzol. After centrifugation at maximum speed for 5 min, 200uL of chloroform was added, mixed thoroughly, allowed to sit for 5 minutes, and centrifuged for another 5 minutes. The resulting aqueous phase was mixed with 50% ethanol and subjected to RNA binding column (Aurum RNA miniprep Kit (732-6820, Bio-Rad) to extract DNA-free high-quality RNA. 25 ng RNA was used to perform gene expression using iTaq Universal One-Step Real-Time qPCR kit (Bio-Rad). The reaction was performed according to the manufacturer's instructions using CFX Connect Real-Time PCR Detection system (1855200, Bio-Rad). The probes used for TaqMan gene expression were as follows: (gene name and code)

BSA-Oleate Complex

0.25 M of oleic acid (OA) in 100% ethanol and 0.5% BSA in DPBS was prepared and incubated in 60°C water bath for 30 minutes. Then, dropwise 800uL of 0.25 M OA was added into 49.2 mL of 0.5% BSA to make 4 mM OA in 5% BSA. The solution was heated for additional 3 hours with vigorous vortexing every 30 minutes until the solution was clear before freezing it. OA BSA conjugate was warmed for 30 minutes in a 60°C water bath before cell treatment.

ITalk and Click Chemistry

AML12 cells were treated with 100 μ M Itaconate-alkyne (ITalk, MedChemExpress, HY-133870) for 12 hours. Cells were then lysed, clicked, and released for mass spectrometry analysis according to the Click-&-Go™ Dde Protein Enrichment Kit (Click Chemistry Tools, 1153).

MS/MS Analysis

Samples were analyzed on a LC-MS/MS system consisting of an Orbitrap Eclipse Mass Spectrometer (Thermo Scientific, Waltham, MA) and a Vanquish Neo nano-UPLC system (Thermo Scientific, Waltham, MA). Peptides were separated on a DNV PepMap Neo (1500 bar, 75 μ m x 500 mm) column for 120min employing linear gradient elution consisting of water (A) and 80% acetonitrile (B) both of which contained 0.1% formic acid. Data were acquired by top speed data-dependent mode where maximum MS/MS scans were acquired per cycle time of 3 seconds between adjacent survey spectra. MS2 scans were repeated with precursor ion subsets isolated by ion mobility using the FAIMS which compensation voltage was set to -45 eV, -55 eV, and -65 eV sequentially. Dynamic exclusion option was enabled where duration was set to 120 seconds. To identify proteins, spectra were searched against the UniProt human protein FASTA database (20,309 annotated entries, Jun 2021) using the Sequest HT search engine with the Proteome Discoverer v2.5 (Thermo Scientific, Waltham, MA). Search parameters were as follows: FT-trap instrument; parent mass error tolerance, 10 ppm; fragment mass error tolerance, 0.6 Da (monoisotopic); enzyme, trypsin (full); # maximum missed cleavages, 2; variable modifications, +15.995 Da (oxidation) on methionine; static modification (only for soluble part), +57.021 Da (carbamidomethyl) on cysteine.

Histological analysis and lipid droplet staining

After undergoing treatments, the cells were rinsed with PBS and fixed in 4% PFA at room temperature for five minutes. Subsequently, the cells were stained with Nile Red for ten minutes at 37°C, washed with PBS, and mounted using Fluroshied with DAPI. The cells were visualized using the ZOETM Fluorescent Cell Imager (Bio-Rad, 1450031).

Liver sections with a thickness of 5µm, were fixed with 4% PFA for 10 minutes at room temperature. Next, the slides were dipped a few times in 60% isopropyl alcohol and then incubated in the working solution of Oil Red O for 10 minutes. The slides were subsequently rinsed a few times in 60% isopropyl alcohol, followed by three rinses with distilled water. The sections were then stained with hematoxylin as a counterstain for 1 minute, followed by three rinses with distilled water. Finally, the slides were mounted using Fluroshied with DAPI and imaged.

Animals and experimental model of sepsis

The C57BL/6N-Acod1^{em1(IMPC)J/J} mice are commercially available from Jackson Laboratories (029340) and were bred within the Quinn Laboratory. Genotype was verified via PCR before inducing experimental sepsis. Male mice were subjected to sepsis via intraperitoneal injection of cecal slurry previously described by Starr et.al²⁸⁶. Female mice in the treatment group were intraperitoneally injected with LPS (5mg/kg body weight) at a dose of 5mg/kg body weight. After 24 hours, the mice were euthanized, and blood and tissue samples were collected for further analysis.

Study approval

All experiments and procedures involving mice were carried out following the approved protocols of the Institutional Animal Care and Use Committee (IACUC) of Wake Forest School of Medicine.

CHAPTER V

Concluding remarks

Studies in Chapter II were done to expand our knowledge of metabolic reprogramming in the liver during sepsis. Through the course of this investigation, we found that:

1. Sepsis leads to impairment in hepatic mitochondrial metabolism and leads to TCA cycle remodeling and dysregulation of REDOX homeostasis.
2. Sepsis leads to dysregulation of global hepatic lipid metabolism which in turn causes dyslipidemia and ultimately leads to the development of hepatosteatosis.
3. Reversing hepatic metabolic dysfunction during sepsis by targeting the PDH/PDK axis can lead to the restoration of metabolic manifestations observed during the condition. This finding suggests that pharmacological interventions aimed at restoring mitochondrial metabolic reprogramming and bioenergetics during over-inflammation are protective in rodent models of sepsis.

However, a major limitation of this study was that the molecular regulators of hepatic dyslipidemia were not identified. Furthermore, we could not conclude whether this was a protective or maladaptive response. Therefore, the studies in Chapter IV focused on understanding the hepatic molecular regulators of lipid metabolism, specifically the role of sepsis-related factors such as TCA cycle-derived metabolites in the regulation of hepatic lipid metabolism and the role of

these lipids in modulating the systemic response to sepsis. Through the course of this investigation, we found that:

1. Septic *Irg1* mice lacking the ability to synthesize itaconate in response to sepsis display overt hepatosteatosis and lipid burden compared to WT septic controls.
2. *In vivo*, itaconate is an anti-steatotic molecule as it directly modulates lipid handling and burden in hepatocytes.
3. Itaconate stabilizes Cpt1a by inhibiting its proteasomal degradation which enhances mitochondrial uptake of free fatty acids and β -oxidation which is underlying the anti-steatotic effects.
4. At a systemic level, septic *Irg1* KO mice display impaired oxidation of fatty acid for fuel. Additionally, *Irg1* KO mice display signs of brown adipose tissue dysfunction such as impaired UCP1 expression and overt-hypothermia in response to LPS-induced sepsis compared to WT septic counterparts. Collectively, this highlights the potential role of itaconate in the regulation of systemic energy metabolism with consequences on thermogenesis in response to inflammation.

Collectively, the data presented so far supports the growing body of literature suggesting the role of itaconate in the regulation of not just hepatic but systemic lipid and energy homeostasis. However, much work still needs to be done to better understand the functional relevance and mechanism underlying this regulation by itaconate. Some of them are:

1. Identification and verification of adaptor E1, E2, and E3 ubiquitin enzymes responsible for the ubiquitination of CPT1a are necessary. While few of these proteins were identified in ITalk proteomics (NEDD4, UBA1, UBE2L3), it is not known if these proteins are involved in the ubiquitination of CPT1a. Based on itaconate's electrophilic properties and the fact that reactive cysteine residues of CPT1a were found to not be itaconated, it can be hypothesized that functional loss of proteins of the three-tiered enzymatic cascade necessary for ubiquitination could be potentially underlying this. Covalent modification of catalytic cysteine domains of E1, E2, and E3 proteins is known to inhibit the proper transfer of ubiquitin onto target substrate proteins. Once the appropriate protein is verified, experiments utilizing genetic tools/manipulation can be used to verify the regulatory role of these proteins in the proteasomal turnover of CPT1a. Furthermore, this would all for verifying the functional role of itaconate in regulating lipid burden in hepatocytes indeed through its inhibitory effects on ubiquitination enzymes.
2. To investigate the mechanism by which itaconate governs thermogenesis during inflammation, it would be of interest to explore whether the lack of hepatic substrates, such as acylcarnitines, contributes to overt hypothermia in Irg1 KO septic mice. In response to sepsis, there is an increased accumulation of acylcarnitines in the liver and circulation, but it is unclear if this supports systemic adaptations like thermogenesis during inflammation. Moreover, the hepatic lipid burden is high in the absence of Irg1 in

response to septic conditions. We believe this is due to the impairment of FAO, given the significant decrease in fatty acid oxidation (FAO) protein expression in the absence of endogenous itaconate production during inflammation. This would further decrease substrate production for thermogenesis. To better understand the physiological relationship between endogenous itaconate and the regulation of hepatic FAO to support thermogenesis and systemic energy metabolism, rescues, and restoration studies, such as the administration of bolus acylcarnitine or overexpression of CPT1a to increase FAO and hepatic output of acylcarnitine in septic *Irg1* KO mice, would be helpful.

3. Alternatively, it has been reported that brown adipose tissue (BAT) tissues express *Irg1* in response to LPS-induced inflammation. However, the biological function of itaconate in BAT tissue function and thermogenesis has not been studied. It would be pertinent to investigate this, especially if we find that hepatic restoration of CPT1a or administration of acylcarnitines to *Irg1* KO mice still display signs of BAT tissue dysfunction and overt hypothermia. Perhaps the impaired expression of UCP1 and increase in gross BAT weight is a local consequence of the loss of *Irg1* within the BAT, which culminates in the dysfunction of local FAO needed to fuel thermogenesis.

In summary, our cellular studies have identified the immunometabolite itaconate as playing a role in the regulation of hepatic lipid metabolism, with potential systemic effects on energy metabolism and thermogenesis

during inflammation. Although we have not fully teased out the mechanism underlying this anti-steatotic phenotype of itaconate, we have identified and validated that it is a result of enhanced CPT1a stability by itaconate. This enhancement results in an increase in mitochondrial uptake of FA for FAO, leading to a lower intracellular lipid burden. By addressing the limitations of our study that were previously mentioned, future research has the potential to reveal Irg1/itaconate as a promising therapeutic target for restoring and preserving hepatic function in sepsis.

RABINA MAINALI

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Education

Ph.D. Candidate, Integrative Physiology and Pharmacology, *Expected Graduation: August 2023*

Wake Forest University, Graduate School of Arts & Sciences, Winston-Salem, NC

Bachelor of Science in Biochemistry, Salem College, Winston-Salem, NC, *Graduation: December 2018*

Skills Summary

- Extensive experience in mammalian and bacterial cell culture, including primary hepatocyte isolation, and bioenergetics studies using Agilent Seahorse XF technology.
- Comprehensive knowledge of *in vitro* and *in vivo* experimental techniques, cell-based molecular biology assays, gene expression analysis (RT-PCR, electrophoresis), protein isolation and purification (Western blot, Co-IP), immunological staining, and DNA/RNA isolation.
- Proficient in statistical analysis software, including GraphPad Prism and Excel, histological specimen preparation and storage, light and fluorescent microscopy, and mouse handling and colony management.
- Knowledgeable in the foundations of GMP, with the ability to troubleshoot and optimize experimental protocols and designs and development and validation of methods.
- Highly skilled in critical thinking and problem-solving, with a proven ability to build and maintain strong relationships with collaborators and colleagues.
- Experienced in market research and able to effectively apply this knowledge to inform experimental design and strategy.

Professional Experience

Wake Forest School of Medicine, NC

May 2019 - Current

Ph.D. Candidate: Mentor-Dr. Matthew Quinn

- Research the dysregulation of metabolism that is associated with the chronic phase of sepsis.
 - More specifically, investigate and analyze hepatic metabolic reprogramming that results from inflammation-driven liver injury using *in vitro* & *in vivo* experimental models.
 - Additionally, study the role of mitochondrial metabolite itaconate in the modulation of hepatic lipid metabolism and host survival during overt inflammation like sepsis.
- Demonstrate research findings with efficacy and professionalism to diverse audiences at national and international conferences.
- Exhibit exceptional proficiency in scientific writing, including the development of comprehensive grant proposals, manuscripts, and technical reports.
- Mentor and supervise a team of 5-7 college students in the lab environment, ensuring seamless research operations and high-quality output.

Wake Forest Innovation, NC*February 2022 - Current**Business Technology Intern*

- Conducted extensive market analysis and prior art search to ensure comprehensive due diligence before proceeding with provisional patent filing.
- Demonstrated exceptional proficiency in statistical analysis and data management, utilizing cutting-edge cloud-based applications such as Sophia to ensure precise and accurate results.
- Acquired comprehensive training in the drafting of invention disclosures and invention agreements, allowing for seamless and efficient patent filing procedures.

Wake Forest School of Medicine, N*January 2020 - May 2020**Rotating graduate student: Mentor-Dr. Cristina Furdui*

- Conducted comprehensive evaluations on the impact of silver nanoparticles on mitochondrial dynamics, bioenergetics, and oxidative stress across four distinct lung cancer cell types.
- Provided exceptional support in the evaluation and preparation of samples for mass spectrometry analysis, demonstrating proficiency in sample handling and preparation procedures.

Microbiology & Immunology, Wake Forest School of Medicine, NC*January 2019 - May 2019**Technician*

- Demonstrated exceptional proficiency in maintaining and caring for a colony of mice per IACUC's rigorous regulatory standards.
- Provided essential facilitation and support in a preclinical study focused on investigating the effects of dietary polysaccharides on gut microbiome dysbiosis and metabolic dysfunction in obese mice.
- Conducted comprehensive evaluations of the rheological and sensory profiles of probiotic yogurt formulated with in-house-isolated probiotic bacteria, utilizing cutting-edge techniques and methodologies.
- Provided exceptional assistance in the extraction and analysis of bioactive polysaccharides from acorns and sago.

Center for Diabetes, Obesity & Metabolism*September 2017 - May 2018***Wake Forest School of Medicine, NC***Undergraduate Intern*

- Conducted comprehensive research on the effects of short-chain fatty acids on the feeding behavior of *Caenorhabditis elegans*, utilizing cutting-edge techniques and methodologies to investigate the underlying mechanisms of action.
- Demonstrated exceptional proficiency in managing routine lab operations, including organizing experiments, setting up equipment, and managing inventory of essential laboratory reagents.

- Provided invaluable contributions to the isolation and screening of fecal bacterial strains with potential probiotic properties, demonstrating extensive knowledge and experience in microbiological techniques and procedures.

Residential Life, Salem College

August 2017 - December 2018

Head Residential Assistant

- Demonstrated exceptional leadership and management skills while supervising a team of 14 residential assistants in providing support to on-campus students by developing community-building programs, responding to complaints, requests, and emergencies, and ensuring a safe and welcoming living environment.
- Provided invaluable contributions to the coordination of the enforcement of campus policies and the processing of reports of violation, demonstrating comprehensive knowledge of the relevant regulatory standards and procedures.
- Directed campus-wide events promoting and supporting diversity, utilizing innovative and engaging strategies to promote inclusivity and celebrate diversity within the campus community.

Academic & professional membership

- | | |
|--|-----------|
| • Accelerate to Industry™, Workforce Development Program | Feb 2021- |
| Current | |
| • American Society of Biochemistry & Molecular Biology | Oct 2020- |
| Current | |
| • Beta Beat Beta Biological Society | Aug 2016- |
| Dec 2018 | |

Presentation & Awards

- Funding: T32 GM127261 through National Institute of General Medical Sciences, June 2021-Current
- *Itaconate is a regulator of hepatic lipid metabolism during inflammation*- Selected talk: EMBO workshop (Thiol oxidation in biology: Biochemical mechanisms to physiological outcomes), October 2022
- *Itaconate is a negative regulator of hepatic lipid metabolism during sepsis* – Spotlight Session, Annual Experimental Biology (ASBMB section) conference, April 2022
- *Infection-induced itaconate and its role in metabolic reprogramming*- Salem College, Women in Science and Mathematics Seminar, March 2022
- European Molecular Biology Organization Registration and Travel Award, October 2022
- Wake Forest School of Medicine Alumni Travel Award, April 2022
- American Society of Biochemistry & Molecular Biology Graduate Researcher Travel Award, April 2022

Mentorship

- As a Ph.D. candidate, trained 3 undergraduates and 2 high school students on molecular laboratory techniques, science communication, and data analysis.

Technical Skills

- | | | |
|--|--|--|
| • Mammalian/bacterial cell culture | • Primary hepatocyte isolation | • Bioenergetics studies (Agilent Seahorse XF) |
| • Mice colony management (plus <i>In vivo</i> experiment and dissection) | • Gene expression analysis (RT-PCR, electrophoresis) | • Protein isolation & purification (Western blot, Co-IP) |
| • DNA & RNA isolation | • Immunological staining | • <i>C. elegans</i> management |
| • Statistical analysis (GraphPad Prism & Excel) | • Histological specimen preparation & storage | • Microscopy (Light & fluorescent) |
| • Market research | • Troubleshooting & Optimization | • Experimental design |
| • Critical thinking | • Problem-solving | • Relationship building |

Publications

- **Mainali, R.**, Zabalawi, M., Long, D., Buechler, N., Quillen, E., Key, C. C., ... & Quinn, M. A. (2021). Dichloroacetate reverses sepsis-induced hepatic metabolic dysfunction. *Elife*, 10, e64611.
 - Oh, T., Hutchins, D., **Mainali, R.**, Goslen, K., & Quinn, M. A. (2022). "Itaconate and Its Derivatives Repress Early Myogenesis In Vitro and In Vivo." *Frontiers in Immunology* 13 (2022): 748375-748375.
 - Miller, B., **Mainali, R.**, Nagpal, R., & Yadav, H. (2021). A Newly Developed Synbiotic Yogurt Prevents Diabetes by Improving the Microbiome—Intestine—Pancreas Axis. *International journal of molecular sciences*, 22(4), 1647.
 - Ahmadi, S., Wang, S., Nagpal, R., **Mainali, R.**, Soleimani-Zad, S., Kitzman, D., & Yadav, H. (2019). An In Vitro Batch-culture Model to Estimate the Effects of Interventional Regimens on Human Fecal Microbiota. *Journal of visualized experiments: JoVE*, (149).
 - Nagpal, R., **Mainali, R.**, Ahmadi, S., Wang, S., Singh, R., Kavanagh, K., ... & Yadav, H. (2018). Gut microbiome and aging: Physiological and mechanistic insights. *Nutrition and healthy aging*, 4(4), 267-285.
 - Ahmadi, S., **Mainali, R.**, Nagpal, R., Sheikh-Zeinoddin, M., Soleimani-Zad, S., Wang, S., ... & Yadav, H. (2017). Dietary polysaccharides in the amelioration of gut microbiome dysbiosis and metabolic diseases. *Obesity & control therapies: open access*, 4(3).
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