Immune-metabolic Regulation by the Transsulfuration Pathway

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Abstract

Immune-Metabolic Regulation by the Transsulfuration Pathway

Aileen Heeyeon Lee

2021

Pro-longevity interventions such as caloric restriction (CR) and methionine restriction (MR) can improve metabolic health. However, despite decades of extensive studies, the mechanisms that underlie the benefits of these dietary interventions are still being characterized. Particularly, the effect of these diets on immune cells in mediating their metabolic benefits has been largely unexplored. This work aimed to expand our understanding of how these dietary interventions may regulate immune responses and identify novel immunomodulatory pathways that could be harnessed during inflammatory and metabolic pathologies.

In line with other studies in animal models, we identified that the transsulfuration pathway (TSP) was induced in humans with CR. The TSP controls the synthesis of cysteine from methionine, and produces byproducts such as hydrogen sulfide (H₂S). We find that during MR, NLRP3 inflammasome-mediated inflammation can be inhibited by the induction of autophagy due to low availability of sulfur-containing amino acids (SAA). Additionally, the TSP can inhibit NLRP3 inflammasome through the production of H₂S. This highlights SAA availability mediated by the TSP as an immunoregulatory checkpoint that may be harnessed to reduce inflammasome-mediated inflammation. However, MR failed to reduce white adipose tissue inflammation in mouse models of acute endotoxemia,
Muckle Wells Syndrome, and aging, suggesting that more direct interventions of specific TSP metabolites may need to be explored.

In addition to regulation of inflammation, we have found that single amino acid deficiencies can drive extreme thermogenesis in mice, and pathways that protect critical amino acid levels such as the TSP can serve as important checkpoints to defend against unchecked thermogenesis. We find that cysteine deficiency in mice lacking the TSP leads to rapid loss of fat mass and robust adipose tissue browning. This is mediated by an increase in sympathetic signaling in the adipose tissue, in part through a reduction in the expression of catecholamine degrading enzymes in adipose tissue macrophages.

Altogether, this work highlights that regulation of cysteine availability through the TSP serves as an important check point in metabolic and inflammatory homeostasis, and may underlie many of the metabolic benefits during CR. Manipulation of cysteine availability may thus potentiate novel therapeutic strategies for combating inflammatory metabolic diseases such as aging and obesity.
Immune-metabolic Regulation by the Transsulfuration Pathway

A Dissertation
Presented to the Faculty of the Graduate School
Of
Yale University
In Candidacy for the Degree of
Doctor of Philosophy

By
Aileen Heeyeon Lee

Dissertation Director: Vishwa Deep Dixit, Ph.D.

June 2021
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<th>Description</th>
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<tbody>
<tr>
<td>2AB</td>
<td>2-aminobutyric acid</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ATGL</td>
<td>adipose tissue triglyceride lipase</td>
</tr>
<tr>
<td>ATM</td>
<td>adipose tissue macrophage</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BCAA</td>
<td>branched chain amino acid</td>
</tr>
<tr>
<td>BCAAR</td>
<td>branched chain amino acid restriction</td>
</tr>
<tr>
<td>BIRDS</td>
<td>biosensor imaging of redundant deviation in shifts</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophage</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cryopyrin-associated periodic syndromes</td>
</tr>
<tr>
<td>Casp-1</td>
<td>caspase-1</td>
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<tr>
<td>CAT</td>
<td>cysteine aminotransferase</td>
</tr>
<tr>
<td>CBS</td>
<td>cystathionine β-synthase</td>
</tr>
<tr>
<td>CBT</td>
<td>core body temperature</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CLS</td>
<td>crown like structure</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-o-methyl transferase</td>
</tr>
<tr>
<td>CR</td>
<td>caloric restriction</td>
</tr>
<tr>
<td>CSE</td>
<td>cystathionine-γ lyase</td>
</tr>
<tr>
<td>CTRL</td>
<td>control</td>
</tr>
<tr>
<td>CysF</td>
<td>cystine free</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEG</td>
<td>differentially expressed gene</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obese</td>
</tr>
<tr>
<td>DKO</td>
<td>double knock out</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obese</td>
</tr>
<tr>
<td>EAA</td>
<td>essential amino acid</td>
</tr>
<tr>
<td>EE</td>
<td>energy expenditure</td>
</tr>
<tr>
<td>EFAT</td>
<td>epididymal fat</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FCAS</td>
<td>familial cold autoinflammatory syndrome</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>GCN2</td>
<td>general control nonderepressible 2</td>
</tr>
<tr>
<td>GHRKO</td>
<td>growth hormone receptor knock out</td>
</tr>
</tbody>
</table>
GSH  glutathione
GSSG  oxidized glutathione
H₂S  hydrogen sulfide
Hcys  homocysteine
HFD  high fat diet
HRI  heme-regulated inhibitor
HSL  hormone sensitive lipase
i.p.  intraperitoneally
i.v.  intravenous
IIMR  integrated immune-metabolic response
ILC  innate lymphoid cell
iNK  invariant natural killer
IRS  insulin receptor substrate
LAM  lipid associated macrophage
LCFA  long chain fatty acid
LCMV  lymphocytic choriomeningitis virus
LPS  lipopolysaccharide
MAOA  monoamine oxidase A
MPST  3-mercaptopyruvate sulfur transferase
MR  methionine restriction
MRI  magnetic resonance imaging
MWS  Muckle-Wells syndrome
NAM  nerve associated macrophage
NBD  nucleotide binding domain
NE  norepinephrine
NIH  National Institutes of Health
NLRP3  Nacht, LRR, and PYD domains containing protein 3
NOMID  neonatal-onset multisystem inflammatory disease syndrome
OA  ophthalmic acid
OXPHOS  oxidative phosphorylation
PBMC  peripheral blood mononuclear cell
PCA  principle component analysis
PERK  PKR like endoplasmic reticulum kinase
PGC-1α  peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PIP3  Phosphatidylinositol 3,4,5 triphosphate
PKA  protein kinase A
PKR  protein kinase R
PPG  propargylglycine
PR  protein restriction
PRR  pattern recognition receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PTM</td>
<td>posttranslational modification</td>
</tr>
<tr>
<td>PVM</td>
<td>perivascular macrophage</td>
</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
</tr>
<tr>
<td>RFAT</td>
<td>retroperitoneal fat</td>
</tr>
<tr>
<td>SAA</td>
<td>sulfur amino acid</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endo plasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SFAT</td>
<td>subcutaneous fat</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TREC</td>
<td>T cell receptor excision circle</td>
</tr>
<tr>
<td>TRPM8</td>
<td>transient receptor potential cation channel subfamily M member 8</td>
</tr>
<tr>
<td>TSP</td>
<td>transsulfuration pathway</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>Ut/OvFAT</td>
<td>peri-uterine/ovarian fat</td>
</tr>
<tr>
<td>VAT</td>
<td>visceral adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
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</tbody>
</table>
Chapter 1: Introduction

Aging is associated with systemic low-grade inflammation in the absence of apparent infection, and is the single greatest risk factor for chronic diseases including cardiovascular disease, type II diabetes, cancer, and neurodegenerative diseases. With the exception of viral pandemics such as the current COVID-19 crisis, as the population of Americans over the age of 65 has increased, there has been a shift in the leading causes of death from infectious diseases and acute illness to non-communicable chronic diseases and degenerative illnesses. By 2030, people aged 65 and older are projected to account for 20 percent of the total U.S. population, and by 2056, are projected to outnumber individuals under the age of 18 (Colby and Ortman, 2014). Over the next 20-30 years, the number of people over the age of 65 will double to ~80 million in the U.S. with healthcare costs projected to be 2 trillion dollars (Ortman et al., 2014). In the 2013 report from the Centers for Disease Control and Prevention, “The State of Aging in America 2013,” more than a quarter of all Americans suffered from multiple chronic conditions, such as heart disease, cancer, chronic lower respiratory diseases, Alzheimer’s disease, and diabetes, accounting for 66% of the national health care burden (CDC, 2013). Furthermore, these age-related conditions are also associated with high morbidity and mortality in the context of infectious diseases, putting the elderly at even higher risks. Particularly, studies of the 2009 H1N1 pandemic, the MERS outbreak, and current COVID-19 pandemic have revealed that aging, obesity, and diabetes to be high risk factors for disease severity and mortality (Drucker, 2020; Kulcsar et al., 2019; Pastor-Barriuso et al., 2020; Perez-Saez et al., 2020; Van Kerkhove et al., 2011). It has thus become crucial to understand the process of aging and
develop therapeutic interventions to increase healthspan and delay the onset of age-associated disease.

**Aging and Inflammation**

While many facets contribute to the decline in health during aging, inflammation is an important driver of most age-related diseases (Ferrucci et al., 2005; Franceschi and Campisi, 2014). One contributor of chronic inflammation is the accumulation of damaged macromolecules and cell debris that increase with age. These include damaged cellular organelle components, reactive species from oxidative stress, and cellular components indicative of dying cells such as extracellular ATP, ceramides, saturated fatty acids, urate and cholesterol crystals, amyloid fibrils, succinate, and advanced glycation end products, which act as danger associated molecular patterns (DAMPs); these DAMPs are sensed by innate sensors called pathogen recognition receptors (PRRs) (Goldberg and Dixit, 2015; Medzhitov, 2008). One such sensor of molecular DAMPs is the NACHT, LRR and PYD domains containing protein 3 (NLRP3) inflammasome. While the NLRP3 inflammasome normally plays an important role in defense against bacterial infections, sustained NLRP3 activation becomes a major driver of age-related inflammation. Consistently, ablation of NLRP3 delays the onset of age associated diseases such as bone loss, thymic demise, insulin insensitivity, development of cataracts, and memory loss (Youn et al., 2013).

Activation of the NLRP3 inflammasome is tightly regulated in a two-step process. The first priming step involves sensing of cytokines or pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) by PRRs, leading to NF-κB mediated transcription of the NLRP3 inflammasome complex, including NLRP3, IL-1β, and IL-18.
Once the inflammasome is primed, sensing of additional PAMPs or DAMPs such as extracellular ATP, microbial toxins, or crystalline structures by NLPR3 acts as the second signal that leads to the assembly and oligomerization of the inflammasome complex involving NLRP3, ASC, and caspase 1 (Cai et al., 2014; Kanneganti and Dixit, 2012; Lu et al., 2014; Mangan et al., 2018). This assembly initiates the proteolytic cleavage of pro-caspase 1 into its active form, which can further process pro-IL-1β and pro-IL-18 into their biologically active forms that are secreted out of the cell and mediate inflammation. Activation of the NLRP3 inflammasome further leads to the activation of gasdermin D which mediates pyroptotic cell death, a rapid inflammatory form of cell death (Aglietti et al., 2016; Shi et al., 2015). How the NLRP3 inflammasome can uniquely sense and respond to such a plethora of PAMPs and DAMPs has been an outstanding question in the field. Studies have revealed that these stimuli commonly induce cellular stress responses which lead to intracellular signaling events such as potassium and chloride ion efflux, calcium ion influx, mitochondrial reactive oxygen species (ROS) and DNA release, and lysosomal disruption which then drive NLRP3 inflammasome activation (Di et al., 2018; Domingo-Fernandez et al., 2017; Hornung et al., 2008; Lee et al., 2012; Murakami et al., 2012; Zhang et al., 2010; Zhong et al., 2018; Zhou et al., 2011). However, exactly how NLRP3 senses these diverse intracellular signaling events is still unknown.

Activation of NLRP3 is particularly increased in the adipose during aging and obesity (Vandanmagsar et al., 2011; Youm et al., 2013). Amongst myeloid cells, macrophages are important regulators of adipose tissue homeostasis through NLRP3 inflammasome dependent regulation of insulin-action and lipolysis (Vandanmagsar et al.,
In healthy adipose tissue, macrophages are skewed towards an anti-inflammatory phenotype and play important roles in the removal of dying adipocytes, lipid processing and buffering, antigen presentation, modulating angiogenesis, and regulating sympathetic tone (Camell et al., 2017; Haka et al., 2016; Hill et al., 2018; Jaitin et al., 2019; Lee et al., 2013; Oodegaard et al., 2007; Silva et al., 2019; Xu et al., 2013). But with obesity, an increased infiltration of macrophages is accompanied by a switch towards a pro-inflammatory phenotype, expressing TNF-α, iNOS, and IL-6 (Weisberg et al., 2003). Furthermore, NLRP3 activation by these inflammatory macrophages mediates subsequent insulin resistance in obesity (Vandanmagsar et al., 2011). With aging, known activators of the NLRP3 inflammasome such as reactive oxygen species, urate crystals, cholesterol crystals, and saturated fatty acids are increased (Dostert et al., 2008; Duewell et al., 2010; Martinon et al., 2006; Vandanmagsar et al., 2011). Transmission electron microscopy of macrophages in the thymus, where adiposity is increased with age, have revealed that aged macrophages accumulate these activators intracellularly in the form of large lipid droplets, crystals, protein aggregates, and electron dense materials in the lysosome (Youm et al., 2016). As such, ablation of NLRP3 reverses the metabolic defects in glycemic control in aged mice (Youm et al., 2013). Conversely, growth hormone receptor knock out (GHRKO) mice that are notable for their longevity, are characterized by reduced levels of NLRP3 activation (Spadaro et al., 2016). Importantly, analysis of multi-omics profiling of young and older adult peripheral blood samples from the Stanford-Ellison longitudinal aging study found an age-dependent increase in inflammasome gene modules; furthermore, follow up of the study found the high expression of inflammasome modules was associated with all-cause mortality (Furman et al., 2017).
More recent studies have found that activation of NLRP3 in adipose tissue macrophages drives the expression of catecholamine degradation enzymes, causing the lipolysis resistance that is characteristic of reduced metabolic function in age and obesity (Camell et al., 2017; Pirzgalska et al., 2017). Additionally, NLRP3 activation in macrophages further recruits and maintains B cells in tertiary lymphoid structures called fat associated lymphoid clusters, and drive metabolic dysfunction exhibited as lipolysis resistance and cold intolerance in aged animals (Camell et al., 2019). These with other studies have highlighted a tight network of interactions that link metabolic function with inflammatory processes. Further understanding of these interactions will allow for therapeutic strategies to improve metabolically driven chronic diseases that occur with age. This is particularly important because the prevalence of metabolic disorders such as obesity and diabetes in adults aged 65 and over has increased continuously between 1999 and 2010 (Fakhouri et al., 2012).

**Dietary Interventions can Extend Lifespan and Restrain Age-related Inflammation**

One of the most successful interventions to delay aging and extend healthspan and lifespan has been caloric restriction (CR). Since the first studies by Osborne et al. and McCay et al. to show that decreasing food intake slowed growth and increased lifespan of rats (McCay et al., 1935; Osborne et al., 1915), subsequent studies have demonstrated that CR extends lifespan in many species including mice, yeast, fruit flies, nematodes, fish, hamsters, and monkeys (Masoro, 2005; Sanchez-Roman and Barja, 2013). In addition to increasing lifespan, CR has been shown to delay the onset of most age-associated diseases, such as neoplastic disease, cardiomyopathy, degenerative diseases, and metabolic disorders.
Besides CR, other, more targeted dietary interventions such as restricting protein intake, or restricting specific amino acids can also increase lifespan (Lee et al., 2008; Orentreich et al., 1993; Richardson et al., 2021; Solon-Biet et al., 2014), suggesting the restriction of protein derived macronutrients may be an important factor for their health benefits. Indeed, protein quality and amino acid composition of diet have been more strongly associated with metabolic and age associated health (Solon-Biet et al., 2014). Independently of caloric intake, low protein and high carbohydrate diets were found to have the strongest extension of lifespan in mice (Solon-Biet et al., 2014). In addition to dietary protein restriction (PR), some studies suggest it is the restriction of essential amino acids (EAAs) that drive beneficial effects, as supplementing dietary restriction with EAAs but not non-essential amino acids were sufficient to reverse pro-longevity effects (Grandison et al., 2009; Yoshida et al., 2018). Diets restricting specific EAAs have been extensively characterized to increase metabolic homeostasis and promote healthy aging; these diets include the restriction of sulfur amino acids (SAAs), methionine and cysteine, also known as methionine restriction (MR), and restriction of the branched chain amino acids, leucine, isoleucine, and valine, also known as branched chain amino acid restriction (BCAAR) (Fontana et al., 2016; Lee et al., 2016; Solon-Biet et al., 2014). These dietary interventions commonly lead to reduced adiposity and inflammation, increased insulin sensitivity and lipolytic gene signatures, and reduction of lipogenesis in adipose tissue and liver (Kitada et al., 2019). Although much less characterized, restriction of tryptophan can also lead to increased lifespan and increases recovery to cold stress, suggesting an increase in thermogenic capacity (Segall and Timiras, 1975, 1976; Zapata et al., 2018). Surprisingly,
these studies indicate that deficiencies of specific essential amino acids drive reprograming of metabolic tissues to promote health. Despite numerous studies investigating the mechanisms driving increased longevity during these dietary interventions, there have been a lack of studies investigating the alterations to the immune system and their role in tissue reprogramming.

**Autophagy and Inflammation**

One pathway that is increased during dietary protein and amino acid restriction is the integrated stress response-mediated autophagy. The integrated stress response allows for adaptive responses to various stresses such as starvation and ER stress. Stressors are sensed by four different sensors: general control nonderepressible 2 (GCN2), protein kinase R (PKR), heme-regulated inhibitor (HRI), and PKR like endoplasmic reticulum kinase (PERK) (Pakos-Zebrucka et al., 2016). GCN2 senses amino acid depletion by sensing the accumulation of uncharged tRNAs (Gallinetti et al., 2013). HRI recognizes heme deficiency, while PKR senses viral infection through activation by double stranded RNA (Pakos-Zebrucka et al., 2016). Finally, PERK is activated by ER stress (Pakos-Zebrucka et al., 2016). Activation of these sensors are integrated by the activation and phosphorylation of eIF2α leading to global translational arrest (Pakos-Zebrucka et al., 2016). One central component of the integrated stress response is autophagy (Kroemer et al., 2010). Autophagy, which literally means “self-eating” in Greek, is a process that delivers cellular components such as organelles and proteins to lysosomes for degradation and recycling of amino acids. This process is mediated by a broad network of proteins, many of which are called autophagy related genes (ATGs) (Tsukada and Ohsumi, 1993).
The most well characterized initiator of autophagy is deprivation of amino acids. In addition to GCN2-sensing of uncharged tRNAs, depletion of EAAs leads to interaction of their specific sensors such as SAMTOR (SAM sensor), sestrin 2 (leucine sensor), and CASTOR (arginine sensor) with GATOR1 or GATOR2 to prevent mTORC1 activation (Kim and Guan, 2019). The inhibition of mTORC1 leads to the dissociation of ULK1 from mTORC1 (Hosokawa et al., 2009). ULK1 then proceeds to phosphorylate itself and other complex members such as ATG13 and FIP200, ultimately leading to the formation of the phagophore that undergoes further expansion, maturation and fusion with the lysosome (Dikic and Elazar, 2018).

As an ancient, evolutionarily conserved pathway, autophagy has been adapted to mediate multiple critical functions. In addition to acting as a homeostatic process that is activated to replenish amino acids during starvation, autophagy was more recently discovered to play a cytoprotective role through the elimination of damaged and dysfunctional organelles such as mitochondria and protein aggregates in a process called selective autophagy (Rui et al., 2015). Selective autophagy is in part mediated by p62, which is also known as sequestosome 1 (SQSTM1); p62 acts as an adaptor between LC3 and ubiquitin chains to allow for recruitment of specific targets that have been ubiquitinated for autophagy (Bjorkoy et al., 2005; Narendra et al., 2010). In addition to p62, NDP52 can also act as an adaptor protein that not only binds to ubiquitin chains, but also galectin-8, which marks damaged endosomes and lysosomes (Thurston et al., 2012).

Autophagy can also act as a type of primitive immune response via another process of selective autophagy called xenophagy, through the capture and degradation of intracellular pathogens such as *Mycobacterium tuberculosis, Shigella flexneri*, and
Salmonella typhimurium (Levine 2005). Conversely, autophagy can also regulate immune function. Multiple groups have now shown that autophagy can inhibit inflammasome activity through several mechanisms. Initially, Saitoh et al. showed that depletion of autophagosome machinery such as ATG16L1, ATG7, or treatment with the autophagy inhibitor, 3-methyladenine (3-MA), lead to increased production of IL-1β in response to LPS (Saitoh et al., 2008), suggesting that autophagy negatively regulates inflammasome activation. Follow up studies have found that autophagy can promote the labeling of inflammasome components with ubiquitin by parkin for proteasomal degradation (Shi et al., 2012). Autophagy was also found to promote mitophagy in a p62 dependent manner to reduce mitochondrial ROS production and subsequent inflammasome activation (Zhong et al., 2016; Zhou et al., 2011). These studies underscore amino acid starvation induced autophagy as a potential mechanism to regulate inflammation to mediate the effects of protein restricting interventions. In a model of colitis, PR and BCAAR reduced inflammation through the inhibition of NLRP3 inflammasome and reduction of Th17 differentiation in the gut. This response was dependent on amino acid sensing through GCN2 (Ravindran et al., 2016). Furthermore, treatment with halofuginone, which activates starvation responses by inhibiting prolyl tRNA charging, also inhibits differentiation of Th17 cells, which play pathogenic roles in a multitude of autoimmune diseases (Sundrud et al., 2009). These studies highlight the induction of amino acid starvation responses in immune cells as potential strategies against inflammation driven pathological states such as obesity, aging, and autoimmunity.
The Transsulfuration Pathway as a Novel Immunomodulatory Metabolic Pathway

Another pathway that could potentially mediate the effects of these dietary interventions is the transsulfuration pathway (TSP). Recent studies from our lab have revealed that Cth expression, a gene that encodes for cystathionine-γ lyase (CSE) and is part of the TSP, is induced in the adipose tissue of humans that underwent 14% CR over two years (CALERIE II, Dixit lab, unpublished). The TSP involves the metabolism of sulfur amino acids (SAAs), beginning with the catabolism of methionine to generate intermediates such as the methyl donor S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), homocysteine, and cystathionine. Additionally, TSP also allows for the generation of cysteine through the action of CSE which can further provide important metabolites and byproducts such as glutathione, taurine, pyruvate, and hydrogen sulfide (H$_2$S). Interestingly, recent studies have linked the TSP to longevity. The lifespan extension induced by CR in *Drosophila* was abolished when treated with the inhibitor of CSE, propargylglycine (PPG) (Kabil et al., 2011), and overexpression of another TSP enzyme, cystathionine β-synthase (CBS) increased median lifespan in *C. elegans* (Hine et al., 2015). Furthermore, a recent study evaluating the effects of CR across two different mouse strains, and sex, found that 20% and 40% CR induces increased production of H$_2$S, one of the end products of the TSP (Mitchell et al., 2016). Finally, treatment of yeast with H$_2$S donors, NaHS and GYY4137, is sufficient to extend median lifespan of yeast, i.e. delay replicative senescence (Hine et al., 2015). These studies suggest that TSP mediated H$_2$S production is an important mediator of lifespan benefits.

H$_2$S is a gas molecule that has recently gained attention as a physiologically relevant signaling molecule. While the role of H$_2$S in controlling vasodilation by its action
on endothelial smooth muscle is the most established (Paul and Snyder, 2015), one study reports that H$_2$S treatment can induce suspended animation in mice, leading to reduced metabolic rate and decreased body temperature (Blackstone et al., 2005). Other studies have begun to uncover a role for H$_2$S in immune processes. In T cells, H$_2$S is required for the differentiation of regulatory T cells by promoting demethylation at the Foxp3 locus by Tet1 and Tet2 (Yang et al., 2015). An anti-inflammatory role for H$_2$S has been implicated by its action on NF-κB to augment its antiapoptotic transcriptional activity in macrophages (Sen et al., 2012), and its action on antioxidant gene regulation (Yang et al., 2013). Given that both regulatory T cells and anti-inflammatory macrophages play important roles in promoting metabolic homeostasis of adipose tissue, the increased localized production of H$_2$S may play important roles in regulating immune cells to mediate the effects of dietary interventions.

**Harnessing Adaptive Thermogenesis**

One other effect of pro-longevity dietary interventions is the activation of adipose tissue thermogenesis (Kim et al., 2017; Wanders et al., 2017). Brown adipose tissue (BAT) along with skeletal muscle are the main thermogenic organs in the body. BAT can generate heat by inducing energy-consuming futile cycles. Brown adipocytes express high amounts of mitochondria and the uncoupling protein 1 (UCP1) which allows the uncoupling of mitochondrial oxidative phosphorylation (OXPHOS) to generate heat instead of ATP (Kozak and Harper, 2000). BAT is mainly activated in response to cold, where sensory neurons expressing transient receptor potential cation channel subfamily M member 8 (TRPM8) sense the cooling of the skin and signal to the central nervous system (CNS).
(Bautista et al., 2007). Within the CNS, sensory signals are conveyed to the hypothalamus, which integrates these signals to activate sympathetic signaling that directs vasoconstriction of cutaneous blood vessels to reduce heat loss through the skin (Bini et al., 1980), and subsequent activation of thermogenic organs to increase energy expenditure and generate heat via non-shivering mechanisms (Nakamura and Morrison, 2007, 2008). During early cold exposure, when non-shivering thermogenic mechanisms are insufficient to maintain body temperature, shivering thermogenesis mediated by the involuntary contraction of muscle primarily generates heat (Israel and Pozos, 1989). When cold exposure becomes chronic, reliance on shivering mechanisms is reduced and an increase in non-shivering thermogenic capacity by BAT and white adipose tissue (WAT) occurs (Betz and Enerback, 2018; Foster and Frydman, 1979). Indeed, acclimatization to cold increases the density of noradrenergic fibers to increase response to sympathetic signaling in both BAT and WAT (Chi et al., 2018; Murano et al., 2009). Furthermore, recent studies have reminded the field that current standard housing conditions for mice at 20-22°C are lower than their thermoneutral temperatures (28-33°C), such that mice are constantly in a state of chronic cold stress and activate adaptive thermogenesis (Gaskill et al., 2009). Accordingly, housing mice at 30°C reduces the activation of non-shivering thermogenesis, and comparison of housing at 20°C vs. 30°C suggests that nearly half of the total energy expenditure of mice at 20°C is utilized to maintain core body temperature (Skop et al., 2020). In this way, BAT and WAT acts as adaptive thermogenic organs that allows maintenance of core body temperature in response to environmental temperatures.

BAT is highly innervated by sympathetic nerve fibers and highly vascularized, allowing for its rapid activation by the CNS and dispersion of heat throughout the body via
the circulation (Rosell and Belfrage, 1979). In contrast to white adipocytes, brown adipocytes store lipids in multiple smaller lipid droplets and harbor a large number of mitochondria. While in rodents, BAT is prominently found in the interscapular region, in response to long term or repeated cold exposure, additional brown-like adipocytes called ‘beige adipocytes’ are induced in the WAT, particularly in subcutaneous white adipose tissues (SFAT) (Wu et al., 2012). This process where there is an increase in thermogenic capacity of the SFAT is called ‘browning’ or ‘beiging’. UCP1 is uniquely expressed within the inner mitochondrial membrane of brown and beige adipocytes, and forms a channel allowing protons to flow into the mitochondrial matrix from the cytosol (Kozak and Harper, 2000). This uncouples the proton gradient of the electron transport chain, allowing for uncoupling of OXPHOS from ATP generation, ultimately converting energy stored as triglycerides into heat (Kozak and Harper, 2000). Activation of UCP1 begins with the stimulation of β3-adrenergic receptors on adipocytes by catecholamines released by sympathetic nerves (Jimenez et al., 2003). This leads to the upregulation of the thermogenic program including UCP1, and mitochondrial biogenesis by induction of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in a p38 mitogen-activated protein kinase (MAPK) dependent manner (Cao et al., 2001). β3-adrenergic signaling also activates lipolysis through the action of protein kinase A (PKA), a cyclic AMP activated protein kinase (Fricke et al., 2004). PKA phosphorylates perilipin A and hormone-sensitive lipase (HSL), where phosphorylated perilipin A dissociates with lipid droplets and acts as a scaffold for the phosphorylation of HSL, which is involved in the cleavage of stored triglycerides into fatty acids and glycerol. Another adipose tissue lipase, adipose tissue triglyceride lipase (ATGL) also induces lipolysis semi-independently of
PKA, through its interaction with CGI-58 and perilipin 5 (Lass et al., 2006; Wang et al., 2011a). ATGL favors triglycerides as substrates as it catalyzes the first rate-limiting step of lipolysis, whereas HSL is thought to primarily function more downstream as a diacylglycerol lipase (Bezaire et al., 2009; Haemmerle et al., 2002; Holst et al., 1996; Zimmermann et al., 2004). The released free fatty acids (FFAs) are then utilized as fuels for beta oxidation and to increase flux through the electron transport chain. As UCP1 acts as a long chain fatty acid (LCFA)/proton symporter, the release of long chain fatty acid anions and their association with UCP1 drives stable uncoupling of the electron transport chain (Fedorenko et al., 2012). And in a futile cycle as ATP production becomes uncoupled, accumulation of ADP continues to drive the activation of OXPHOS, further increasing heat production (Wikstrom and Springett, 2020).

While UCP1 was long thought to be the primary mediator of non-shivering thermogenesis, the discovery that \textit{Ucp1}^{-/-} mice are able to adapt to long term cold exposure uncovered the possibility of alternative thermogenic mechanisms independent of UCP1 (Ukropec et al., 2006). One such mechanism is through creatine futile cycling. Utilizing \textit{Ucp1} deficient animals, Kazak and colleagues identified increased levels of mitochondrial creatine kinases, CKMT1 and CKMT2, upon acute cold exposure, specifically in the beige adipocytes of browning WAT (Kazak et al., 2015). Blocking the transport of creatine reduced oxygen consumption in both human and \textit{Ucp1}-deficient beige adipocytes, suggesting that mobilization of creatine in and out of the mitochondria were important for thermogenic respiration (Kazak et al., 2015). The conceptual framework behind the futile creatine cycle is as follows: mitochondrial creatine kinases are positioned within the inner mitochondrial membrane so that they can immediately utilize the ATP produced from
OXPHOS to phosphorylate creatine and generate phosphocreatine and ADP. However, through increased creatine transport, increased CKMT1 and CKMT2 activity drive an excess of ADP over ATP, driving increased respiration to produce additional ATP in a futile cycle (Kazak et al., 2015). While limited expression of CKMT1 and CKMT2 to beige adipocytes might have suggested that creatine futile cycling was limited to browning WAT, recently, another mitochondrial creatine kinase, creatine kinase B (CKB) was found to drive creatine cycling in the BAT as well (Rahbani et al., 2021). Other studies have begun to identify roles for non-shivering thermogenesis in muscle resulting from calcium cycling by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) where sarcolipin uncouples the hydrolysis of ATP from Ca^{2+} transport (Bal et al., 2012; Smith et al., 2002), or its expression of UCP1 and UCP3 (Riley et al., 2016). While UCP1-independent thermogenic mechanisms are still being discovered and verified, their redundancy highlights the importance of mechanisms to maintain and defend core body temperature.

Given that cold-induced thermogenesis essentially increases energy expenditure by burning fat, there has been great interest in whether these pathways can be harnessed as therapeutic strategies in the context of obesity. Until the last decade, the presence of BAT in human adults had been controversial. However, once the existence of BAT was confirmed by multiple studies in 2009 (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009), the therapeutic prospect of increasing browning processes as a means to reduce fat mass and improve metabolic homeostasis has gained much excitement. Given that human BAT exhibits more heterogeneous molecular signatures resembling both classic and beige adipocytes of mice (Sun et al., 2020), understanding the mechanisms that activate not only BAT, but also browning of WAT will
be important in developing novel therapeutic strategies. BAT function decreases in rats as well as in humans with age and obesity (Cypess et al., 2009; McDonald and Horwitz, 1999; Pfannenberg et al., 2010; Saitoh et al., 2008; Vijgen et al., 2011). This decrease in thermogenic capacity with age is in part mediated by the NLRP3 inflammasome (Camell et al., 2019; Camell et al., 2017). Interestingly CR and MR delays the loss of BAT that occurs during aging and promotes thermogenesis of WAT (Valle et al., 2008; Wanders et al., 2015). Additionally, intermittent fasting of animals even on a high fat diet (HFD), where mice were fed HFD 2 days and fasted for 1 day, increased WAT browning to improve metabolic homeostasis (Kim et al., 2017). The fact that thermogenesis is increased in the context of MR where mice are reported to increase their food intake may suggest that a decrease in caloric intake is not the driver of the shift from energy storage towards energy expenditure (Hasek et al., 2010). How and why these dietary perturbations lead to increased thermogenesis is poorly understood. Understanding the mechanisms that drive this response may lead to novel therapeutic strategies that can be targeted in lieu of undergoing broad dietary restrictions that are difficult to maintain.

**Immune Cells in Regulating Thermogenesis**

One emerging area of organismal regulation of thermogenesis is the crosstalk of different resident tissue cell types within the adipose tissues. As the homeostatic role of immune cells in adipose tissue have been continually characterized, some studies have revealed a role for immune cells in regulating thermogenic processes. For example, macrophages in close contact with the numerous sympathetic nerves that densely innervate the BAT and SFAT can control thermogenesis by controlling the bioavailability of
catecholamines (Camell et al., 2017; Chi et al., 2018; Pirzgalska et al., 2017). Inhibition of monoamine oxidase A (MAOA), an enzyme that degrades catecholamines such as norepinephrine (NE) and serotonin in macrophages, increased local NE content in adipose tissue, leading to increased lipolysis and thermogenesis by upregulation of UCP1 (Camell et al., 2017). Moreover, type 2 innate lymphoid cells (ILC2s), eosinophils, invariant natural killer (iNK) T and γδ T cells have also been implicated in increasing energy expenditure and UCP1 (Brestoff et al., 2015; Hu et al., 2020; Lynch et al., 2016; Qiu et al., 2014). Notably, FGF21, which gets induced during dietary restriction, can promote the expression of CCL11 by adipocytes to recruit eosinophils, suggesting that immune cells are actively recruited and regulated during adaptive adipose tissue remodeling (Huang et al., 2017). Conversely, a recent study has identified lymphocyte derived IL-10 to inhibit thermogenesis (Rajbhandari et al., 2019). Further studies are required to determine how exactly specific immune cells affect UCP1 and alternative thermogenic pathways; whether they act directly on adipocytes, or regulate other niche compartments such as the sympathetic nervous system or vasculature to control heat production are still unknown. Identification of such mechanisms may reveal strategies to divert the fate of dietary lipids from storage to heat production through increased mitochondrial uncoupling, thus reducing metabolic diseases emanating from lipotoxicity and obesity.

**FGF21 Integrates Amino Acid Starvation, ER Stress, and Metabolic Adaptations**

Changes in organismal nutritional state, such as starvation, ketogenic diet, or low protein diets drive the expression of FGF21, an endocrine fibroblast growth factor (FGF) (De Sousa-Coelho et al., 2013; Fazeli et al., 2015; Laeger et al., 2014; Reitman, 2007).
FGF21 is expressed in many organs, but the liver is the main contributor to circulating levels of FGF21 (Nishimura et al., 2000). Unlike other members in the FGF family, FGF21 has reduced affinity for heparin sulfate, allowing for it to disperse and act systemically as an endocrine factor and integrate metabolic responses across multiple tissues including the liver, adipose tissue, skeletal muscle, pancreas, and the brain (Kharitonenko et al., 2005).

FGF21 controls glucose and lipid metabolism via binding to its receptor, β-Klotho in complex with its co-receptors FGFR1C, FGFR2C, and FGFR3C (Ogawa et al., 2007). In adipose tissue, FGF21 increases response to insulin by increasing the expression of glucose transporter GLUT1 (Ge et al., 2011), while also increasing energy expenditure through induction of PGC-1α and UCP1 (Fisher et al., 2012; Kwon et al., 2015). More specifically, FGF21 acts through β-Klotho receptors in the central nervous system to induce sympathetic activation of brown adipose tissue in a neuropeptide corticotropin-releasing factor (CRF)-dependent manner (Bookout et al., 2013; Owen et al., 2014). In addition, FGF21 administration in UCP1 KO mice revealed that FGF21 can promote weight loss and improve glycemic control in a UCP1 independent manner by reducing food intake (Samms et al., 2015).

In line with the increased expression of FGF21 during pro-longevity dietary interventions CR, PR, and MR, transgenic overexpression of FGF21 in mice leads to a 50% increase in median lifespan (Zhang et al., 2012). Interestingly, FGF21 seems to be responding specifically to restriction of protein intake, independently of energy intake, as CR diets and ketogenic diets supplemented with isocaloric amounts of protein blocked the induction of FGF21 (Laeger et al., 2014). More specifically, restriction of specific essential amino acids, as such in MR diets, is sufficient to drive robust expression of FGF21.
(Wanders et al., 2017). Indeed, studies utilizing deprivation of leucine induced FGF21 expression in a GCN2 and ATF4 dependent manner (De Sousa-Coelho et al., 2013). However, unlike leucine or BCAAR diets, MR also induces hepatic FGF21 and lipolytic gene signatures independently of GCN2 (Lees et al., 2017). Instead, MR specific hepatic changes are driven by PERK and glutathione depletion (Wanders et al., 2016), suggesting that besides GCN2-mediated ISR, endoplasmic reticulum (ER) stress and oxidative stress may play key roles in regulating FGF21 during amino acid starvation responses.

ER stress activates the unfolded protein response (UPR) which can signal through three separate branches, each representing distinct ER membrane proteins: PERK and ATF4, IRE1α and XBP1, and ATF6. These branches become activated when unfolded or misfolded proteins accumulate within the ER. Activation of PERK results in the phosphorylation of eIF2α and global translational arrest, leading to the activation of ATF4, which regulates the expression of genes involved in amino acid metabolism and antioxidant defense (Pakos-Zebrucka et al., 2016). IRE1α activation leads to the transcription of the potently active form of XBP1, which drives the expression of genes involved in ER biogenesis and turnover (Iwakoshi et al., 2003; Yoshida et al., 2001). Finally, activation of ATF6 leads to its relocation to the Golgi where it is cleaved and is involved in the expression of chaperones (Adachi et al., 2008; Shen et al., 2002). Schaap and colleagues first showed that ER stress stimuli as well as overexpression of ATF4 can induce the expression of FGF21 in hepatocytes, identifying two ATF4 binding sites in the FGF21 promoter (Schaap et al., 2013). This study suggested that FGF21 may be a downstream mediator of the PERK/ATF4/eIF2α branch of the ER stress-driven ISR. Indeed, FGF21 deficient mice exhibited increased hepatic ER stress in response to postprandial and
tunicamycin-induced stress, indicating that FGF21 also directly plays a role in ameliorating ER stress (Maruyama et al., 2018). However, ATF4 is a broad effector of ISRs and also acts downstream of GCN2 mediated amino acid starvation responses (Harding et al., 2000). In addition to ATF4 driven FGF21, Jiang and colleagues found that IRE1α was required for the induction of FGF21 in the context of tunicamycin-induced ER stress as IRE1α KO mice had reduced FGF21 levels without altering ATF4 expression (Jiang et al., 2014). Furthermore, FGF21 directed ERK activation to suppress eIF2α-ATF4-CHOP, indicating that FGF21 can reduce ER stress and subsequent activation of alternative branches of the UPR program (Jiang et al., 2014). The fact that FGF21 is controlled by at least two branches of the UPR supports a role for FGF21 as a mediator of the UPR. Understanding the effector function of FGF21 within the context of ER stress response during pro-longevity diets that have underlying amino acid restriction may lead to the discovery of novel physiological phenomena.

**Summary and Perspectives**

Dietary interventions such as CR, PR, and MR extend lifespan and healthspan to delay the onset of age-associated chronic diseases and metabolic dysfunction. However, practicing these dietary interventions in daily life can be extremely difficult. Studying the mechanisms that drive the effects of these pro-longevity interventions can allow for the identification of novel therapeutic targets specific for different aspects of aging such as inflammation, metabolic perturbations, and immune dysfunction. In particular, the study of MR allows for a simplified model of amino acid restriction that still exhibits the benefits of the broader dietary restriction regimes such as CR and PR. Many of the metabolic
perturbations with age stem from alterations to adipose biology in part caused by increased chronic, low-grade inflammation. Investigating how different responses to pro-longevity dietary restrictions are integrated within the adipose tissue may yield the discovery of novel immunomodulatory metabolic pathways. In this study, we identify the induction of the TSP, a pathway involved in the catabolism of methionine to generate cysteine, as an intriguing node that may play an important role in modulating the effects observed during pro-longevity interventions. This project investigates the role of the TSP in regulating inflammation in the context of MR and aging (Chapter 2), and its action in regulating amino acid metabolism to maintain adipose tissue homeostasis (Chapter 3).
Chapter 2: Regulation of Inflammation by the Transsulfuration Pathway

Introduction

Despite methionine being an essential amino acid, studies by the Orentreich Foundation surprisingly found that restriction of methionine from 0.86% to 0.17% of the diet increased lifespan by up to 45% in rats (Orentreich et al., 1993). Many follow-up studies have further established the effects of methionine restriction (MR) diet in delaying the onset of age-associated diseases, even when started in adult animals (Ables et al., 2012; Lees et al., 2014; Miller et al., 2005; Richie et al., 1994). However, there has been a lack of studies exploring the role of MR in regulating inflammation to mediate its effects as a pro-longevity intervention. Large scale transcriptomics analysis comparing 20 months caloric restriction (CR) and MR across subcutaneous fat (SFAT), brown adipose tissue (BAT), liver, and skeletal muscle in mice suggested proinflammatory signaling pathways were down-regulated in the SFAT and liver of MR mice (Ghosh et al., 2014; Wanders et al., 2014). However, these studies were mostly limited to highlighting pathways involved with immune recruitment, accumulation, and activation, rather than actual inflammatory signatures, and subsequent follow-up studies have failed to confirm and identify clear mechanisms that regulate this reduction in inflammatory profile (Sharma et al., 2019).

One important driver of inflammation in the context of age and metabolic diseases such as obesity and atherosclerosis is the NLRP3 inflammasome (Duewell et al., 2010; Vandannmagasar et al., 2011; Youm et al., 2013). Whether MR can regulate NLRP3 inflammasome activation has not been established. Methionine restriction in rats and mice decreases serum methionine and cysteine levels (Elshorbagy et al., 2010; Mentch et al., 2015). Methionine is an essential amino acid (EAA) that generates s-adenosyl methionine
(SAM), homocysteine, cysteine, creatine, carnitine, and succinyl-coA. Accordingly, MR driven autophagy has been found to be required for lifespan extension in yeast (Ruckenstuhl et al., 2014). Autophagy is a known negative regulator of NLRP3 inflammasome. Zhou et al initially showed that mitochondrial ROS drives increased NLRP3 activation, and disruption of autophagy led to the accumulation of damaged mitochondria and mitochondrial ROS (Zhou et al., 2011). In addition to mitochondrial ROS, DNA released for damaged mitochondria also activates the NLRP3 inflammasome (Nakahira et al., 2011). During NLRP3 inflammasome activation, damaged mitochondria get ubiquitinated by the E3 ubiquitin ligase Parkin, and removed in a p62 dependent manner by selective autophagy (Zhong et al., 2016). Besides damaged mitochondria, NLRP3 inflammasome components can also be directly targeted for degradation. NLRP3 complexes co-localize with autophagosomes, and ASC in the context of the AIM2 inflammasome complex has been shown to be ubiquitinated, and removed in a p62 dependent manner (Shi et al., 2012). This interaction of the NLRP3 complex with p62 via ASC has been suggested to be dependent on phosphorylation of NLRP3, as NLRP3 lacking the phosphorylation site does not interact with p62 and subsequently does not get sequestered into the phagosome (Spalinger et al., 2016). These studies have established that autophagy of NLRP3 inflammasome components and activators such as damaged mitochondria serve as a negative feedback mechanism to prevent excessive inflammation. Whether these pathways play a role in regulating inflammation during MR remains to be investigated.

Alternatively, the transsulfuration pathway (TSP) also gets induced during MR (Hine et al., 2015; Wang et al., 2019b). The transsulfuration pathway allows for the
synthesis of cysteine from methionine (Jackson and Block, 1931). During MR, circulating cysteine levels are reduced, and the TSP likely gets activated to generate cysteine (Elshorbagy et al., 2010). One of the byproducts of TSP activation is hydrogen sulfide (H$_2$S), a gaseous molecule. H$_2$S is produced by cystathionine ß-synthase (CBS), and cystathionine γ-lyase (CSE), cysteine aminotransferase (CAT), and 3-mercaptoppyruvate sulfurtransferase (MPST) (Moore et al., 2003). While a role for H$_2$S has been studied in various contexts of inflammatory diseases, there have been mixed reports of the role of H$_2$S in either inducing or reducing inflammation in the context of pancreatitis, endotoxemia and sepsis, edema, colitis, and burn induced injury (Bhatia, 2015). Given that the role of H$_2$S in regulating macrophage mediated inflammation was largely unexplored at the start of this project, we aimed to explore the role of TSP and H$_2$S in regulating NLRP3 mediated inflammation.

Cryopyrin-associated periodic syndromes (CAPS) are rare hereditary autoinflammatory conditions, clinically characterized by fever and systemic inflammation of the subcutaneous skin, joints, the musculoskeletal system, and the central nervous system. These conditions are caused by autosomal dominant mutations in the NLRP3 gene (Cuisset et al., 1999; Sarrauste de Menthiere et al., 2003). These mutations are mostly located within the NACHT domain (Dode et al., 2002), which includes a nucleotide binding domain (NBD) (MacDonald et al., 2013). There is a spectrum of NLRP3 driven autoinflammatory diseases including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease syndrome (NOMID), with FCAS being the least severe, and NOMID being the most severe in neonates and young children (Neven et al., 2008). MWS patients present with clinical
features similar to FCAS, with periodic fever, chills, and severe fatigue and arthritis, oftentimes being diagnosed when they develop hearing loss. In MWS patients, gain of function mutations in the NACHT domain of the NLRP3 gene leads to excessive activation of the NLRP3 inflammasome (Agostini et al., 2004; Chae et al., 2011). How downstream negative regulatory loops are regulated in CAPS patients not well established. Moreover, during sepsis and endotoxemia, overwhelming proinflammatory responses drive multiorgan damage or failure. This proinflammatory response is driven by the concerted action of numerous immune cells and involves NLRP3 inflammasome activation (Anand et al., 2011; Danielski et al., 2020). In this chapter, we aim to understand whether MR regulates inflammation, specifically mediated by the NLRP3 inflammasome, and whether MR can be harnessed to ameliorate NLRP3 mediated diseases such as MWS and sepsis associated endotoxemia.

Results

**P62 independent autophagy inhibits inflammasome activation during methionine and cysteine restriction.**

To test whether reduction in systemic SAAs could influence inflammasome mediated inflammation, bone marrow derived macrophages (BMDMs) were cultured in media lacking methionine and cysteine. Using LPS as the priming signal 1 that drives transcription of the inflammasome complex, and ATP as the DAMP that serves as signal 2 that drives the assembly of the NLRP3 inflammasome, we observed a decrease in inflammasome activation observed by reduced IL-1β and caspase-1 (casp-1) cleavage when methionine and cysteine were restricted acutely at the time of LPS treatment (Fig
2.1A). Given that methionine is an essential amino acid that cannot be synthesized, and cysteine is a conditional essential amino acid that is synthesized from methionine and serine, macrophages rely on the import of SAAs via SLC7A5 and SLC7A11 (Sato et al., 1999; Yoon et al., 2018). To understand whether induction of autophagy induced by the lack of environmental EAAs was mediating the reduction in inflammasome activation, BMDMs were treated with the class III phosphatidylinositol 3-kinase autophagy inhibitor 3-methyladenine (3-MA) during methionine and cysteine restriction. Indeed, inhibition of autophagy reversed the reduction of inflammasome activation, suggesting that autophagy is mediating the anti-inflammatory effects of methionine and cysteine restriction (Fig 2.1B). Some of the effects of autophagy on inflammasome mediated inflammation are dependent on mitochondrial removal (Harris et al., 2011; Zhong et al., 2016). NLRP3 inflammasome activation leads to the accumulation of defective mitochondria and increased mitochondrial ROS production and DNA release, which gets removed by p62 mediated mitophagy to prevent excessive inflammasome activation (Nakahira et al., 2011; Zhong et al., 2016; Zhou et al., 2011). To understand whether methionine and cysteine restriction induced p62 mediated mitophagy to reduce inflammation, the NLRP3 inflammasome was activated in BMDMs lacking p62 in the presence or absence of SAAs. Interestingly, despite an increase in inflammasome activation caused by p62 deficiency alone, methionine and cysteine restriction still reduced inflammasome activation in p62 deficient macrophages, suggesting that the methionine and cysteine restriction was independent of p62 driven mitophagy (Fig 2.1B). Furthermore, methionine and cysteine restriction did not alter potassium efflux, suggesting that the autophagy mediated effects were downstream of the inflammasome assembly trigger, potassium efflux (Fig 2.1 C). We
next tested the impact of methionine and cysteine restriction in a mouse model of constitutively active NLRP3, where a mutation found in MWS patients was knocked into the \textit{Nlrp3} gene (\textit{Nlrp3}^{A350VneoR}) (Brydges et al., 2009). The mutation found in the NACHT region of the \textit{Nlrp3} gene, causes auto-assembly of the inflammasome once it is expressed, even in the absence of a signal 2 trigger. We found that culturing BMDMs from \textit{Nlrp3}^{A350VneoR} mice in the absence of methionine and cysteine restriction still reduced inflammasome activation, observed by decreased IL-1β cleavage and secretion (Fig 2.1 D). Since the \textit{Nlrp3}^{A350VneoR} mutation leads to auto-assembly of the inflammasome complex even in the absence of any secondary signals, methionine and restriction driven autophagy likely reduces inflammasome activation by increasing the clearance of NLRP3 complex components.

**Exogenous hydrogen sulfide inhibits inflammasome activation**

In addition to autophagy, the TSP is increased in the liver during MR, specifically by an increase in the expression of CSE (Fig 2.4A). To understand whether intrinsic TSP might directly regulate inflammasome activation, we first explored whether macrophages express CSE, the enzyme required for the last step of cysteine production within the TSP. Although, some studies suggest that CSE is induced in macrophages in the context of bacterial infection (Gobert et al., 2019), we find that BMDMs in M1 (LPS + IFNγ) or M2 (IL-4) polarization conditions do not express CSE (Fig 2.2A). Preliminary experiments in peritoneal macrophages had suggested that CSE may be induced with thioglycollate stimulation (Fig 2.3A). However, confirmation with \textit{Cth^−/−} mice revealed that these bands were nonspecific (Fig 2.2B). Gene expression analysis of PBS or thioglycolate induced peritoneal macrophages did not detect \textit{Cth} or \textit{Cbs} expression (data not shown).
Furthermore, published RNA-seq data sets from the ImmGen consortium suggest little to no expression of Cth in various tissue macrophages (Fig 2.3C) (Heng et al., 2008). These analyses establish that macrophages lack cysteine synthesis machinery, and likely import cysteine/cystine extracellularly to maintain cellular homeostasis. Given the lack of CSE expression in macrophages, we explored the effects of exogenously provided H$_2$S in regulating inflammasome activation. We found that treatment with sodium hydrosulfide (NaHS), a H$_2$S donor, reduced Il-1β secretion in a dose dependent manner (Fig 2.2B). Given reports that H$_2$S can modify NF-κB to regulate its role in the transcription of antiapoptotic pathways (Sen et al., 2012), and the role of NF-κB in priming inflammasome activation by transcription of NLRP3 components, Nlrp3, Il1b, and Il18, we investigated whether transcription of NF-κB targets were altered. However, we find that transcription of Nlrp3, Il1b, or Il18 is not changed by exogenous H$_2$S during inflammasome activation (Fig 2.2C). Additionally, potassium efflux is not affected with H$_2$S treatment, suggesting that detection of secondary DAMP signals is not altered by H$_2$S (Fig 2.2 D). Interestingly, H$_2$S treatment is unable to reduce inflammation in Nlrp3$^{A350VneoR}$ BMDMs, suggesting that H$_2$S may be acting during the assembly of the NLRP3 inflammasome complex.

**Methionine restriction improves metabolic health and reduces inflammation during aging**

To investigate the effects of MR *in vivo*, we fed young, 8-week-old mice and old, 18-month-old mice MR diet for three months. We find that MR prevents weight gain in both young and old animals (Fig 2.4 A-B). This reduction in weight gain aligns with a reduction in adiposity, with reduced subcutaneous fat (SFAT) mass (Fig 2.4C), visceral epididymal fat (EFAT) mass (Fig 2.4D), and lipid content in the liver (Fig 2.4G). Additionally, MR
animals had improved glucose and insulin sensitivity (Fig 2.4E-F), suggesting that MR can improve metabolic health, even when started in already aged animals. Consistent with prior studies that have shown that macrophages are the main drivers of inflammation in adipose tissue of aged mice (Camell et al., 2017; Lumeng et al., 2011; Wu et al., 2007), MR reduced EFAT macrophages in both young and old animals, while to a lesser degree, macrophages were reduced in the SFAT in young animals with MR (Fig 2.4H-I). Additionally, transcription of inflammatory cytokines, IL-1β and IL-6 were reduced in the retroperitoneal fat (RPFAT), a visceral adipose depot, with 3 months MR (Fig 2.4J). Similarly, gene expression of NLRP3 inflammasome components, Il1b, Nlrp3, and Il6 is also reduced in the SFAT of animals that underwent PR (Fig 2.4K). However, reduction in inflammation was only observed in the RPFAT of young animals, but not old animals (Fig 2.4J, 2.5E).

To understand these results, we investigated the expression of the TSP in young and old animals. We find that regulation of the TSP during MR is tissue dependent. CSE is most highly expressed in the liver and kidney in mice (Fig 3.3C). In response to MR, CSE is induced specifically in the liver but not the kidney (Fig 2.5A-B). In the RPFAT, expression of CSE is not changed, but MR increases the expression of CBS, another TSP enzyme that can generate H₂S, in young animals, but not old animals (Fig 2.5C-D). This specific increase in the expression of TSP in young animals is consistent with the decreased expression of inflammatory cytokines in young animals. Additionally, though RPFAT is a visceral adipose depot, the contribution of inflammation in RPFAT towards systemic metabolism has not been well characterized. How inflammation and TSP is being regulated in other visceral adipose depots such as EFAT, and liver during MR in young and old animals may be more relevant to the systemic metabolic changes that are observed.
Methionine restriction fails to reduce inflammation in models of NLRP3 inflammasome activation

In order to determine whether MR can reduce inflammation in pathological states we turned to in vivo models of MWS and acute endotoxemia. Inducible $Nlrp^3A350VneoR/+$ CAG-cre mice were fed MR diet 1 week prior to tamoxifen treatment. Mice were injected with tamoxifen once daily for two days. $Nlrp^3A350VneoR/+$ CAG-cre mice begin to lose weight rapidly and in severe cases begin to die (Fig 2.6A). There was no difference in weight loss between CTRL and MR fed animals (Fig 2.6A). Despite reduced splenomegaly due to inflammation in MR fed animals (Fig 2.6B), parameters of inflammation such as gene expression of NLRP3, Casp-1, IFN-γ, TNFα, and IL-18 were not decreased in the spleen, and if anything, IL-1β was increased with MR (Fig 2.6C). Furthermore, there were no differences in serum levels of IL-1β, IL-6, TNF, and MCP-1 between CTRL and MR fed animals (Fig 2.6D). We next tested the impact of MR in a model of acute endotoxemia, where young WT mice were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS) for four hours after being on CTRL or MR diet for three months. Compared to CTRL diet fed animals, MR fed mice showed no differences in metabolic parameters such as decreases in blood glucose (Fig 2.6E), core body temperature (CBT) (Fig 2.6F), or systemic inflammation, measured by serum IL-1β, IL-6, TNF, and MCP-1 (Fig 2.6G). These results suggest that short- and long-term MR may be insufficient to reduce inflammation in NLRP3 inflammasome mediated pathological states.
Summary and Discussion

Through *in vitro* studies utilizing BMDMs, we find that MR can reduce NLRP3 inflammasome mediated inflammation through two separate mechanisms: the first through induction of autophagy due to decreases in systemic SAAs. This induction of autophagy is independent of p62 mitophagy, suggesting that SAA restriction may reduce mitochondrial ROS through alternative mechanisms. SAA restriction can reduce NLRP3 mediated inflammation even in a MWS model of constitutively active NLRP3, indicating that SAA restriction mediated autophagy may act downstream of inflammasome assembly, potentially through increased degradation of inflammasome components. Some groups have shown that pro-IL-1β can be degraded intracellularly by autophagy and proteosomes to reduce IL-1β release (Ainscough et al., 2014; Harris et al., 2011). Indeed, pro IL-1β is reduced with SAA restriction in an autophagy dependent manner (Fig 2.1B), suggesting that increased degradation of pro-IL-1β can also be playing a role in the reduction of inflammasome activation during SAA restriction.

Besides the activation of autophagy to reduce NLRP3 mediated inflammation, we find that exogenously produced H₂S can act on macrophages to reduce inflammasome activation. This action of H₂S is independent of the transcriptional activity of NF-κB in response to signal 1, unlike what has previously been shown about the action of H₂S on NF-κB regulation of antiapoptotic pathways in response to inflammation (Sen et al., 2012). Instead, the action of H₂S seems to be downstream of sensing of signal 2 and potassium efflux, potentially regulating the assembly of the inflammasome complex. H₂S can act to regulate enzymatic activity by posttranslational modification of reactive cysteine thiol groups in a process called sulfhydration (Paul and Snyder, 2015). Particularly of interest,
many different posttranslational modifications (PTMs) have been detected in NLRP3 and have been found to play important roles in NLRP3 inflammasome activation (Shim and Lee, 2018). Besides PTMs involved in the phosphorylation and ubiquitination of NLRP3, other known PTMs involve modifications of cysteine thiol groups. One of these PTMs include alkylation, which was first reported by Juliana et al where inhibitors such as Bay11-7082 acted through direct alkylation of NLRP3 (Juliana et al., 2010). Cys419, which resides in the ATPase catalytic pocket of NLRP3 has been predicted as a potential alkylation site (Cocco et al., 2016), which is consistent with reduced ATP binding affinity and reduced ATPase activity of NLRP3 post-alkylation (He et al., 2014; Juliana et al., 2010). This ATPase activity is important for the oligomerization of NLRP3 with itself as well as with ASC (Duncan et al., 2007). More recently, diarylsulfonylurea NLRP3 inhibitors such as MCC950/CRID3 was shown to bind to the ATP binding pocket of the NACHT domain (Walle et al., 2019). Subsequently, MCC950/CRID3 was unable to reduce circulating IL-1β and IL-18 in CAPS models of mice where there are mutations in the NACHT domain (Walle et al., 2019). Given that H2S was unable to reduce inflammasome activation in BMDMs modeling MWS (Nlpr3A350VneoR), H2S may similarly be acting in the ATP binding pocket to regulate ATPase activity and assembly of inflammasome. More recently, the itaconate derivative, 4-OI was shown to alkylate NLRP3 to block interaction with NEK7, and could reduce inflammasome activation in human CAPS peripheral blood mononuclear cells (PBMCs) (Hooftman et al., 2020). Unlike H2S, 4-OI is much larger, and likely creates steric hindrance to prevent NLRP3 binding with its binding partners.

In addition to our work, other groups have independently found a role for H2S in regulating NLRP3 inflammasome activation. Castelblanco et al. found in BMDMs and
THP1 cells that H₂S treatment via sodium thiosulfate or GYY4137 inhibited MSU or nigericin induced inflammasome via blocking ASC oligomerization (Castelblanco et al., 2018). However, another study investigating H₂S action on human PBMCs and THP1 cells found that NaHS treatment contrarily increased the production of IL-1β and IL-18 (Basic et al., 2017). This last study treated cells with 1mM NaHS which is an extremely high dose of H₂S. And while physiological and toxic levels of H₂S have been controversial given the volatile nature of H₂S, making it difficult to accurately measure, these studies reinforce the notion that a tight balance of H₂S is required to modulate inflammation and studies manipulating H₂S intrinsically, rather than the use of extrinsic donors, will be important to understand the role of H₂S physiologically.

In line with the in vitro results identifying potential pathways to regulate inflammation during MR, we found improved metabolic health in both young and old animals that underwent 3 months MR. In the adipose tissue, there was a reduction of macrophages, the major expressors of NLRP3 inflammasome with MR and a reduction in the expression of NLRP3 mediated inflammatory components in adipose tissue with MR and PR. However, this reduction in adipose tissue inflammation was limited to young animals and not old animals when the RPFAT was examined. Consistently, TSP was only upregulated in the RPFAT of young animals with MR, suggesting that local production of H₂S may have mediated the specific reduction of inflammation in young animals. However, old animals expressed higher basal levels of CBS in the RPFAT on CTRL diet, indicating that absolute expression of TSP genes alone cannot predict inflammatory status in adipose tissue. Importantly, we were unable to evaluate inflammation and the TSP in gonadal visceral tissues in this study, which play important roles in metabolic decline with
Whether MR can reduce inflammation in tissues that are associated with systemic metabolic homeostasis such as EFAT and liver to mediate the improved metabolic parameters in aged animals needs to be further investigated.

Finally, short- and long-term MR diet was unable to reduce inflammation in the *in vivo* models of MWS-NLRP3 inflammasome activation and endotoxemia. Both of these models involve acute activation of the NLRP3 inflammasome, whereas aging is characterized by more chronic low-grade inflammation. Thus, MR as an intervention may not be able to induce autophagy and TSP to levels required to restrict large scale acute inflammation.

In conclusion, we find that pro-longevity interventions such as MR has the potential to reduce NLRP3 inflammasome activation through induction of autophagy and the TSP. MR and PR can indeed reduce NLRP3 associated cytokines, IL-1β and IL-6 in the adipose tissue of young animals, and improve metabolic parameters, even when started at an old age, when age-associated inflammation and metabolic deterioration has already begun. However, MR is unable to reduce inflammation in the context of acute strong inflammatory stimuli such as CAPS and endotoxemia. Whether the pathways identified from our *in vitro* studies can be further harnessed to reduce inflammation in a broader context of inflammatory disease needs to be further investigated.
Figures

A) Differentiated BMDMs were switched to culture media containing or lacking methionine and cysteine and stimulated with LPS for 4 hours and ATP for an additional 1 hour. Caspase 1 and IL-1β were detected by western blot of lysate and supernatant.

B) WT and p62 KO BMDMs were switched to culture media containing or lacking methionine and cysteine and stimulated with LPS for 4 hours and ATP for an additional 1 hour with or without the treatment of 3-MA. IL-1β cleavage was detected in the lysates by western blot.

C) Intracellular potassium levels were detected in BMDMs cultured with or without methionine and cysteine during LPS and ATP mediated inflammasome activation.

D) Nlrp3\textsuperscript{A350V/neoR} Cag-cre BMDMs were activated with 4-OHT (Tam) and cultured in the presence or absence of methionine and cysteine during LPS and ATP mediated inflammasome activation. IL-1β was detected in lysates and supernatant by western blot.

Data representative of at least 3 independent experiments, with the exception of p62 KO BMDM experiments, which was only repeated twice. C) Data expressed as mean±SEM. Statistical differences were calculated by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001
Figure 2. Exogenously produced hydrogen sulfide inhibits inflammasome activation.

A) Differentiated BMDMs were untreated (M0), or polarized with LPS and IFNγ (M1), or IL-4 (M2) for 24 hours. CSE was detected by western blot, with liver lysate as a control. B) BMDMs were treated with 0, 10, 50, 100μM of NaHS with 4 hour LPS and 1 hour ATP stimulation. IL-1β was detected in lysate and supernatant by western blots. C) BMDMs were treated with 0, 10, 50, 100μM NaHS and stimulated with LPS for 4 hours. Inflammasome gene transcripts were quantified by qPCR with Gapdh as the reference gene. D) Intracellular potassium levels were detected in BMDMs cultured with increasing concentrations of NaHS during LPS and ATP stimulation. E) Nlrp3A350VneoR Cag-cre BMDMs were activated with 4-OHT and cultured with increasing NaHS with LPS stimulation. IL-1β was detected in lysates and supernatant by western blot. Data representative of at least 3 independent experiments. C-D) Data expressed as mean±SEM. Statistical differences were calculated by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001
Figure 2. CSE is not expressed in macrophages

A) Expression of CSE in PBS and thioglycollate induced peritoneal macrophages measured by western blot. B) Western blot expression of CSE in Cth\textsuperscript{+/+} and Cth\textsuperscript{−/−} in thioglycollate induced peritoneal macrophages shows band is nonspecific. C) RNA-seq expression of Cth expression in various macrophage and stromal populations from the ImmGen Consortium.
Figure 2. 4 MR reduces inflammation in vivo.

A) Body weight curves of young and old mice on Ctrl or MR diets over 12 weeks (n=10,10,7,5/group). B) Percent gain in body weight over 13.5 weeks quantified. Dissected C) SFAT and D) EFAT mass normalized to body weight. E) GTT post 14 hours fasting and glucose bolus IP in young and old animals after 12 weeks Ctrl or MR diet. F) ITT post 4 hour fast and insulin IP after 13 weeks Ctrl or MR diet. G) H&E histology sections of liver in young and old animals fed 13.5 weeks CTRL or MR diet. White scale bar represents 50um. H) Representative FACs panel of CD11b+ F4/80+ macrophages gated on CD45+ CD3- B220- Siglec F- cells in EFAT. I) Macrophage populations gated as in H) quantified in EFAT and SFAT. J) Inflammatory cytokines measured by qPCR in RPFAT in young animals fed 13.5 weeks Ctrl or MR diet. K) Inflammatory genes measured by qPCR in SFAT of animals after 6 months PR. (I) Each data point is representative of 2 animals pooled. Data expressed as mean±SEM. Statistical differences were calculated by (A, E-F) 2-way ANOVA with Sidak’s correction for multiple comparisons and (B-D, I-K) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001
Figure 2. 5 CSE expression during MR and inflammation in old adipose tissue.

A) Expression of CSE detected by western blot in liver of young and old animals fed Ctrl and MR diets for 13.5 weeks. B) Expression of CSE detected by western blot in kidney of young and old animals fed Ctrl and MR diets for 13.5 weeks. Expression of C) Cth and D) Cbs in RPFAT of young and old animals fed Ctrl and MR diets for 13.5 weeks. E) Inflammatory cytokines measured by qPCR in RPFAT in old animals fed 13.5 weeks Ctrl or MR diet. Data expressed as mean±SEM. Statistical differences were calculated by unpaired t-test.
Figure 2. 6 MR fails to reduce inflammation in models of MWS and LPS.

A) Body weight curves during daily tamoxifen injections after 1 week Ctrl or MR diet in Nlrp3\textsuperscript{A350VneoR} Cag-cre mice. B) Spleen weights in Nlrp3\textsuperscript{A350VneoR} Cag-cre mice after 2 days of tamoxifen injections in mice fed Ctrl or MR diet. C) Expression of inflammatory genes in the spleen of Nlrp3\textsuperscript{A350VneoR} Cag-cre mice measured by qPCR. D) Inflammatory cytokines measured by Luminex in the serum of Nlrp3\textsuperscript{A350VneoR} Cag-cre post tamoxifen injection. E) Percent changes in blood glucose levels after 4 hours of PBS or LPS IP in mice fed Ctrl or MR diet for 3 months. F) Percent changes in core body temperature after 4 hours of PBS or LPS IP in mice fed Ctrl or MR diet for 3 months. G) Inflammatory cytokines measured by Luminex in the serum after 4hour PBS or LPS IP. Data expressed as mean±SEM. Statistical differences were calculated by (A-B, D-G) 2-way ANOVA with Sidak’s correction for multiple comparisons and (C) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001
Chapter 3: Transsulfuration Pathway Mediated Cysteine Metabolism and Thermogenesis

Introduction

The transsulfuration pathway (TSP) is induced during dietary interventions such as caloric restriction (CR) and methionine restriction (MR) (Hine et al., 2015; Mitchell et al., 2016). These diets are associated with improved metabolic health, increased energy expenditure (EE), and reduced adiposity (Bruss et al., 2010; Hasek et al., 2010). While the effects of one of the TSP byproducts, H₂S, has garnered much attention as discussed in Chapter 2, the molecular basis driving the induction of the TSP has not been studied. In this chapter, we aim to investigate the basis for TSP induction, to further the understanding of mechanisms that drive metabolic adaptations caused by sulfur amino acid (SAA) restriction and its impact on organismal energy expenditure.

Results

Human calorie restriction induces the transsulfuration pathway in response to reduced cysteine derived metabolites

To study the mechanisms that drive CR effects in human adipose, we analyzed the subcutaneous adipose tissue (SFAT) of eight participants that maintained CR for 2 years and provided biopsies at baseline, 12 months, and 24 months (Fig 3.1A). Transcriptomic analyses of these tissues revealed that CR increased expression of CTH, which encodes for the enzyme that catalyzes the synthesis of cysteine from cystathionine (called cystathionine γ-lyase, or CSE) (Fig 3.1B). We observed a simultaneous reduction in the expression of
BHMT, which encodes for betaine-homocysteine S-methyltransferase, and converts homocysteine to methionine, away from cysteine production by the TSP (Fig 3.1B). This suggested an activation of the transsulfuration pathway (TSP) to shift SAA metabolism towards cysteine production (Fig 3.1D). To understand the activation of the TSP during CR, we analyzed the metabolites of SFAT after 12 months CR via metabolomics. In line with the reduced expression of BHMT, we identified a reduction in dimethylglycine (DMG), a byproduct of BHMT. Notably, while metabolites upstream of cysteine such as cystathionine, homocysteine, S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM), and methionine were not changed, we found that CR reduced cysteine derived metabolites including cysteine, $\gamma$-glutamyl-cysteine ($\gamma$-Glu-Cys), glutathione (GSH), and cysteinylglycine (Cys-Gly) (Fig 3.1C). Collectively, these results suggest that sustained CR in humans causes activation of the TSP as a metabolic response to maintain cysteine its downstream metabolites (Fig 3.1D).

**Cysteine deficiency drives rapid loss of adipose tissue through an increase in energy expenditure.**

To assess whether cysteine depletion plays an integral role in driving CR effects, we developed a model to induce cysteine deficiency in mice. Cysteine is a conditionally essential amino acid, because cysteine can be generated by CSE. We developed Cth$^{-/-}$ mice which lack CSE, and can therefore no longer produce cysteine, creating a model where cysteine has to be sourced from the diet. Cysteine deficiency was thus induced by feeding Cth$^{-/-}$ mice a custom amino acid diet that contains (CTRL diet) or lacks cystine (CysF diet) as a dietary cysteine source (Appendix 1, Fig 3.2A). The CTRL and CysF diets are isocaloric with almost equal ratios of macronutrients, and equal amounts of all other
essential amino acids. Cystine was replaced with cornstarch in the CysF diet (Appendix 2), but this replacement shifts the balance of calories between protein and carbohydrates by less than 1%. Therefore, feeding Cth⁺/⁻ a CysF diet produces a unique model of systemic cysteine deficiency.

Utilizing this model, we found that mice with cysteine deficiency rapidly lost ~25-30% body weight within 1 week compared to littermate Cth⁺/+ fed a CysF diet or Cth⁻/⁻ fed a CTRL diet (Fig 3.2B). Due to severe weight loss, mice are required to be euthanized within one week. Despite rapid weight loss, mice remained active and did not display any sickness behavior. This weight loss is associated with significant loss of fat mass relative to lean mass (Fig 3.2C). This response is sex-independent, with female cysteine deficient animals losing weight at equal rates to males (Fig 3.3 B). In wild type mice, Cth expression is highest in the liver and kidney, with much lower expression found in the adipose tissues and brain (Fig 3.3C). In order to determine whether tissue specific expression of Cth was important to maintain systemic cysteine levels and prevent cysteine deficiency, we generated Cthfloxflox animals (Fig 3.3D). Depletion specifically in the liver (Fig 3.3E-F), or adipose tissue (Fig 3.3H-I) was insufficient to drive rapid weight loss, suggesting that the expression of Cth in multiple tissues could compensate for a loss of Cth expression in a specific tissue (Fig 3.3 G, J). In addition, Cth⁻/⁻ animals co-housed together with Cth⁺/+ mice still maintained weight loss when fed a CysF diet, suggesting that microbiota is not the driving factor of this weight loss (Fig 3.3K). In all, these results highlight that redundant expression of Cth across multiple tissues defend systemic cysteine levels; loss of cysteine maintenance causes severe weight and fat loss in a sex and microbiota-independent manner.
Consistent with a loss of fat mass, cysteine deficient animals increased their normalized energy expenditure (EE) compared to CTRL diet fed animals, particularly during the dark cycle (Fig 3. 2B). However, the representation of whole-body energy expenditure has been highly debated within the metabolism field, and normalizing metabolic rate to body mass has been criticized because it overcompensates for the mass effect, leading to potential misinterpretation of actual biology (Tschop et al., 2011). Instead, calculation of the analysis of covariance (ANCOVA) or representation of the data as regression between energy expenditure and body mass, have been proposed as better methods for comparing differences in metabolic rates (Tschop et al., 2011). However, these methods have been shown to generate spurious results when mice display no overlap of body weight between groups (Fernandez-Verdejo et al., 2019), such as is between the Cth+/+ and Cth−/− mice fed CysF diet. Indeed, typical light and dark cycle EE averages for 2 days against body mass did not allow for proper comparison of the regression of the two groups, due to a lack of overlap between the two data sets (Fig 3.4 C). Similarly, regression of EE of the light cycle over 2 days against body mass is not significantly different between Cth+/+ and Cth−/− mice (Fig 3.4A). But notably, comparison of the regression of EE during the dark cycle between Cth+/+ or Cth−/− mice is significantly different, suggesting that EE is indeed increased in cysteine deficient animals during the dark cycle (Fig 3.4B). These results are consistent with normalized EE shown in Fig 3.2D-E. Careful examination of the EE over the full week of CysF diet, clearly portrays that the whole EE curve slowly shifts down in cysteine deficient animals that are continuously losing weight (Fig 3.4D). Even as the absolute values of EE are decreasing as the mice lose weight, the amplitude of the peaks remains consistent (Fig 3.4D). This unique, robust weight loss situation highlights the fact
that body mass does indeed contribute to the absolute measurements of EE and may justify the use of normalized EE in this context.

To assess whether the increase in energy expenditure was supported by increased fat utilization, we examined the respiratory exchange ratio (RER). RER is measured by the ratio between carbon dioxide production and oxygen consumption, and serves as an indirect measurement of the respiratory quotient. The respiratory quotient during rest and mild activity indicates the fuel preference that is being utilized for metabolism. An RER of 1.0 indicates a predominant utilization of carbohydrates as the fuel source whereas an RER of 0.7 indicates fat as the predominant fuel source. When we measured RER in Cth^{+/+} and Cth^{-/-} mice fed CysF diet, we found that cysteine deficient animals had significant reduction in RER through both the light and dark cycle (Fig 3.4E-F), indicating increased fat utilization. This supports the decrease in fat mass and increase in EE in cysteine deficient animals, as fat is a much more efficient fuel source than carbohydrates.

Regulation of total body weight is tightly controlled by mechanisms that maintain the balance of energy intake and EE. The weight loss during cysteine deficiency could thus be caused by a reduction in energy intake in addition to increased EE. When we compared food intake of Cth^{+/+} or Cth^{-/-} mice fed CysF diet, we found no significant differences in accumulated food intake (Fig 3.4G). But accurate measurements of food intake were difficult due to cysteine deficient animals having an increased propensity to play with their food at later time points. To definitively determine the contribution of energy intake vs. EE on weight loss, we conducted a paired food intake experiment using a relatively low amount of food provided daily to ensure that all the mice finished the daily allotment of diet. Given the slight caloric restriction this caused, there was a non-significant, but
consistent decrease in body weight in pair fed animals compared to ad libitum fed animals (Fig 3.4H). But despite eating the same amount of food, only cysteine deficient pair fed animals lost significant body weight and fat mass (Fig 3.4H-I). This supports the notion that an increase in EE, rather than a reduction in energy intake was driving the weight loss in cysteine deficient animals.

**Serum metabolite analysis confirms systemic cystine deficiency**

We next conducted serum metabolomics to identify systemic changes in metabolite profile during cysteine deficiency. Principle component analysis (PCA) displayed clear separations of $Cth^{+/+}$ and $Cth^{-/-}$ metabolite profiles with an additional separation between $Cth^{+/+}$ CTRL and CysF fed animals (Fig 3.5A). Fig 3.5B displays the top 25 metabolites changing in $Cth^{-/-}$ CysF animals compared to CTRL fed mice. One of these metabolites is L-cystine, which is significantly reduced in $Cth^{-/-}$ CysF serum (Fig 3.5B, C). Cysteine and its downstream metabolites, $\gamma$-Glu-Cys, and GSH are not altered, suggesting that cysteine deficiency is really maintained by a reduction in systemic cystine levels (Fig 3.5C). This makes sense, as cystine is the predominant form of cysteine in the circulation. Interestingly, with the exception of cystathionine which is significantly increased in CysF diet fed $Cth^{-/-}$ mice compared to CTRL fed animals, other SAA metabolites such as methionine, SAM, SAH, and Hcys, are not significantly changed (Fig 3.5C). Instead, analogs of cysteine and GSH associated metabolites, such as 2-aminobutyric acid (2AB) and ophthalmic acid (OA) are increased in the serum of cysteine deficient mice (Fig 3.5C). OA is a GSH analog where cysteine is replaced by 2AB, and this increased flux towards OA production vs GSH production may be indicative of systemic cysteine deficiency (Fig 3.5D). We explored the potential bioactivity of several metabolites increased with cysteine deficiency including
OA, 2AB, and uridine in the context of lipid mobilization. To do this, we conducted an *ex vivo* lipolysis assay on adipose explants. We were unable to find repeatable effects of these metabolites in inducing lipolysis *ex vivo*, suggesting that these metabolites are not directly involved in driving lipid mobilization through action on adipocytes (data not shown). Additional studies are required to definitively assess a functional role of these metabolites in lipid metabolism. Regardless, serum metabolomics was able to identify that cysteine deficiency was indeed being induced in this model, maintained by a decrease in circulating cystine.

**Cysteine deficiency drives thermogenesis of adipose tissue.**

The decrease in fat mass during cysteine deficiency is driven by a loss of all major adipose depots including subcutaneous fat (SFAT), visceral epididymal fat (EFAT), and brown adipose tissue (BAT) (Fig3.6A). Histological analyses revealed this reduction in adipose tissue size is associated with a complete transformation of white adipose depots into a BAT-like appearance, with the formation of multilocular adipocytes and enlarged nuclei, a phenomenon known as browning (Fig3.6B). Surprisingly, these morphological changes were quite robust in the EFAT as well, which is normally not considered to be an adipose depot amenable to browning (Fig3.6B). Given these robust changes in the adipose tissues, we conducted total RNA-sequencing (RNA-seq) of the major adipose depots, SFAT, EFAT, and BAT, to broadly assess the molecular basis of the changes occurring in adipose tissue during cysteine deficiency. PCA analysis reveals that cysteine deficiency drives clear transcriptomic changes in all three tissues (Fig 3.6C), with almost binary transcriptional changes occurring in the SFAT and EFAT as displayed by heatmap (Fig 3.6D). Gene set enrichment analysis comparing *Cth*+/− CysF vs CysF CTRL identified that
the top downregulated pathways are involved in extracellular matrix and collagen deposition, highlighting the broad remodeling of the adipose tissue that is also evident from the histology (Fig 3.6E). In addition, multiple metabolic pathways appear to be regulated by cysteine deficiency within the SFAT with ‘respiratory electron transport chain and heat production’ as the top pathway induced during cysteine deficiency (Fig 3.6E).

Indeed, numerous genes identified by the ‘thermogenesis’ GO-term pathway are differentially expressed comparing Cth−/− CTRL and CysF in the SFAT (Fig 3.7A). Brown adipocytes are unique from white adipocytes in that they contain high density of mitochondria, which are densely packed with cristae and increased respiratory-chain components (Himms-Hagen and Desautels, 1978). This increased mitochondrial capacity, along with the expression of UCP1, which uncouples oxidative phosphorylation, allows for increased heat production. Compared to Cth−/− CTRL, Cth−/− CysF have increased proportions of reads that map to the mitochondria in the SFAT, suggesting an increase in mitochondrial content like brown adipocytes (Fig 3.7B). Additionally, UCP1, which is normally not expressed in white adipose tissues, becomes widely expressed with cysteine deficiency in both the SFAT and EFAT as measured by UCP1 staining in paraffin embedded sections (Fig 3. 7C). This increase in UCP1 was confirmed by WB and qPCR in the SFAT (Fig 3. 7D-E). The induction of numerous thermogenic genes that were identified by RNA-seq including Cidea, Cox7a1, Cox8b, Dio2, Eva1, Pgc1, Elovl3, and Slc27a2, was also verified by qPCR (Fig 3.7E-F). These results indicate that surprisingly, cysteine availability acts as a thermogenic checkpoint.

To identify the metabolic pathways important for the tissue remodeling observed during cysteine deficiency, we utilized GAM-clustering to identify pathways commonly
changing in all three tissues. GAM-clustering is a recently developed, network-based computational approach for unbiased identification of key metabolic subnetworks from transcriptional profiles generated by RNA-seq. It was recently refined to allow for the comparison of multiple groups (Gainullina et al., 2020). GAM-clustering applied to the RNA-seq of all three tissues (SFAT, EFAT, and BAT) with all four sample conditions ($Cth^{+/+}$ and $Cth^{-/-}$ fed CTRL or CysF diet) identified 8 distinct metabolic modules (Fig 3.8A). Some of these modules identified tissue specific metabolic features, such as modules 1 and 2 which were more specific to BAT, whereas modules 3, 5, and 7 were more specific to the white adipose depots, SFAT and EFAT. Within those white adipose tissue modules, module 5 was most enriched in EFAT whereas module 3 was most enriched in SFAT (Fig 3.8A). Interestingly, module 2, which was strongly enriched in the BAT of all animals, was also slightly enriched in the SFAT and EFAT of cysteine deficient animals specifically. Given the browning of white adipocytes that occur with cysteine deficiency, this module may highlight metabolic pathways directly related to thermogenic capacity. Pathways enrichment analysis identified branched chain amino acid (BCAA) degradation, TCA cycle, and fatty acid (FA) metabolism to be key features in module 2 (Fig 3.8B, E). The enrichment of BCAA degradation is quite interesting, as a recent study found that BCAAs were actively taken up by BAT upon cold exposure to fuel mitochondrial oxidation (Yoneshiro et al., 2019). Similarly, during cysteine deficiency, increased BCAA catabolism in the adipose tissues may support mitochondrial oxidation to drive thermogenesis.

Module 4 represented metabolic features that were downregulated by cysteine deficiency across all three tissues (Fig 3.8A, C). This module was characterized by steroid
and cholesterol biosynthesis, suggesting that cholesterol biosynthesis is decreased in the adipose tissues during cysteine deficiency (Fig 3.8 C, F). Conversely, module 6 represented metabolic features that were increased by cysteine deficiency across all three tissues (Fig 3.8A, D). This module was specific to lipid metabolism (Fig 3.8D, G). These unbiased analyses point towards increased changes in lipid metabolism and BCAA metabolism to support increased mitochondrial oxidation and thermogenic capacity of adipose tissues during cysteine deficiency.

**Redox status of adipose tissue during cysteine deficiency**

One important aspect of cysteine metabolism is its role in redox homeostasis. Cysteine is a component of glutathione, one of the major antioxidants that neutralize oxidative damage. Chouchani et al have demonstrated that mitochondrial ROS is acutely increased during cold challenge, and is required for UCP1 activity. They showed that sulfenylation of UCP1 due to increased mitochondrial ROS exposure is required to activate UCP1 from its purine inhibited state (Chouchani et al., 2016). Similarly, forcing oxidative redox state by treatment with a glutathione depleting agent, buthionine sulfoximine, increases the expression of thermogenic programs in adipose tissue (Barbato et al., 2015). These studies suggest that increased oxidative stress is an additional regulator of thermogenesis in the adipose tissues. Given the requirement of cysteine for glutathione synthesis, oxidative stress could be induced during cysteine deficiency. We performed metabolomics in the SFAT to assess the impact of cysteine deficiency on glutathione and cysteine metabolism in the adipose tissue undergoing a thermogenic response. Surprisingly, we detected no differences in glutathione in its reduced form (GSH) by orbitrap type mass spectrometry (Fig 3.9A). However, the oxidized form of glutathione...
(GSSG) was decreased in the SFAT, suggesting that oxidative status during cysteine deficiency in the adipose tissue may actually be reduced (Fig 3.9A). Furthermore, cysteine levels were increased while cystine levels in the adipose tissue are not altered, supporting a lack of GSH depletion in adipose tissue and a less oxidative redox state (Fig 3.9B). However, in contrast to our results where we do not find serum or tissue reductions of GSH, other groups that have investigated cysteine deficiency, report reductions of GSH in the plasma (Mani et al., 2011), and liver (Ishii et al., 2010; Mani et al., 2011). Further evaluation of redox status by measuring ROS production and confirmation of GSH levels through other methods may be important to make definitive conclusions of the redox status in adipose tissues during cysteine deficiency. Ophthalmic acid (OA) has been suggested as an oxidative stress biomarker indicative a hepatic GSH consumption (Soga et al., 2006). In addition to serum OA, we find OA is increased in the SFAT during cysteine deficiency (Fig 3.9D). Gene expression of enzymes involved in the synthesis of GSH and OA, Gclc and Gss were elevated (Fig 3.9C), suggesting that there may indeed be increased pressures to maintain GSH and OA during cysteine deficiency. However, cysteine deficiency also increases threonine levels in the SFAT, which is an upstream metabolite that generates 2AB and OA (Fig 3.9D-E). Thus, substrate availability could drive the activation of this pathway and OA production independently of glutathione synthesis. Collectively, metabolomics of SFAT during cysteine deficiency suggests that redox homeostasis is shifted towards a more reduced state, rather than oxidative state, and thus unlikely to be a driving mechanism of thermogenesis in this context.
scRNA-seq of SFAT stromal vascular fraction suggests increased mobilization of preadipocytes.

To understand the cellular basis of adipose tissue remodeling during cysteine deficiency, we isolated the SFAT stromal vascular fraction by enzymatic digestion and conducted single cell RNA sequencing (scRNA-seq) by 10x Genomics. We isolated cells from Cth+/+ and Cth−/− fed CTRL or CysF diet for 4 days to identify changes occurring before full remodeling of the tissue, with each sample pooled from 4 animals (Fig 3.10A). A total of 4,666 cells in Cth+/+ CTRL; 5,658 cells in Cth+/+ CysF; 4,756 cells in Cth−/− CTRL; and 3,786 cells in Cth−/− CysF were analyzed for scRNA-seq (Fig 3.10D). By unbiased clustering, we were able to identify a total of 15 cell populations, including 3 adipocyte progenitor populations; endothelial and pericytes; mixed T cell populations including αβ T cells, γδ T cells, ILC2s, and NK T cells; B cells; reticulocytes; mesothelial-like cells; Schwann cells; and several myeloid clusters (Fig 3.10B-C). Comparison of Cth−/− CysF with other groups revealed dramatic changes in cellular composition and transcriptional state (Fig 3.10D).

Particularly, a significant loss of clusters 0, 1, and 2 were apparent during cysteine deficiency (Fig 3.10D-E). Furthermore, these clusters contained the highest numbers of differentially expressed genes (Fig 3.11E), highlighting them as important populations in mediating the effects of cysteine deficiency. By expression of Pdgfra, we were able identify these clusters as adipocyte progenitors (Fig 3.10C, F). To understand the differential changes amongst these clusters, we conducted pseudotime analysis to place these clusters on a trajectory to help allocate their cell lineage. Trajectory analysis based on pseudotime suggested that cluster 2 differentiates into two separate preadipocyte
clusters, cluster 0 and 1 (Fig 3.10G-H). Cth\textsuperscript{+/−} CysF animals proportionally lost Clusters 0 and 1, while relatively maintaining cluster 2 compared to the other groups (Fig 3.10D-E), suggesting that more differentiated preadipocytes were becoming mobilized and lost during cysteine deficiency. Indeed, cluster 2 expressed Dpp4, an early progenitor marker that has been shown to give rise to different committed preadipocytes (Merrick et al., 2019)(Fig 3.10I). Cluster 0 was enriched for both Icam1 and F3, which have been shown to be expressed by committed adipogenic, and antiadipogenic preadipocytes, respectively (Hepler et al., 2018; Merrick et al., 2019)(Fig 3.10I). Cd9, a fibrogenic marker in preadipocytes (Hepler et al., 2018; Marcelin et al., 2017), along with the collagen gene, Col5a3, were broadly expressed across clusters 0 and 1, and was specifically lost by day 4 of inducing cysteine deficiency (Fig 3.10I). We next sought to identify beige/brown adipocyte precursors in our scRNA-seq dataset to understand whether there was an increased commitment towards brown adipocytes. To do this, we examined the expression of a CL-316,243 β3-adrenergic receptor agonist-stimulated gene signature reported by Burl et al, because β3-adrenergic signaling induces the differentiation of brown adipocytes (Burl et al., 2018). We found that cluster 1 had the most pronounced expression of this gene signature (Appendix 5, Fig 3.11B), and indeed Tagln, or Sm22, which have been previously described in beige adipocytes (Berry et al., 2016; Long et al., 2014; Oguri et al., 2020), is specifically expressed by a subset of cells in cluster 1 (Fig 3.10I). Interestingly, these Tagln expressing cells are completely lost with cysteine deficiency (Fig 3.10I). Given the strong browning phenotype observed at day 6, it is possible that these cells become mobilized and differentiate early on during cysteine deficiency, leading to the absence of these cells. Indeed, when we performed pathways analysis on cluster 1, comparing gene
expression of \(Cth^{+/}\) CysF with \(Cth^{+/}\) CTRL, we found that one of the top upregulated pathways was ‘adipogenesis’ (Fig 3.11C). Furthermore, when we examined the expression of stem associated markers and mature adipocyte markers in the adipocyte progenitor clusters, we observed a clear downregulation of stem markers and increase in mature adipocyte markers, suggesting that cysteine deficiency was driving the maturation of progenitor cells, which are not captured within the stromal vascular fraction (Fig 3.11A). However, given the robust transformation of the adipose tissue during cysteine deficiency, where significant portions of the white adipose tissue have become brown, it is unlikely that mobilization of brown precursors alone is mediating this response. Trans-differentiation of mature white adipocytes into brown adipocytes can also contribute to the browning of SFAT and needs to be further examined.

**Cysteine deficiency increases lipolysis via activation of sympathetic nervous system**

To determine the mechanism of adipose thermogenesis post-cysteine deficiency, we next investigated the processes upstream of increased fatty acid oxidation. Activation of the sympathetic nerves leads to release of catecholamines such as norepinephrine. Norepinephrine acts on adipocytes through \(\beta_3\)-adrenergic receptors and stored triglycerides are hydrolyzed and converted to free fatty acids (FFAs) in a catabolic process called lipolysis. These FFAs subsequently fuel mitochondrial respiration and ATP production or uncoupling by UCP1 to generate heat (Fig 3.12A). Lipolysis is mainly regulated by two lipases in white adipose tissue, hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Zechner et al., 2009). When we measured the activation of lipolysis in the SFAT by pHSL and ATGL by western blot, we found that
cysteine deficiency increases ATGL expression without affecting pHSL levels (Fig 3.12B). ATGL preferentially catalyzes the first step of triglyceride hydrolysis whereas HSL has a much broader range of substrates with a preference for diacylglycerols and cholesteryl esters (Haemmerle et al., 2002; Zimmermann et al., 2004). Our data suggests that mobilization of lipids during cysteine deficiency is mainly mediated by ATGL, without significant activation of HSL. Given a dramatic browning response in WAT during cysteine deficiency, the increased ATGL expression is consistent with work that shows BAT relies heavily on the action of ATGL to mobilize lipid substrates for uncoupling and heat production (Morak et al., 2012).

Upstream of lipolysis, non-shivering thermogenesis is mainly activated by the sympathetic nervous system and thyroid hormone. We find that norepinephrine levels are significantly increased in $Cth^{+/-}$ CysF SFAT, compared to cysteine sufficient controls, $Cth^{-/-}$ CTRL, $Cth^{+/-}$ CysF, and $Cth^{-/-}$ CTRL (Fig 3.12C), indicating that sympathetic activation is significantly increased in the SFAT of cysteine deficient animals. Furthermore, when we examined the RNA-seq of SFAT during cysteine deficiency, we found that the expression of norepinephrine degradation enzymes, $Comt$ and $Maoa$ were decreased in cysteine deficient mice (Fig 3.12D-E). Confirmation by qPCR revealed that $Maoa$ expression was indeed decreased (Fig 3.12G). However, the expression of $Comt$ was not consistently decreased in the SFAT with cysteine deficiency when measured by qPCR (Fig 3.12F). Despite these disparities between different methods of accessing gene expression of $Comt$, protein expression of COMT was lowered in cysteine deficient SFAT when measured by western blot (Fig 3.12H). This reduction was more apparent in the membrane bound form of COMT (MB-COMT). Furthermore, we found increased levels
of S-adenosyl homocysteine (SAH) in the SFAT of cysteine deficiency, which can inhibit the enzymatic activity of methyltransferases like COMT (Fig 3.12I). In all, these results suggest the decreased degradation of norepinephrine during cysteine deficiency increases catecholamine bioavailability together with sympathetic activation to promote lipolysis and thermogenesis in SFAT (Fig 3.12J). Consistent with this, application of imaging mass spectrometry to detect norepinephrine localization in BAT sections identified increased concentrations and distribution of norepinephrine throughout the BAT tissue of cysteine deficient animals (Fig 3.12K). These results suggest that cysteine deficiency drives increased sympathetic activation, in part by a decrease in catecholamine degradation enzymes. This increases lipolysis, mediated mainly by an increase in ATGL, which provides the substrates required for mitochondrial respiration.

**Cysteine deficiency leads to a loss of monocyte derived macrophages but enrichment of resident macrophages.**

Previously, it was shown that macrophages expressing catecholamine degradation enzymes, COMT and MAOA, could regulate sympathetic activation in the context of aging and obesity (Camell et al., 2017; Pirzgalska et al., 2017). Given the increased sympathetic activation of SFAT and BAT during cysteine deficiency, we next aimed to understand whether this process was being regulated by tissue resident macrophages. Besides a loss in adipocyte progenitor populations, scRNA-seq of the stromal vascular fraction revealed a decrease in multiple myeloid populations, such as cluster 8 and 11, as well as an enrichment of a more resident macrophage population, cluster 4 (Fig 3.10E). Furthermore, in addition to adipocyte progenitors, resident macrophages expressed a large number of differentially
regulated genes, suggesting that they may play an important role in the remodeling of adipose tissue during cysteine deficiency (Fig 3.11 E). When we examined macrophage populations by flow cytometry, we were able to confirm a 50% reduction in macrophages after 6 days of CysF diet in Cth+/− compared to Cth+/+ animals (Fig 3.13A).

To further understand the dynamic changes occurring in the macrophage population, we performed scRNA-seq of resident myeloid cells after induction of cysteine deficiency. Given that cysteine deficiency is accompanied by a loss of macrophages in an already much smaller tissue, we chose a relatively early timepoint of 3 days of diet, and pooled cells from 5 animals per group, to ensure we had enough cells for analysis. To target resident cells within the tissue parenchyma, we utilized short-term intravenous (i.v.) labeling of CD45 to exclude circulating cells, along with additional markers CD3, B220, and Siglec F, to exclude T cells, B cells, and eosinophils, and CD11b that were stained after single cell suspension to sort resident myeloid cells specifically (Fig 3.13B). We performed scRNA-seq on a total of 7,877 cells from Cth+/+ and Cth+/− (Fig 3.13C), with 2,383 cells from Cth+/− CTRL and 2,679 cells from Cth+/− CysF (Fig 3.13D). Cth+/+ conditions were processed on a separate day from Cth+/− conditions, and we achieved much fewer cells with 1,616 cells from the CTRL, and 1,199 cells in the CysF group (data not shown). Of note, dendritic cell (DC) populations resembling conventional DCs, Cluster 5 and 6, were only identified in the Cth+/+ samples, suggesting potential inconsistencies in the processing of these samples (Fig 3.13C). Due to the biases introduced by processing on different days, we only show data from the Cth+/− from here onward.

In the scRNA-seq analysis of Cth+/− samples, we identified 5 other clusters of macrophages, including (0) lipid associated macrophages that express high levels of Cd36;
(1) *Cd209* and *Cd9* expressing macrophages/DCs; (2) *Ly6c2* expressing monocyte derived macrophages; (3) *Lyve1* expressing resident macrophages; and (4) proliferating macrophages (Fig 3.1C, 3.14A). Given our previous work identifying macrophages tightly associating with nerve fibers and bundles (Camell et al., 2017), we hypothesized that amongst the tissue resident macrophages, these nerve-associated macrophages (NAMs) would play important roles in taking up and metabolizing catecholamines released by sympathetic nerves to limit the availability of catecholamines to surrounding adipocytes. Alterations to the function of NAMs may thus play important roles in controlling sympathetic activation during cysteine deficiency. Currently, there are no known markers for NAMs in the adipose tissue. To try to identify these macrophages in our scRNA-seq datasets, we identified genes commonly expressed in NAMs of other various tissues, such as the lung, gut, skin, and sciatic nerves (Chakarov et al., 2019; Gabanyi et al., 2016; Kolter et al., 2019; Mueller et al., 2003; Pirzgalska et al., 2017; Ural et al., 2020). When we overlayed this gene set (Appendix 6) onto our scRNA-seq data, these genes were enriched in cluster 3 (Fig 3.13F). This cluster also specifically expressed *Maoa*, one of the enzymes that degrade norepinephrine, supporting the notion that NAMs are present within cluster 3 (Fig 3.13 G). Besides *Maoa*, cluster 3 macrophages also expressed *C1qa, Gas6, Mertk*, and *Lyve1* (Fig 3.13E, Fig 3.14A).

To verify an increase in NAMs during cysteine deficiency, we looked for cell surface markers unique to cluster 3. We found that CD163 is highly expressed in cluster 3 (Fig 3.13H), and was able to verify an increase of CD163+ macrophages after 6 days of inducing cysteine deficiency by flow cytometry (Fig 3.13I). However, it is important to note that CD163 is not exclusively expressed in NAMs and can also be expressed by other
resident macrophages in clusters 1 (Fig 13.H). In fact, transcriptionally, cluster 3 seemed to be quite heterogeneous, with other markers such as Lyve1 present, which have been described to be expressed in perivascular macrophages within the fat (Silva et al., 2019), and Trem2 (Fig 14.A), which have been described in lipid associated macrophages that increase in obesity (Jaitin et al., 2019). Indeed, when we examined the expression of CD163 and Lyve1 by confocal whole mount imaging, we found that both CD163 and Lyve1 were expressed by NAMs directly in contact with nerves, as well as parenchymal macrophages that were further away from nerves (Fig 3.14B). Furthermore, the expression of MAOA was detected in both NAMs as well as CD163+ macrophages that were associated with adipocytes (Fig 3.14C-D). Collectively, these data suggest that macrophages in the adipose tissue are not easily classified into distinct functional subsets based on previously described markers. Nonetheless, the expression of Comt is widely decreased in all macrophages with cysteine deficiency, suggesting that decreased catecholamine degradation in macrophages may contribute to the increased sympathetic tone in SFAT (Fig 3.13J).

To further understand the changes in the resident macrophage cluster where NAM-like macrophages appear to be enriched, we examined the pathway enrichment of differentially regulated genes with cysteine deficiency in cluster 3 (Fig 3.14E). We found that some of the top pathways increased in resident macrophages included ‘phosphodiesterases in neuronal function’ and ‘complement cascade’ (Fig 3.14E). Particularly, recent work has found that microglia interact with neurons and synapses to regulate neuronal function through the expression of complement and complement receptors (Hong et al., 2016; Wang et al., 2020). Cluster 3 is indeed enriched in the
expression of complement genes such as *C1qa* (Fig 3.14A). These pathways suggest NAMs could be altering their interaction with nerves in the context of cysteine deficiency, to support increased sympathetic activation (Fig 3.14 E).

**UCP1 is not necessary for thermogenesis during cysteine deficiency**

Given the robust activation of thermogenic pathways during cysteine deficiency, we next investigated whether cysteine deficiency triggers adaptive thermogenesis to defend core body temperature. We first evaluated whether core body temperature was being altered during cysteine deficiency. Utilizing temperature data loggers surgically implanted into the peritoneal cavity, we measured core body temperature of *Cth*+/− mice on CTRL or CysF diet over 6 days. We found that core body temperature was not significantly different between the two groups (Fig 3.15A-B). A recent study investigating the thermoneutral point in mice, found that after ambient temperatures reached the thermoneutral point for mice, energy expenditure remained constant at higher temperatures (Skop et al., 2020). Instead, the body temperature of the mice began to increase as ambient temperatures increased beyond the thermoneutral point (Skop et al., 2020). This study suggests that once core body temperature is defended, energy expenditure levels are maintained at a minimal level for basic bodily functions independent of core body temperature maintenance in mice. During cysteine deficiency, mice have increased energy expenditure, but not higher core body temperatures, suggesting that the increased energy expenditure is being utilized to generate heat and defend core body temperature. To confirm that energy expenditure was actually being utilized to generate heat, we utilized a magnetic resonance imaging (MRI) method called BIRDS (Biosensor Imaging of Redundant Deviation in Shifts) to measure
temperatures of BAT in $Cth^{+/+}$ and $Cth^{-/-}$ animals after 6 days of CysF diet (Fig 3.15C).

This method relies on differing relaxation times of the non-exchanging protons of an exogenous contrast agent, thulium and macrocyclic chelate, which becomes altered by temperature. The chemical shifts that occur paramagnetically are detected by BIRDS and converted into physicochemical parameters to obtain absolute temperatures with high sensitivity (Coman et al., 2013; Coman et al., 2010). We adapted this method to measure *in vivo* temperature differences of BAT in the context of cysteine deficiency. We found that the relative temperature of the BAT compared to its surrounding tissue was higher in $Cth^{-/-}$ mice compared to $Cth^{+/+}$ mice (Fig 3.15C-D), suggesting that the adipose tissue was increasing energy expenditure to indeed generate heat and defend core body temperature.

Cysteine deficiency seems to increase susceptibility to cold such that it is required to increase heat production to maintain core body temperature. Considering that in animal facilities, mice are normally housed at temperatures below their thermoneutral zone, mice are constantly under thermogenic stress. To further confirm that mice were indeed inducing thermogenesis to defend core body temperature due to increased susceptibility to cold, we housed mice at 30°C in addition to 22°C. We found that housing at 30°C reduced the browning phenotype of SFAT when cysteine deficiency was induced by 6 days of CysF diet (Fig 3.15E-F). In addition, gene expression of *Ucp1*, required for uncoupling mediated heat production, was reduced when housed at 30°C (Fig 3.15G), suggesting that indeed a need to generate heat significantly drove thermogenic browning of the SFAT. However, surprisingly, despite reduced fat mobilization and heat production, cysteine deficient animals still significantly lost weight at 30°C (Fig 3.15E). Furthermore, transcriptional expression of some mitochondrial enzymes involved in fatty acid oxidation such as *Acadm*
and Cox8b were even further increased at 30°C with cysteine deficiency (Fig 3.15G), suggesting that the browning responses are only attenuated and additional pressures to mobilize fat metabolism and weight loss may be present during cysteine deficiency.

Given that these results suggested that cysteine deficiency somehow increased pressures to defend core body temperature, we next tested whether UCP1 was required to maintain core body temperature during cysteine deficiency. To do this, we generated Cth−/− Ucp1−/− double KO (DKO) mice. Interestingly, we found that Cth+/− Ucp1+/− DKO mice lost weight at a similar rate to its Cth+/− littermates on a CysF diet (Fig 3.16A), but had increased fat mass, suggesting that less lipids were being mobilized and utilized for heat production in the Cth+/− Ucp1+/− DKO animals (Fig 3.16B). This increase in fat mass particularly came from the SFAT and BAT, whereas loss of visceral gonadal peri-uterine/ovarian fat (Ut/OvFAT) was not significantly different (Fig 3.16C). This reduction in browning is evident from histology, where the SFAT from Cth+/− Ucp1+/− DKO post 6 days of CysF diet shows slightly larger lipid droplets compared to the Cth−/− mice (Fig3.16D). However, robust browning of the SFAT is still observed by the formation of multilocular adipocytes in Cth+/− Ucp1+/− DKO on CysF diet (Fig 3.16D), suggesting that lipid mobilization is still highly activated even in the absence of UCP1 during cysteine deficiency. In line with the amelioration of fat loss in the Cth+/− Ucp1+/− DKO mice compared to Cth+/−, Cth+/− Ucp1+/− DKO have reduced energy expenditures measured by indirect calorimetry in metabolic chambers (Fig 3.16E-F). Given the similar body weights in these animals, it was possible to represent the data without normalization, as is the gold standard (Fig 3.16E), and conduct linear regression analysis of EE against body mass to show significant decreased EE in Cth+/− Ucp1+/− DKO compared to Cth−/− animals (Fig 3.16F). Despite this reduction in EE of
Cth\(^{+/−}\) Ucp1\(^{−/−}\), surprisingly, Cth\(^{+/−}\) Ucp1\(^{−/−}\) mice were able to maintain core body temperature identically to the Cth\(^{+/−}\) mice on CysF diet (Fig 3.16G). This indicated that UCP1 was not required for the maintenance of core body temperature.

Furthermore, RER dropped similarly over time in both Cth\(^{+/−}\) and Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO, indicating that they both increased utilization of fat as the major fuel source for metabolism during cysteine deficiency (Fig 3.17A). If anything, Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO had even lower RER on the very last dark cycle on the diet, suggesting greater fat utilization (Fig 3.17B). Consistent with this lower RER, Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO mice expressed higher levels of ATGL and tyrosine hydroxylase (TH) measured by western blot in the SFAT (Fig 3.17C-E). These results suggest that Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO mice increase their mobilization of lipids through increased lipolysis. TH is the rate limiting enzyme for norepinephrine production and is predominantly expressed in sympathetic nerves innervating the adipose tissue. Increased TH expression suggests that Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO may potentially have increased sympathetic activation compared to Cth\(^{+/−}\) at 6 days post-cysteine deficiency. However, TH is also increased in Ucp1\(^{−/−}\) independently of cysteine deficiency (data not shown), and further evaluation of sympathetic tone by measuring catecholamines is required.

Despite a lack of UCP1, gene expression of activators of the thermogenic program such as Ppargc1, Cidea, Cpt1 are significantly increased in Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO compared to Cth\(^{+/−}\) in the BAT after 6 days of CysF diet (Fig 3.17F). Furthermore, gene expression of other mediators of the thermogenic program such as Acadm, Cox7a1, Elovl3, and Slc27a are also significantly increased in Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO mice compared to Cth\(^{+/−}\) (Fig 3.17F). These data suggest that Cth\(^{+/−}\) Ucp1\(^{−/−}\) DKO mice are increasing their mobilization of lipids
for mitochondrial respiration, even more so than the \( Cth^{+/−} \), which already rely on significantly elevated mitochondrial respiration in response to cysteine deficiency. The lack of UCP1 may force \( Cth^{+/−} Ucp1^{+/−} \) DKO mice to rely on less efficient mechanisms of thermogenesis, which could potentially explain the increase in FA metabolism and mitochondrial respiration.

Several UCP1 independent thermogenic mechanisms have been identified in recent years. We evaluated one such mechanism, creatine futile cycling, by measuring gene expression of the enzymes involved in this process, \( Gatm, Gamt, Ckmt1, Ckmt2, \) and \( Slc6a8 \). While the expression of creatine synthesis genes, \( Gatm \) and \( Gamt \) were not differentially induced with cysteine deficiency in \( Cth^{+/−} Ucp1^{+/−} \) DKO mice compared to \( Cth^{−/−} \) animals, the expression of one of the creatine kinases that utilize ATP, \( Ckmt2 \), was significantly increased and the transporter for creatine, \( Slc6a8 \), albeit not significantly, was slightly increased in the BAT (Fig 3.17G). These results indicate that creatine futile cycling may be one mechanism that becomes activated to generate heat in the absence of UCP1 in \( Cth^{+/−} Ucp1^{+/−} \) DKO mice. In all, it seems that UCP1 is not strictly necessary to maintain core body temperature during cysteine deficiency. However, the reduction in energy expenditure despite increased activation of FA metabolism leaves many questions unanswered. Furthermore, weight loss is maintained even when energy expenditure is reduced. More studies will be necessary to fully understand the dynamic metabolic changes occurring during cysteine deficiency.
Summary & Discussion

We find that during CR, the TSP is induced to defend against reduced cysteine levels. We developed a model to study the effects of cysteine deficiency by knocking out the gene, Cth, to force a requirement for cysteine from the diet. Surprisingly, we discovered that cysteine deficiency drives rapid mobilization and utilization of lipids in adipose tissues to increase heat production via browning of SFAT and EFAT. Activation of browning in these white adipose depots is mediated through an increase in sympathetic signaling that drives increased lipolysis in the SFAT and BAT. We find that increased sympathetic activation is in part mediated by a decrease in degradation of norepinephrine; the expression of COMT and MAOA is decreased in the adipose tissues. Particularly, COMT expression is decreased in resident macrophages during cysteine deficiency.

Whether macrophages are indeed playing a critical role in regulating sympathetic tone and browning during cysteine deficiency still needs to be verified. And if so, whether specific populations of macrophages are mediating its effects is unclear. Recent studies have begun to redefine macrophages within the adipose tissue. While early studies had purely divided macrophages into proinflammatory and anti-inflammatory macrophages (Lumeng et al., 2007a; Weisberg et al., 2003), more recent studies utilizing scRNA-sequencing have started to categorize adipose macrophages according to their function and niche (further discussed in Chapter 4). Given an increase in sympathetic activity, we aimed to study whether NAMs mediate neuro-immune crosstalk to impact adipose metabolism during cysteine deficiency. Indeed, our data demonstrates that residential macrophages were enriched during cysteine deficiency in the SFAT, and their expression of Comt was decreased. However, due to the heterogeneity within cluster 3, and limitations
of sensitivity of scRNA-seq, we were unable to identify exclusive iron-clad markers for NAMs in the SFAT. Instead, we found that this population of resident macrophages were likely a mixed population, including perivascular macrophages, parenchymal adipocyte adjacent macrophages, and nerve associated macrophages, and thus may be acting through several different mechanisms. This heterogeneity of residential macrophages in adipose tissue may highlight the complexity of the adipose tissue niche, where nerves and blood vessels have been found to be highly integrated with adipocytes, and nearly every adipocyte has been suggested to be in close contact with a nerve (Cao et al., 2018; Chi et al., 2018; Wolf et al., 2017). We plan to further investigate whether these macrophages are important in mediating the remodeling of adipose tissue during cysteine deficiency by depleting macrophages using clodronate liposomes. Recently, a study found that adipose tissue macrophages were important in mediating a model of thermogenesis where adipocytes were deficient in fatty acid synthase (Henriques et al., 2020). This study suggested that macrophages can promote browning independently of mediating sympathetic activation, and additional detailed studies are required to determine if indeed NAMs or parenchymal residential macrophages can regulate the thermogenic response in the context of cysteine deficiency.

Given the significant decrease in monocyte derived macrophages in addition to the enrichment of residential macrophages during cysteine deficiency, loss of specific adipose tissue macrophages could also play a role in adipose tissue remodeling. Ideally, depletion of resident macrophages specifically would delineate the role of enriched resident macrophages vs the loss of monocyte derived macrophages during cysteine deficiency. Pilot studies attempting to deplete adipose resident macrophages utilizing CD169-cre
iDTR mice in our lab have not been successful and require further optimization to successfully deplete resident macrophages in the adipose tissue. However, if macrophage depletion by clodronate liposomes further enhances weight loss during cysteine deficiency, it may suggest that alternatively, macrophages, including monocyte-derived macrophages are required to maintain adiposity, and loss of these macrophages during cysteine deficiency promote unchecked thermogenesis.

Our results suggest that cysteine is an important metabolite in maintaining core body temperature, and loss of mechanisms to maintain cysteine levels severely challenges homeostatic thermoregulation. While it is still unclear why cysteine deficiency requires the activation of thermogenic processes, it appears that thermogenesis can be successfully maintained even in the absence of UCP1. Moreover, it appears that even when fat mass loss is ameliorated by housing at thermoneutrality, weight loss is still maintained, suggesting that lean mass is significantly lost during cysteine deficiency as well. In agreement with our cysteine deficiency studies, recently, a group studying the effects of protein content in diet ranging from 20% to 1%, found that mice preferentially lost adipose depots including subcutaneous adipose, epididymal adipose, mesenteric adipose, retroperitoneal adipose, and brown adipose in response to low protein diet (Wu et al., 2021). They also report that mice on low protein diets lost the mass of reproductive organs, pancreas, and liver while other lean organs such as the brain, lungs, heart, kidneys, and spleen were protected (Wu et al., 2021). And while this study did not measure skeletal muscle, in a study that deleted Cth in a 129/SvJ mouse background, mice fed a low cysteine diet before adulthood (3 weeks of age), prevented developmental growth, and developed severe paralysis of the lower extremities after one week (Ishii et al., 2010). This paralysis
was caused by increase autophagy in the skeletal muscle, suggesting that cysteine deficiency during development promoted degradation or atrophy of the skeletal muscle (Ishii et al., 2010). Evaluating how these different lean organs are changing during cysteine deficiency in adult mice may help us understand the biology driving weight loss and adipose thermogenesis.

Particularly, the increase in BCAA degradation pathways in the adipose tissue is intriguing. Normally, BCAAs are primarily oxidized in the skeletal muscle. However, recent studies utilizing mouse models lacking BAT (UCP1-cre x PPARγ) found that in addition to skeletal muscle, BAT plays an active role in clearing BCAAs from the circulation (Yoneshiro et al., 2019). Clearance of BCAAs from the circulation is important, as buildup of these amino acids and byproducts are toxic to the brain and other organs, and can lead to serious health problems associated with maple syrup urine disease (Dancis et al., 1960; Menkes et al., 1954). Because we do not actually detect differences in BCAAs in the serum of cysteine deficient mice, investigation of adaptations in the skeletal muscle, such as protein turnover and autophagy may provide important clues to how and why BCAA metabolism gets enriched in adipose tissue. Similarly, we observe an increased flux towards OA production in the SFAT during cysteine deficiency. While this could be interpreted as an increased pressure to generate glutathione, we also observe increased levels of threonine in the adipose tissues, which give rise to 2-oxybutric acid and subsequently 2AB. Thus, rather than regulation of glutathione production, the induction of this pathway may serve as a mechanism to clear amino acids that have been released by skeletal muscle.
In conclusion, our data demonstrates that cysteine availability is a previously unidentified key checkpoint in maintaining core body temperature. Through the generation of CSE deficient animal fed a cysteine free diet, we show that when cysteine levels are not maintained, thermogenic pathways are activated in the adipose tissues, leading to rapid weight loss to the point of mortality, as mice are required to be euthanized at 30% weight loss. This response is mediated by an increase in sympathetic signaling in the adipose tissue. How this metabolic response in the adipose tissue is being integrated by adaptations of other metabolic organs such as the skeletal muscle, liver, and hypothalamus remains to be characterized and may provide further understanding of how cysteine metabolism guards thermogenic homeostasis.
Figures

A) Schematic of human CALERIE II study. Participants provided subcutaneous adipose (SFAT) biopsies at baseline, 12 months, and 24 months of CR for RNA-seq and metabolomics (n=8 per timepoint). B) Normalized expression of changing transsulfuration pathway genes CTH and BHMT in human SFAT at baseline, 12 months, and 24 months CR. Adjusted p-values (padj) were calculated in the differential gene expression analysis (n=8). C) Selected metabolites involved in cysteine metabolism measured by MS/MS in human SFAT at baseline and 12 months CR. Significance was calculated using paired t-tests. *p<0.05, **p<0.01, ***p<0.001. D) Schematic summary of changing TSP genes and metabolites from baseline to 12 months CR that were measured in human SFAT. Green lines represent measured, but unchanging metabolites, blue arrows indicate significantly decreasing, and red arrows indicate significantly increasing metabolites or genes via paired t-test (p<0.05).

Figure 3. 1 Human calorie restriction activates the transsulfuration pathway
Figure 3. 2 Cysteine deficiency in mice drives rapid fat loss

A) Schematic of mouse model used to achieve cysteine deficiency utilizing Cth<sup>-/-</sup> fed a Cystine free (CysF) diet. B-C) Male Cth<sup>+/+</sup> and Cth<sup>-/-</sup> were fed CTRL or CysF diets for 6 days (n=5 Cth<sup>+/+</sup> CTRL, n=12 Cth<sup>+/+</sup> CysF, n=8 Cth<sup>-/-</sup> CTRL, n=17 Cth<sup>-/-</sup> CysF, 3 expts pooled). B) Percent body weight over 6 days of diet. C) Fat mass and lean mass measured by EchoMRI at day 6 of diet. D) Energy expenditure measured in metabolic cages at day 4 and 5 of diet and E) quantified day and night averages, normalized by daily body weight (n=10 Cth<sup>+/+</sup> CysF, n=12 Cth<sup>-/-</sup> CysF, 3 expts pooled). Data expressed as mean±SEM. Statistical differences were calculated by (B,D,E) 2-way ANOVA with Sidak’s correction for multiple comparisons and (C) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001
Figure 3. Whole body depletion of Cth is required for cysteine deficiency response

A-B) Female Cth+/+ and Cth-/- were fed CTRL or CysF diets for 6 days (n=5 Cth+/+ CTRL, n=5 Cth+/+ CysF, n=6 Cth-/- CTRL, n=8 Cth-/- CysF, 2 expts represented). A) Body weight curve and B) fat mass and lean mass measured by EchoMRI at day 6 of diet. C) Expression of Cth in various tissues of WT mice (n=3). D) Schematic of Cth conditional deletion used to cross to either Alb-cre or Adipoq-cre. Protein expression of CSE in Alb-cre Cth+/+ and Cth-/- mice measured by western blot in E) liver and F) kidney to confirm deletion. G) Body weight over 6 days of CTRL or CysF diet in Alb-cre Cth+/+ and Cth-/- mice. H) Protein expression of CSE in Adipoq-cre Cth+/+ and Cth-/- mice measured by western blot in H) SFAT and I) liver to confirm deletion. J) Body weight over 6 days of CTRL or CysF diet in Adipoq-cre Cth+/+ and Cth-/- mice. K) Body weight over 6 days of CysF diet in co-housed Cth+/+ and Cth-/- mice (n=4/group, 2 expts represented). Data expressed as mean±SEM. Statistical differences were calculated by (B) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 4 Metabolic parameters during cysteine deficiency

A-G) Metabolic cage data of Cth^{+/+} and Cth^{-/-} on 6 days of CysF diet after 3 days acclimation (n=10 Cth^{+/+}, n=12 and Cth^{-/-}). Linear regression analysis of unnormalized average energy expenditure measured by indirect calorimetry against body mass in Cth^{+/+} and Cth^{-/-} on days 4 and 5 of CysF diet A) for the light cycle, B) dark cycle, and c) light and dark cycle combined. D) Unnormalized EE over the full course of 6 days on CysF diet. E) RER over the full course of 6 days on CysF diet. F) Area under the curve quantified for RER. G) Accumulated food intake over the full course of 6 days. H-I) Cth^{+/+} and Cth^{-/-} pair fed CTRL or CysF diet over 6 days (n=4 Cth^{+/+}CysF ad lib, n=4 Cth^{+/+}CysF pair fed, n=6 Cth^{-/-} CTRL pair fed, n=5 Cth^{-/-} CysF pair fed). H) Percent body weight over 6 days, and I) fat mass and lean mass measured by EchoMRI on day 6. Data expressed as mean±SEM. Statistical differences were calculated by (B) linear regression analysis, by (H) 2-way ANOVA with Sidak’s correction for multiple comparisons, and by (F, I) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 5 Serum metabolomics of Cth\textsuperscript{+/+} and Cth\textsuperscript{-/-} mice fed CTRL or CysF diet
Figure 3. 6 Transcriptional response of adipose tissues to cysteine deficiency

A) Principal component analysis (PCA) of serum metabolomic profile measured by orbitrap MS/MS in Cth$^{+/+}$ or Cth$^{-/-}$ mice fed CTRL or CysF diet for 6 days (n=5 Cth$^{+/+}$ CTRL, n=6 Cth$^{+/+}$ CysF, n=4 Cth$^{-/-}$ CTRL, n=5 Cth$^{-/-}$ CysF). B) Normalized heat map of top 25 metabolites changing in the serum of Cth$^{-/-}$ mice fed CTRL or CysF diet for 6 days. C) Box plots of select metabolites involved in TSP from serum of Cth$^{+/+}$ mice fed CTRL or CysF diet for 6 days. D) Schematic summary of changing metabolites in the serum of Cth$^{+/+}$ mice fed CTRL or CysF diet for 6 days. Green lines represent measured, but unchanging metabolites, blue arrows indicate significantly decreasing, and red arrows indicate significantly increasing metabolites. Statistical differences were calculated by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.

A) Representative subcutaneous, visceral, and brown adipose depots of Cth$^{+/+}$ and Cth$^{-/-}$ post 6 days of CysF diet. B) Representative H&E histology images of SFAT and EFAT of Cth$^{-/-}$ mice fed CTRL or CysF diet for 6 days. C-D) Whole tissue RNA-seq of SFAT, EFAT, and BAT of Cth$^{+/+}$ and Cth$^{-/-}$ fed 6 days of CTRL or CysF diet (n=4/group). C) PCA and D) heat map highlighting changes specifically occurring in cysteine deficiency. E) Select top pathways being up- and down-regulated in Cth$^{-/-}$ CysF vs CTRL in the SFAT.
Figure 3. Cysteine deficiency induces thermogenesis in white adipose tissue

A) Heatmap of representative genes involved in thermogenesis that are differentially regulated by cysteine deficiency in the SFAT detected by RNA-seq (n=4/group). B) Percentage of reads coming from nuclear and mitochondrial transcripts. C) Representative images of SFAT and EFAT stained for UCP1 from Cth−/− mice fed CTRL or CysF diet at day 6. D) Western blot detection of UCP1 in the SFAT of Cth−/− mice fed CTRL or CysF diet at day 6. E-F) qPCR analysis of SFAT expression of thermogenic genes after 6 days of CTRL or CysF diet. Statistical differences were calculated by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 8 GAM-clustering analysis of RNA-seq
A-D) High expression is represented as red, low expression is represented as blue. Each column represents a different sample. A) GAM-clustering algorithm was applied to RNA-seq data of BAT, EFAT, and SFAT. 8 different metabolic modules were identified. B) Highlight of subnetworks within module 2 by pathways enrichment analysis. Heat map of representative genes within BCAA degradation, TCA cycle, and FA metabolism are shown. C) Module 4 pathways enrichment analysis identifies cholesterol/steroid biosynthesis as top correlated pathway. Heat map of representative genes within cholesterol biosynthesis are shown. D) Module 6 pathways enrichment analysis identifies lipid metabolism as top correlated pathway. Heatmap of representative genes within lipid metabolism are shown. E-G) Corresponding subnetwork schematic diagrams for Modules 2, 4, and 6. Edges of modules are attributed with color according to correlation of its enzyme’s gene expression to this particular module pattern, and thickness according to its score. Red represents high correlation and green represents low correlation. E) Subnetwork generated for module 2 highlighting several different metabolic nodes such as amino acid metabolism, TCA cycle, FA metabolism. F) Subnetwork generated for module 4, highlighting cholesterol and steroid biosynthesis. G) Subnetwork generated for module 6, highlighting lipid metabolism. (BCAA: Branched chain amino acids; TCA: Tricarboxylic acid cycle; FA: Fatty acid; SAA: Sulfur amino acids; PIP3:Phosphatidylinositol 3,4,5 triphosphate)
Figure 3. 9 Increased production of ophthalmic acid in the SFAT during cysteine deficiency.

A, B, D) Metabolites measured by Orbitrap type mass spectrometry in the SFAT of Cth⁻/⁻ CTRL and Cth⁻/⁻ CysF (n=6/group). Arbitrary units represent area under peak/internal standard/tissue weight. A) Measurements of GSH and GSSG in the SFAT of Cth⁻/⁻ CTRL and Cth⁻/⁻ CysF. B) Measurements of cysteine and cystine in the SFAT of Cth⁻/⁻ CTRL and Cth⁻/⁻ CysF. C) RNA-seq expression of Got1, Gclc, and Gss in the SFAT of Cth⁻/⁻ CTRL and Cth⁻/⁻ CysF. (n=4/group) D) Measurements of OA, 2AB, and threonine in the SFAT of Cth⁻/⁻ CTRL and Cth⁻/⁻ CysF. D) Schematic showing changes in gene and metabolite expression in glutathione and ophthalmic acid synthesis pathway. Significantly increased have red arrows, significantly decreased have blue arrows, and unchanging have green dashes. Each dot represents a single mouse. Statistical differences were calculated by unpaired t-test. *p<0.05, **p<0.01, ***p<0.001. (2OB: 2-oxalobutyric acid; 2AB: 2-aminobutyric acid; Cys: Cysteine; OA: ophthalmic acid; GSH: Glutathione (reduced); GSSG: Glutathione (oxidized); γGlu-Cys: γ-Glutamyl-Cysteine; γGlu-2AB: γ-Glutamyl-2-Aminobutyric acid; AST: Aspartate transaminase (Got1); γGCS: Gamma-Glutamylcysteine synthetase (Gclc); GS: Glutathione synthetase (Gss)).
Figure 3. 10 scRNA-seq of stromal vascular fraction SFAT during cysteine deficiency.

A) Schematic of cell processing of SFAT stromal vascular fraction (SVF) for scRNA-seq. B) t-SNE plot of SFAT stromal vascular fraction with cluster identities. C) Heat map of normalized gene expression of select markers to identify major cell lineages. D) t-SNE plot showing SVF of Cth<sup>+/+</sup> CTRL, Cth<sup>+/+</sup> CysF, Cth<sup>-/-</sup> CTRL, and Cth<sup>-/-</sup> CysF diet. Population statistics of select clusters have been overlayed. E) Bar chart showing population fold changes in relative abundance of each cluster comparing Cth<sup>-/-</sup> CysF vs. Cth<sup>+/+</sup> CysF. F) t-SNE plot displaying Pdgfra expression in red across all populations. G) Monocle analysis clusters 0, 1, and 2, with coloring by pseudotime to show right most cluster giving rise to two separate clusters. H) Monocle analysis with each cluster represented by color in Cth<sup>+/+</sup> CTRL, and Cth<sup>-/-</sup> CysF. I) t-SNE plots displaying Dpp4, C9, Icam1, Col5a3, F3, and Tagln expression in red across all populations in Cth<sup>+/+</sup> CTRL, and Cth<sup>-/-</sup> CysF samples.
Figure 3. 11 scRNAseq of stromal vascular fraction of SFAT during cysteine deficiency.

A) Heatmap of gene expression of select stem and mature adipocyte genes in cells of clusters 0, 1, and 2 in all samples. B) Enrichment of CL-316,243 activated gene set overlaid on all populations in all samples. C) Select top pathways from gene set enrichment comparing Cth−/− CysF vs. Cth+/− CysF in cluster 1. D) Volcano plot of differentially expressed genes (DEGs) comparing Cth−/− CysF vs. Cth+/− CysF in cluster 1. E) Table of number of DEGs per cluster when compared to Cth+/− CysF.
Figure 3. 12 Cysteine deficiency increases sympathetic tone by reducing degradation of norepinephrine.

A) Schematic showing activation of browning in adipose tissue via sympathetic activation and lipolysis. B) Western blot analysis of lipolytic proteins, pHSL and ATGL, in SFAT of Cth+/+ mice fed CTRL or CysF diet at day 6. C) Measurement of norepinephrine by orbitrap MS/MS in the SFAT of Cth+/+ and Cth−/− fed 6 days CTRL or CysF diet (n=5 Cth+/+ CTRL, n=5 Cth+/+ CysF, n=6 Cth−/− CTRL, n=6 Cth−/− CysF). Boxplots of D) Comt and E) Maoa FPKMs from RNA-seq of SFAT (n=4/group). Gene expression of F) Comt and G) Maoa in the SFAT measured by qPCR (n=8 Cth+/+ CysF, n=10 Cth−/− CysF). H) Measurement of COMT by western blot in SFAT. I) SAH measured by orbitrap MS/MS J) Schematic of norepinephrine degradation K) Imaging mass spec of norepinephrine in the BAT. Data expressed as mean±SEM. Statistical differences were calculated unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 13 scRNA-seq of SFAT residential myeloid cells during cysteine deficiency.

A) Schematic depicting cell sorting strategy of SFAT resident myeloid cells for scRNA-seq. B) t-SNE plot of SFAT resident myeloid cells with cluster identities of Cth\textsuperscript{+/+} CTRL, Cth\textsuperscript{+/+} CysF, Cth\textsuperscript{+/−} CTRL, and Cth\textsuperscript{−/−} CysF. B) t-SNE plots of resident myeloid cells from Cth\textsuperscript{−/−} CTRL and Cth\textsuperscript{−/−} CysF with proportional population statistics displayed for each cluster. D) Heat map of normalized gene expression of select markers to identify major cell lineages. E) Enrichment NAM gene signature overlaid on all populations in all samples. F) t-SNE plot displaying Maoa expression in all samples. G) t-SNE plot displaying Cd163 expression in all samples. H) CD11b\textsuperscript{+} F4/80\textsuperscript{+} macrophages with quantification. I) CD163\textsuperscript{+} macrophages with quantification. J) Violin plots of Comt expression within all cells from each group.
Figure 3.14 Analysis of resident macrophage cluster in SFAT during cysteine deficiency.
A) t-SNE plots showing expression of genes enriched in each cluster. B) Volcano plot of DEGs comparing Cth<sup>−/−</sup> CysF vs. Cth<sup>+/+</sup> CTRL in cluster 3. C) Selected top pathways identified by pathways enrichment analysis of cluster 1, comparing Cth<sup>−/−</sup> CysF vs. Cth<sup>+/+</sup> CTRL. D-F) Representative confocal image of wild type -whole mount SFAT, stained for CD163 (yellow), Tubb3 (green), Lyve1 (red), and MAOA (blue). D) NAMs and adipose tissue macrophages near a nerve. Yellow arrow indicates CD163+ adipose macrophage that is not a NAM. White arrows indicate NAMs that stain both CD163 and Lyve1. Red arrow indicates a Lyve1+ macrophage away from the nerve. E) NAM expressing CD163, Lyve1, and intracellular MAOA. F) Adipose tissue macrophage expressing CD163 and Lyve1, and intracellular MAOA. B-D) Image was generously provided by Elsie Gonzalez-Hurtado.
Figure 3. 15 Thermogenesis is induced to maintain core body temperature.

A) Core body temperatures (CBT) measured in the peritoneal cavity by implantation of Star oddi loggers over 6 days of diet in male Cth\(^+/+\) mice fed CTRL or CysF diet. Recordings were taken every 30 min (n=11 Cth\(^+/+\) CTRL, n=12 Cth\(^+/+\) CysF, 3 expts represented). B) Average day and night temperatures quantified.

C) Measurement of BAT temperature, utilizing MRI imaging technique, BIRDS and D) quantification. E-F) Cth\(^+/+\) and Cth\(^+/−\) housed at 22°C or 30°C while on CysF diet for six days. E) Percent body weight curves. F) Representative H&E histology images of SFAT at day6 of CysF diet. G) Expression of thermogenic genes measured by qPCR. Data expressed as mean±SEM. Statistical differences were calculated by (B,E,D) 2-way ANOVA with Sidak’s correction for multiple comparisons, and by (D) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 16 Cth⁻/⁻ Ucp1⁻/⁻ induce reduced thermogenesis in response to cysteine deficiency.

A-D) Female Cth⁻/⁻ and Cth⁺/⁻ Ucp1⁺/⁻ fed a CysF diet for 6 days (n=8 Cth⁻/⁻, n=8 Cth⁺/⁻ Ucp1⁺/⁻). A) Percent body weight, and B) fat mass measured by echoMRI. C) Dissected gonadal, subcutaneous, and brown adipose tissue weights. D) Representative H&E histology images of SFAT after 6 days of diet. E) EE measured in metabolic cages on day 4-5 of CysF diet ((n=8 Cth⁻/⁻, n=8 Cth⁺/⁻ Ucp1⁺/⁻) and F) linear regression analysis of average EE day4-5 against body mass. G) Core body temperatures (CBT) measured in the peritoneal cavity by implantation of Star oddi loggers over 6 days of diet in male Cth⁻/⁻ mice fed CTRL or CysF diet. Recordings were taken every 30min and representative day 4 shown (n=7 Cth⁻/⁻, n=5 Cth⁺/⁻ Ucp1⁺/⁻). Data expressed as mean±SEM. Statistical differences were calculated by (A, G) 2-way ANOVA with Sidak’s correction for multiple comparisons, by linear regression analysis (F), and by (B, C) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. 17 Cth<sup>−/−</sup> Ucp1<sup>−/−</sup> display increased lipolytic and creatine futile cycling signature

A) RER measured in metabolic cages over 6 days of CysF diet (n=8 Cth<sup>−/−</sup>, n=8 Cth<sup>−/−</sup> Ucp1<sup>−/−</sup>) and B) day and night averages quantified. C) Western blot analysis of ATGL, TH, and UCP1 in BAT of Cth<sup>−/−</sup> and Cth<sup>−/−</sup> Ucp1<sup>−/−</sup> fed a CysF diet for 6 days and D) quantified against tubulin. F) Gene expression analysis of thermogenic genes in BAT of Cth<sup>−/−</sup> Ucp1<sup>−/−</sup> fed a CysF diet for 6 days, measured by qPCR. G) Gene expression analysis of creatine futile cycling genes in BAT of Cth<sup>−/−</sup> Ucp1<sup>−/−</sup> fed a CysF diet for 6 days, measured by qPCR (n=8 Cth<sup>−/−</sup>, n=10 Cth<sup>−/−</sup> Ucp1<sup>−/−</sup>). Data expressed as mean±SEM. Statistical differences were calculated by (B, F, G) 2-way ANOVA with Sidak’s correction for multiple comparisons, and by (D, E) unpaired t-test. *p<0.05, **p<0.01, ***p<0.001.
Chapter 4: Dietary regulation of Immunity

The following chapter was published as a review in Volume 53, Issue 3 in *Immunity*, 2020 (Lee and Dixit, 2020). This review was written together with Vishwa D. Dixit.

**Figure 4.1 Dietary Regulation of Immunity**
SUMMARY

Integrated immunometabolic responses link dietary intake, energy utilization, and storage to immune regulation of tissue function and is therefore essential for the maintenance and restoration of homeostasis. Adipose resident leukocytes have non-traditional immunological functions that regulate organismal metabolism by controlling insulin-action, lipolysis, and mitochondrial respiration to control the usage of substrates for production of heat versus ATP. Energetically expensive vital functions such as immunological responses may have thus evolved to respond accordingly to dietary surplus and deficit of macronutrient intake. Notably, chronic positive energy balance manifested as obesity compromises immunity, increasing morbidity and mortality from infections including Covid-19. Conversely, negative energy balance induced by glucose deprivation leads to fatty acid utilization and ketogenesis, promoting longevity and reducing disease burden. Here, we discuss the immunometabolic checkpoints that promote healthspan and highlight how dietary fate and regulation of glucose, fat, and protein metabolism may impact immunity.

Introduction

The hominids that were destined to become humans evolved with larger and intensive energetic demands for brain function. Host survival thus required mechanisms that balance the energetic costs of essential functions such as successful immune response against infections and tissue repair. Accordingly, humans have developed an integrated immunometabolic response (IIMR) that involves sensing of nutrient balance by neuronal (sympathetic and sensory innervation) and humoral signals (e.g., hormones like insulin,
incretins, FGF21, GDF15, ghrelin and leptin) between the hypothalamus and peripheral tissues to allow the host to prioritize storage and/or utilize substrates for tissue growth, maintenance and immune responses. The evolutionary pressure to maintain healthy adiposity concurrently with tissue protective inflammatory responses to injuries and infections are important drivers of IIMR. It has been hypothesized that because energy rich nutrients have been scarce throughout most of human evolution, dominant genetic pathways evolved to favor increasing intake of calorie rich diets and storage of energy as triglycerides in adipose tissue. Within this evolutionary pressure to store calories, consumption of energy rich diets in the modern world has given rise to obesity. Excessive adiposity or obesity with a body mass index (BMI) of > 30, is a multisystem disorder resulting from chronic caloric intake and perturbation of IIMR. A large body of literature suggests that altered IIMR in obesity causes a chronic proinflammatory state that is linked to diseases such as Type-2 diabetes, non-alcoholic steatohepatitis, and cardiovascular disease (Christ et al., 2019). In addition, obesity impairs adaptive immune responses and is a major risk factor for mortality and morbidity from H1N1 influenza (Van Kerkhove et al., 2011).

In opposition to obesity, negative energy balance without malnutrition is induced by restriction of calories, and is one of the most effective means for lifespan and healthspan extension in multiple species (Anderson et al., 2017; Weindruch and Sohal, 1997). Caloric restriction (CR) limits glucose availability and therefore the host must engage fatty acid oxidation to generate ATP. The effector immune functions that rely on glucose as their primary substrates are likely modulated by such dietary interventions and such interventions could be promising avenues to prevent or treat diseases that involve
hyperinflammatory responses. It is recognized that severe reduction in nutrient and energy intake may cause tradeoffs in non-essential functions. As posited by the disposable soma theory, this involves diversion of resources from host growth and reproduction toward cellular maintenance, with the evolutionary rationale that when nutrients become available again, the conserved pathways dedicated for host defense are preserved and can be re-engaged (Kirkwood et al., 2000). Which scenarios induce such a tradeoff and whether the immune system is indeed dispensable when energy is limiting are open questions. However, emerging evidence suggest that immune response to infectious challenges is maintained during moderate dietary restriction (Collins et al., 2019; Jordan et al., 2019; Nagai et al., 2019) and is boosted when carbohydrate deficient, high fat diet intake induces ketogenesis (Goldberg et al., 2019). Surprisingly, restriction of proteins, including the restriction of essential amino acid methionine in adult life extends lifespan in rodents by almost 40-50% (Orentreich et al., 1993; Solon-Biet et al., 2014). The biochemical metabolic pathways engaged by such dietary alterations may hold the keys to identifying as of yet unknown mechanisms that could be harnessed to control reparative responses, inflammation, and immune-surveillance. In this review we discuss the interaction of dietary intake of macronutrients and their metabolic fate on immune system including adipose tissue resident leukocytes in regulation of inflammation and organismal metabolism.

**Immune Defense in Diet Induced Obesity**

Modern diets are rich in saturated fats and processed carbohydrates, such as high fructose corn syrup, and are deficient in fiber, vitamins, and minerals, while containing high levels of salt. These diets are a leading cause of the emergence of obesity associated
chronic diseases, the majority of which are linked to chronic inflammation (Bray et al., 2017). A vast number of studies have described a predominant role of inflammation, originating from visceral adipose tissue and lipotoxicity, in engaging innate immune cells that lead to development of type 2 diabetes through their production of TNFα, IL-1β and IL-6 (Donath and Shoelson, 2011; Hotamisligil, 2006; Saltiel and Olefsky, 2017). There is however, also increasing evidence of obesity as an independent risk factor for dysregulated adaptive immune response against various infections. The retrospective analyses post 2009 H1N1 pandemic across the globe revealed obesity to be comorbid with influenza in nearly one-third of hospitalized patients with increased fatality post infection (Van Kerkhove et al., 2011). The mechanism by which obesity disrupts immune response and recovery from influenza are manifold and represent the impact of chronic metabolic perturbation on multiple cell types. Diet-induced obese (DIO) mice have reduced levels of H1N1-specific antibodies as well as lower neutralization response during influenza infection (Honce et al., 2020). Whether obesity impacts germinal center reactions, class-switching of antibodies, or the B cell repertoire remains unknown. There are data that suggest that obesity restricts T cell receptor (TCR) repertoire diversity (Yang et al., 2010) and decreases the number of T cell receptor excision circles (TRECs) in blood of obese and diabetic individuals suggesting reduced thymic generation of naïve T cells (Yang et al., 2009b).

In addition, the differentiation of naïve T cells (CD4 and CD8) into an effector state upon antigen recognition is dependent on glucose and glutamine (Jacobs et al., 2008; Michalek et al., 2011; Pearce et al., 2009; Wang et al., 2011b). It is thus likely that hyperglycemia and dyslipidemia seen in obesity affects T cell immune activation against
influenza infection. Interestingly, high-fat diet feeding expands γδ T cells in lungs that are IL-17 competent but lack a tissue protective transcriptional profile and fail to confer protection against influenza (Goldberg et al., 2019). Whether altered lipid versus glucose metabolism drives dysfunctional γδ T responses in obesity remains to be characterized.

Furthermore, effective memory T cell response relies on efficient transition from glycolytic to a more oxidative metabolism (Pearce et al., 2009), although this distinction may not be binary in vivo. Recent studies using 13C-based stable isotope labeling techniques in T cells highlight that glucose use by CD8+ cells is dynamically regulated during the course of an immune response to Listeria infection and identified that glucose dependent serine biosynthesis as a key metabolic program for optimal T cell expansion in vivo (Ma et al., 2019). How diet-induced obesity impacts the metabolic reprogramming of T cells in vivo in bacterial or viral infection requires further investigation. Initial analyses suggest that indeed altered glycolytic and oxidative respiration in memory T cells of obese animals is a likely mechanism influencing influenza disease susceptibility (Rebeles et al., 2019). In addition, Mauro et al found that saturated fatty acid-induced metabolic stress affects T cell differentiation and causes preferential trafficking of CD4+ T cells to non-lymphoid effector sites in obesity. Palmitate drove CD4+ T cells to acquire a specific CD44hi CCR7lo CD62Llo CXCR3+ LFA1+ pro-inflammatory dysfunctional phenotype via the activation of a PI3K p110δ-Akt-dependent pathway (Mauro et al., 2017). Interestingly, adipose tissue serves as a reservoir of memory T cells specific to several viruses including but not limited to lymphocytic choriomeningitis virus (LCMV) and HIV (Damouche et al., 2015), as well as bacteria like Yersenia, and protozoa like Toxoplasma (Han et al., 2017). It is possible that antigen-specific T cells may enter the adipose tissue to potentially resolve
the infection but then remain as a memory population distinct from memory T cells in classical sites of immune response. Formal studies would be required to test the hypothesis that adipose microenvironment shapes the specific transcriptional signatures of memory phenotype that is compromised in obesity. It is however known that in obese mice, upon re-challenge to infection, adipose memory T cells upregulate lipases and cause severe disease including calcification of adipose tissue, pancreatitis, and reduce survival (Misumi et al., 2019). Concernedly, data from the COVID-19 pandemic has identified obesity and diabetes as a major risk factor in disease severity and mortality (Drucker, 2020) (Box 1). Given that in the US, over one third of adults are obese, urgent research efforts are required to determine immunoregulatory mechanisms that impact immune response, disease tolerance, treatment, and potential vaccination failure in this large high-risk population.
**Box1 Immunometabolic regulators of COVID-19 severity:** In addition to old age, metabolic syndrome, diabetes, and obesity are independent risk factors for increased SARS-CoV2 induced mortality and morbidity. Given the prevalence of obesity is 10% among younger adults aged 20-39, 45% among adults aged 40-59 years and 43% among older adults aged 60 and over (Hales et al., 2020), there is urgent need for studies that define the mechanism of aberrant immune response that promotes predisposition to infection and disease. Recent analyses show that 47% of cases of COVID-19 hospitalization represented obese patients with BMI >30. Moreover, 85% of patients above the BMI of 35 developed hyperinflammatory response in lungs that required mechanical ventilation to boost oxygen saturation (Simonnet et al., 2020). Macrophage activation and neutrophil influx mediate hyperinflammatory response in lungs in COVID-19 (Zhou et al., 2020). Given obesity’s known effects on increasing inflammation, future studies will be required to determine the immunological mechanism of diet driven complications of SARS-CoV2 viral infection. Of note, NLRP3 inflammasome, a myeloid cell expressed multiprotein complex that senses pathogen associated molecular patterns (PAMPS) and danger associated molecular patterns (DAMPS) to cause IL-1β and IL-18 release, is activated in obesity, type-2 diabetes, atherosclerosis, and aging (Camell et al., 2015; Goldberg and Dixit, 2015). There is increasing evidence that SARS-CoV2 infection activates the NLRP3 inflammasome (Siu et al., 2019) with increased levels of IL-18 and lactate dehydrogenase (LDH) levels due to inflammasome mediated pyroptotic cell death (Lucas et al., 2020; Zhou et al., 2020). Interestingly, SARS-CoV open reading frame 3a (ORF3a) and ORF8b activates the NLRP3 inflammasome (Siu et al., 2019) by inducing ER stress and lysosomal damage (Shi et al., 2019), mechanisms that are independently shown to be elevated in obesity. It is now known that increased glycolysis, which also induces transcriptional upregulation of inflammasome machinery, causes aberrant activation of myeloid cells and worsens COVID-19 (Codo et al., 2020). This raises the question whether dietary approaches that mimic a glucoprivic state by feeding of a low carbohydrate, high fat diet, which necessitates the switch from glycolysis to production of ketone metabolites, can be employed to stave off COVID-19. Indeed, ketone bodies inhibits the Nlpr3 inflammasome (Youn et al., 2015) and ketogenic diet protects against influenza-induced lethality in mice by expanding γδ-T cells (Goldberg et al., 2019). Thus, raising the possibility that harnessing specific immunometabolic checkpoints, such as glycolytic-to-ketogenic switch maybe relevant to several infections, including SARS-CoV2.

**Adipose Tissue as an Immunological Organ**

Of the metabolic organs responding in the IIMR during chronic positive energy balance (Figure 4.2), adipose tissue has been best characterized and strongly contributes to the
pathogenesis of obesity. Obese adipose contains approximately 2 to 5 million stromal-vascular cells per gram, of which approximately 60-70% are immune cells. In severe obesity, adipose tissue can expand to constitute up to 30-50% of total body mass (Kanneganti and Dixit, 2012). Thus, the adipose tissue represents an underappreciated immunological organ that harbors distinct subpopulations of immune cells that localize to specific niches to perform potentially unique functions.

Even before it was recognized that immune function could be regulated by cellular metabolism (Buck et al., 2015; Frauwirth et al., 2002; O'Neill et al., 2016), the discovery that obesity-induced insulin resistance was directly caused by elevated TNF levels in adipose tissues launched the field of immunometabolism, pioneering the association of the immune system with metabolic processes (Hotamisligil et al., 1993). Although, initially thought to be derived from adipocytes, later studies clarified that accumulation of macrophages in the adipose were the main drivers of inflammatory cytokine production (Lumeng et al., 2007b; Weisberg et al., 2003). It has now been elucidated that during obesity, in addition to macrophages, there are large changes in the adipose immune landscape as certain cells are recruited and activated while other cell types are depleted and replaced, leading to a more proinflammatory environment that drives type 2 diabetes and chronic disease progression (Figure 4.3). Mechanistically, proinflammatory cytokines such as TNF, IL-6 and IL-1β have been shown to block insulin receptor signaling through inhibition of insulin receptor substrates (IRSs), by increasing their serine phosphorylation (Hotamisligil et al., 1996; Werner et al., 2004). Conversely, gain of function of IKKβ in the liver was found to reduce ER stress by elevated XBPI activity leading to an actual improvement in insulin-sensitivity (Liu et al., 2016). Additional studies are required to
determine the delicate balance of chronic pro- and anti-inflammatory cytokine signaling required to repair and restore metabolic homeostasis.

**Adipose Tissue Macrophages in Obesity**

It is now understood that macrophages are resident immune cells found in almost every tissue, and play key roles in maintaining homeostasis (Lavin et al., 2014; Okabe and Medzhitov, 2014). In healthy adipose tissue, macrophages are thought to constitute about 10% of the hematopoietic cell composition. However, it is possible that enzymatic digestion protocols of adipose tissue are not releasing all macrophages from their tissue niches and thus current analyses may be underestimating the total quantity of cells residing in fat. During obesity, an infiltration of monocytes and macrophages, local proliferation, reduced egress, and increased cell longevity leads to a further accumulation of macrophages (Amano et al., 2014; Hill et al., 2015; Nagareddy et al., 2014; Oh et al., 2012) so that more than 50% of the cells in the fat depot can be made up of macrophages (Lumeng et al., 2007b; Weisberg et al., 2003).

Proinflammatory ATMs drive obesity induced insulin resistance by activating kinases such as IκB Kinase (IKK) and JNK (Arkan et al., 2005; Han et al., 2013; Solinas et al., 2007). ATMs that accumulate at crown-like structures (CLSs) further secrete exosomes containing miR155 that act on adipocytes to block insulin signaling (Ying et al., 2017). Ablation of proinflammatory CD11c⁺ ATMS during DIO reduces local and systemic proinflammatory cytokine levels, reduces CLSs and infiltrating macrophages, and leads to rapid normalization of insulin sensitivity, thus highlighting the role of inflammatory ATMs in driving metabolic disease (Patsouris et al., 2008). However, ATMs
can be divided into various populations of diverse lineages and functionality that respond dynamically to local metabolic cues within their unique niches. While these macrophage subsets are still being defined, careful examination of the transcriptional profiles of different ATM groups have clarified that there are numerous ways to define macrophage subsets and most infiltrating macrophages in adipose tissue in chronic inflammation do not fit a simple M1/M2 polarization model (Camell et al., 2017; Kratz et al., 2014).

In lean adipose tissue, macrophages are a mixed heterogeneous population of yolk sac derived, self-maintaining macrophages and bone marrow (BM) derived monocytes and macrophages that are continuously replenished (Amano et al., 2014; Hassnain Waqas et al., 2017; Schulz et al., 2012; Silva et al., 2019). Silva et al have recently described 4 different groups of lean visceral ATMs. The group of macrophages which make up the majority of ATMs in lean adipose tissue express high levels of CD206 and are CD11b^+ MHCII^+ Tim4^+ (Silva et al., 2019). Through bone marrow chimera experiments, these macrophages were found to be largely tissue resident with minimal replenishment from the periphery (Amano et al., 2014; Silva et al., 2019). Surprisingly, these macrophages were found to be laden with lipids even in the lean state (Silva et al., 2019). One of the roles of macrophages in lean adipose is to clear dead adipocytes. ATMs form CLSs around dying adipocytes to clear the dying cell and lipids in a contained manner (Murano et al., 2008). Because adipocytes are too large to fully be engulfed by macrophages, ATMs release acidic exosomes laden with lysosomal enzymes that can take up and degrade large adipocyte fragments which can then be taken up by the macrophages in a process called exophagy (Haka et al., 2016). This process allows for macrophages to internalize large amounts of lipids. During obesity, this process becomes increasingly overburdened as there is an
increase in the number of dying adipocytes and CLSs. The accumulation of macrophages in response to high fat diet is mostly attributed to an influx of peripheral BM derived monocytes that quickly differentiate and proliferate locally (Amano et al., 2014; Hill et al., 2018; Jaitin et al., 2019; Nagareddy et al., 2014; Oh et al., 2012; Silva et al., 2019). The macrophages that accumulate with obesity are also heterogeneous and further studies may clarify the specific roles of each subset in disease progression (Hill et al., 2018; Jaitin et al., 2019).

Most of the infiltrating macrophages are found at CLSs and proliferate locally. They are largely CD9+ and are enriched with signatures for lipid metabolism, lysosomal biosynthesis, and phagocytosis (Hill et al., 2018; Jaitin et al., 2019; Xu et al., 2013). Transcriptomic profiling has identified that CD9+ macrophages that accumulate with diet-induced obesity also express CD163 and Trem2. Due to their association with enlarged adipocytes at CLSs, these macrophages have been named lipid associated macrophages, or LAMs (Jaitin et al., 2019). Depletion of TREM2+ LAMs led to reduced lipid uptake by macrophages and increased adipocyte hypertrophy and weight gain, and a worsening of glucose homeostasis, challenging the idea that infiltrating ATMs during obesity purely cause disease progression, but also play critical protective roles in the adaptation to metabolic stresses that occur during obesity (Jaitin et al., 2019).

Adipose tissue is highly vascularized. As adipocytes increase in size and number via hypertrophy and hyperplasia to store excess calories as triglycerides, hypoxia ensues as the diffusion limit of oxygen is reached. Pockets of local hypoxia leads to angiogenesis to meet the oxygen demands required for hypertrophy and increased mitochondrial respiration. Lyve1+ macrophages express MMP-7,-9, and -12 to remodel the ECM and
promote angiogenesis (Cho et al., 2007). In lean adipose, macrophages are found tightly wound around blood vessels as a subset of perivascular macrophages, or PVMs (Hilgendorf et al., 2019; Silva et al., 2019). These self-maintaining PVMs that are in direct contact with the vasculature make up a large proportion of macrophages in healthy adipose and express high levels of CD206 (Silva et al., 2019). Surprisingly, PVMs store lipid droplets even in the lean state, and rapidly take up dextran and OVA from the circulation, suggesting that they are constantly surveilling and buffering lipids within the adipose circulation and may play important roles in presenting antigen and maintaining T cells within the adipose tissue (Silva et al., 2019). During obesity, PVMs change in morphology from an elongated shape to a rounded shape and have a reduction in their capacity to take up dextran, suggesting that their homeostatic functions are diminished in response to high-fat diet (Silva et al., 2019). As PVMs may play important roles as APCs, how this may impact the interaction of PVMs with other immune populations during DIO remains to be elucidated.

Adipose tissue is also densely innervated by both sympathetic and sensory nerves which are important in controlling lipolysis and thermogenesis (Bartness et al., 2014; Chi et al., 2018; Slavin and Ballard, 1978). Density of innervation varies amongst different fat depots, with brown adipose having the most, then subcutaneous adipose depots, and various visceral adipose tissues such as the mesenteric adipose and gonadal adipose tissues. Release of catecholamines such as norepinephrine (NE) by sympathetic nerves is essential for hydrolysis of triglycerides into fatty acids, a process called lipolysis that provides substrates for energetic and synthetic purposes. (Bartness et al., 2010). Interestingly, macrophages also reside on nerves in the adipose tissue as nerve associated macrophages, or NAMs (Camell et al., 2017; Pirzgalska et al., 2017; Wolf et al., 2017). While a
significant portion of brown adipose NAMs are self-maintaining, long lived resident macrophages, white adipose NAMs are more frequently replenished by BM derived macrophages (Pirzgalska et al., 2017; Wolf et al., 2017). NAMs lack tyrosine hydroxylase and therefore do not produce catecholamines (Fischer et al 2017. Pirzgalska et al 2017, Spadaro et al 2017). Instead, they express the canonical catecholamine transporter, Slc6a2, allowing for the uptake of catecholamines from the local environment and express catecholamine degradation enzymes such as monoamine oxidase A (MAOA) and catechol-o-methyl transferase (COMT) to control local availability of catecholamines (Camell et al., 2017; Pirzgalska et al., 2017). Furthermore, the expression of MAOA and COMT in NAMs is upregulated by NLRP3 mediated inflammation during obesity and aging, leading to increased catecholamine degradation by NAMs to contribute to metabolic dysregulation (Camell et al., 2017). How NAMs integrate metabolic and immune sensing in adipose tissue in response to diet is not well understood and is likely to reveal important non-traditional functions of macrophages in maintaining tissue homeostasis.

Impact of Diet Induced Obesity on Adipose Leukocytes

Besides macrophages, many other immune cell types have been described in adipose tissue and in the context of obesity. Adipose tissue is relatively enriched with innate lymphoid cells such as eosinophils, innate lymphoid cells (ILCs), iNKT cells, and γδ T cells and have been well described in other reviews (Kane and Lynch, 2019; Mraz and Haluzik, 2014). In brief, mast cells, ILC1s, γδ T cells, and neutrophils all increase in adipose tissue with obesity and are associated with inflammation. Mast cells increase in the visceral adipose tissue (VAT), particularly around CLSs and actively degranulate to
release TNFα, IL-6, and IFNγ to promote obesity and diabetes (Altintas et al., 2011; Liu et al., 2009). Recently, Zhang et al. have demonstrated that dietary cholesterol leads to activation of mast cells, suggesting mast cells may be directly sensing and responding to dysregulated metabolites (Zhang et al., 2019). Neutrophils are transiently increased during obesity, and are one of the earliest cells to infiltrate adipose tissue and secrete elastase to contribute to pathology (Elgazar-Carmon et al., 2008; Talukdar et al., 2012). ILC1s or NK cells were found to contribute to obesity by producing IL-2 and IFNγ to promote M1 skewing in macrophages, and displayed higher killing activity towards M2 like macrophages over M1 like macrophages (Boulenouar et al., 2017; O'Sullivan et al., 2016). In humans, CD1c+, CD11c+CD83+ DCs increase in obese adipose and induce Th17 differentiation (Bertola et al., 2012).

γδ T cells are tissue resident and increase during diet induced obesity (Mehta et al., 2015). Mice lacking γδ T cells have reduced accumulation of proinflammatory macrophages and significant reductions in systemic insulin resistance, suggesting that γδ T cells play a role in driving the proinflammatory response during diet induced obesity (Mehta et al., 2015). However, γδ T cells may also serve homeostatic functions in healthy adipose tissue as IL-17A production by γδ T cells stimulates the secretion of IL-33 in stromal cells to maintain the expansion of Tregs in adipose tissue during development (Kohlgruber et al., 2018). Conversely, iNKT cells, ILC2s, eosinophils are reduced in the adipose tissue during obesity and their presence is associated with a reduction in metabolic inflammation, increased insulin signaling and promotion of thermogenesis (Lynch et al., 2016; Lynch et al., 2009; Molofsky et al., 2015; Wu et al., 2011).
Besides innate immune cells, B and T cells also become dysregulated during obesity. B cells in healthy adipose tissue are thought to express IL-10 to negatively control adipose tissue inflammation (Nishimura et al., 2013). During obesity, although adipose B are fewer than T cells, there overall numbers are increased and contribute to insulin resistance by secreting autoreactive, pathogenic IgG antibodies and cytokines that drive inflammatory macrophages, Th17 polarization, and CD8+ T cell cytotoxicity (DeFuria et al., 2013; Duffaut et al., 2009; Winer et al., 2011). Cytotoxic CD8 T cells increase with high fat diet even before macrophages accumulate (Rausch et al., 2008) and along with increased Th1 cells contribute to insulin resistance during obesity (Nishimura et al., 2009; Pacifico et al., 2006; Winer et al., 2009). The expansion of T cells before infiltration of peripheral monocytes and macrophages occurs through antigen expression of resident ATMs, especially within CLSs, but the increase in T cells may not be exclusively through clonal expansion during obesity (Morris et al., 2013). Regulatory T cells and iNKT cells largely contribute to the homeostasis of adipose tissue and produce anti-inflammatory factors such as IL-10, IL-4, and TGF-β to promote metabolic homeostasis (Feuerer et al., 2009; Lynch et al., 2012; Schipper et al., 2012). During obesity, both Tregs and iNKT cells are reduced in adipose tissue (Feuerer et al., 2009; Lynch et al., 2009). How many of these immune cells are sensing metabolic dysregulation and macronutrient availability remains to be established.

**Immune Cells Regulate Thermogenesis**

Maintenance of core-body temperature in homeotherms through heat production is a high priority metabolic event that is crucial for survival. The invaginations of the inner
mitochondrial membrane that forms cristae are the sites of oxidative phosphorylation (OXPHOS) complexes that generate ATP (Kozak and Harper, 2000). In addition to ATP synthesis, mitochondrial OXPHOS generates heat as an energy byproduct. To enhance adaptive thermogenesis, adipocytes upregulate the expression of UCP1 (uncoupling protein 1) that forms pores in the inner mitochondrial membrane to allow protons to pass through without producing ATP (Kozak and Harper, 2000). Therefore, in the uncoupled mitochondria, the rate of OXPHOS is significantly boosted and releases heat instead of ATP. While UCP1 was long thought to be the primary mediator of non-shivering thermogenesis, the discovery that UCP1−/− mice are able to adapt to long term cold exposure exposed the possibility of alternative thermogenic mechanisms independent of UCP1 (Ukropec et al., 2006). More recently, studies have revealed that other mechanisms such as futile creatine cycling, sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) mediated calcium cycling, and UCP3 can also contribute to fat thermogenesis (Ikeda et al., 2017; Kazak et al., 2015; Riley et al., 2016).

Macrophages control thermogenesis by controlling the bioavailability of catecholamines. Inhibition of MAOA in macrophages, increases NE content in adipose tissue leading to increased lipolysis and thermogenesis by upregulation of UCP1 (Camell et al., 2017). Moreover, ILC2s, eosinophils, iNK T and γδ T cells have also been implicated in increasing thermogenesis and upregulating UCP1 (Brestoff et al., 2015; Hu et al., 2020; Lynch et al., 2016; Qiu et al., 2014). Notably, FGF21, which gets induced during dietary restriction, can promote the expression of CCL11 by adipocytes to recruit eosinophils, suggesting that immune cells are actively regulated during adaptive adipose tissue remodeling (Huang et al., 2017). Future studies are required to determine whether immune
cells directly affect UCP1 and alternative thermogenic pathways, differentiation of de novo brown/beige adipocytes, or regulate the sympathetic nervous system to control heat production. Identification of such mechanisms may reveal strategies to divert the fate of dietary lipids from storage to heat production through increased mitochondrial uncoupling, thus reducing metabolic diseases emanating from lipotoxicity and obesity.

**Inflammation and Adipose Tissue Fibrosis**

Metabolic perturbations such as diet induced obesity or dietary restriction lead to rapid remodeling of the adipose tissue, where alterations in extracellular matrix (ECM) proteins play an important role in maintaining adipose tissue homeostasis. During obesity, there are dynamic changes in the deposition of ECM components such as collagens, fibrillins, proteoglycans, and specific matrix metalloproteases. While obesity is associated with an excessive accumulation of hypoxia driven fibrosis that directly leads to poor metabolic output (Halberg et al., 2009), the regulation and interaction of adipose ECM and the immune system is still being established. Chronic inflammation had been thought to be the main driver of fibrosis and metabolic dysfunction during obesity, but studies have revealed that fibrosis may precede and occur independently of tissue inflammation (Halberg et al., 2009). Furthermore, repression of fibrosis alone can improve metabolic parameters without largely affecting inflammation (Hasegawa et al., 2018; Khan et al., 2009). However, immune cells may contribute to the continued pathologic fibrosis during chronic obesity. Wang and colleagues have demonstrated that IFNγ producing ILC1s can increase TGF-β output by macrophages to increase fibrosis, while sensing of dying adipocytes and hypoxia by Mincle and HIF1-α in macrophages within CLSs can further drive adipose tissue
fibrosis (Henegar et al., 2008; Tanaka et al., 2014; Wang et al., 2019a). Recent studies suggest that Mincle may potentially be sensing cholesterol moieties released by dying adipocytes, as human Mincle can bind crystalline cholesterol and Mincle was shown to sense cholesterol sulfate in mouse skin (Kiyotake et al., 2015; Kostarnoy et al., 2017). Further understanding of how different ECM components interact with the immune system to mediate adipose homeostasis and pathology remains to be elucidated.

**Obesity is not accelerated aging of adipose resident immune system**

Both obesity and aging are characterized by NLRP3 inflammasome driven low grade chronic inflammation and metabolic dysregulation. Despite these shared similarities, obesity is not an accelerated model of aging. While aging is commonly associated with visceral obesity, there are distinct immunological changes that underlie the pathological disease progression of each condition (Figure 4.3).

In adipose tissue, obesity leads to an increase in most of the defined macrophage subsets (Jaitin et al., 2019; Silva et al., 2019). During aging, however, the opposite trend is observed where macrophage numbers modestly decline (Camell et al., 2017; Lumeng et al., 2011). Age associated VAT ATMs are enriched for NLRP3 dependent senescence and catecholamine catabolism gene signatures, with GDF3 being the most highly upregulated gene with age (Camell et al., 2017). While an accumulation of senescent pre-adipocytes, endothelial cells, and T cells have been described with age in adipose tissue, these signatures have yet to be highlighted in highly proliferative obese ATMs (Salvestrini et al., 2019). Careful investigation of how these newly defined subsets of macrophages and their
niches change with age may help identify therapeutic targets in ameliorating metabolic pathogenesis in aging.

In contrast to the reduction of innate macrophage populations with aging, adaptive B and T cells increase in fat with age (Camell et al., 2019; Lumeng et al., 2011). While there is also an increase in T and B cells in obese adipose tissue, during aging these cells accumulate in specific niches called fat associated lymphoid clusters (FALCs). Hence, FALCs increase their size and number with age (Lumeng et al., 2011). FALCs are a form of non-classical lymphoid structures that are not encapsulated like traditional lymph nodes but can form germinal centers and respond to immunological challenges (Cruz-Migoni and Caamano, 2016). While sterile peritoneal inflammation induced by zymosan increases the size and numbers of FALCs in visceral adipose depots, an increase in FALC formation is not associated with inflamed obese fat (Benezech et al., 2015). During aging, there is a NLRP3 dependent accumulation of adipose tissue resident B cells within FALCs that produce inflammatory mediators such as IL-1β and CCL2. Depletion of adipose B cells in aged mice improves lipolytic signaling and insulin sensitivity, identifying aged adipose B cells as important mediators of age associated insulin resistance, lipolysis resistance, and adipose dysfunction (Camell et al., 2019).

Tregs play important regulatory roles in protecting metabolic homeostasis in the adipose of young animals and their dramatic reduction during obesity contributes to pathology (Feuerer et al., 2009). Conversely Tregs accumulate in adipose with age (Bapat et al., 2015; Lumeng et al., 2011). Unlike young Tregs, the accumulation of aged adipose Tregs drive insulin resistance and depletion of Tregs in aged animals improves metabolic health (Bapat et al., 2015). However, when aged animals are challenged with HFD to
induce obesity, metabolic homeostasis is not maintained by depletion of Tregs, confirming that distinct mechanisms drive the pathophysiology of age and obesity associated insulin resistance (Bapat et al., 2015).

Other adipose resident immune cells which have been characterized in healthy and obese adipose tissue still require additional examination in the context of aging. While eosinophils which are associated with promoting homeostasis in healthy adipose are decreased in obese fat, there seem to be no substantial changes during aging (Bapat et al., 2015; Wu et al., 2011). Innate T lymphocytes such as iNKT cells are decreased during obesity whereas iNKTs significantly increase with age, and γδT cells increase during both obesity and age in the visceral adipose tissue (Kohlgruber et al., 2018). Surprisingly, ILC2s which reside in visceral adipose tissues, including in FALCs, and produce IL-5 and IL-13 to recruit and maintain eosinophils are also reduced with aging (Molofsky et al., 2015; Moro and Koyasu, 2010). In fact, many of the age associated immunological changes that drive metabolic pathology predominantly occur within the visceral adipose. While immune composition remains to be characterized within the BAT, the depletion of macrophages, and accumulation of FALCs and Tregs with age occur mainly in the visceral adipose depots. Similarly, during obesity, changes in pro-inflammatory subsets such as macrophages are much stronger in visceral adipose depots compared to subcutaneous adipose tissue (Weisberg et al., 2003). These depot specific immune perturbations likely drive tissue differences as visceral adiposity is strongly associated with increased risk for metabolic dysfunction and disease whereas SFAT has been considered to be more beneficial by acting as a metabolic sink that can buffer daily influx of nutrients to prevent ectopic lipid storage in visceral organs (Chusyd et al., 2016). As the immune system within
adipose continues to be explored, it is becoming clear there are complex crosstalk between different immune cell types. How these homeostatic interactions become dysregulated during obesity and aging, and their specific tissue niches, remain to be carefully investigated.

**Dietary Protein and Amino Acid Restriction**

While the mechanisms of CR are not completely understood, protein quality and amino acid composition of diet have been more strongly associated with metabolic and age associated health. Independently of caloric intake, low protein and high carbohydrate diets were found to have the strongest extension of lifespan (Solon-Biet et al., 2014). In addition to dietary protein restriction (PR), some studies suggest it is the restriction of essential amino acids (EAAs) that drive beneficial effects, as supplementing dietary restriction with EAAs but not non-essential amino acids were sufficient to reverse prolongevity effects (Grandison et al., 2009; Yoshida et al., 2018). Restriction of specific EAAs, such as the restriction of sulfur-containing amino acids (SAAs), methionine and cysteine, or methionine restriction (MR), and deficiency of the branched chain amino acids, leucine, isoleucine, and valine, or branched chain amino acid restriction (BCAAR), have been extensively characterized to increase metabolic homeostasis and promote healthy aging (Fontana et al., 2016; Lee et al., 2016; Solon-Biet et al., 2014). These dietary interventions commonly lead to reduced adiposity and inflammation, increased insulin sensitivity and lipolytic gene signatures, and reduction of lipogenesis in adipose tissue and liver (Kitada et al., 2019). Although much less characterized, restriction of tryptophan can also lead to increased lifespan and increases recovery to cold stress, suggesting an increase in
thermogenic capacity (Segall and Timiras, 1975, 1976; Zapata et al., 2018). Surprisingly, these studies indicate that deficiencies of specific essential amino acids drive remodeling and programming of metabolic tissues to promote health. Despite numerous studies investigating the mechanisms driving increased longevity during these dietary interventions, alterations to the immune system and their role in tissue reprogramming have been lacking.

One of the pathways that are induced with CR, PR, and amino acid restriction, is the transsulfuration pathway (TSP) (Hine et al., 2015; Mitchell et al., 2016). The TSP involves the metabolism of SAAs and involves the catabolism of methionine to generate intermediates such as the methyl donor S-adenosylmethionine (SAM), S-Adenosylhomocysteine (SAH), homocysteine, and cystathionine. The TSP also allows for the generation of cysteine through the action of cystathionine-γ lyase (CSE) which can further provide important metabolites and byproducts such as glutathione, pyruvate, and hydrogen sulfide (H₂S) (Figure 4.4). Some TSP metabolites such as SAM and homocysteine are increased with obesity and aging and have been implicated with inflammation and disease risk (Elshorbagy et al., 2013; Obata and Miura, 2015; Rodriguez et al., 2006; Vaya et al., 2012).

Another exciting, relevant byproduct of the TSP is H₂S. Hydrogen sulfide is a gaseous signaling molecule produced by three mammalian enzymes, cystathionine-gamma lyase (CSE), cystathionine-beta synthase (CBS), and 3-mercaptoppyruvate sulfurtransferase (MPST), but some studies have shown that H₂S may be produced via non enzymatic reactions (Glorieux et al., 2020). Of note, lifespan extension induced by CR in Drosophila was abolished when treated with the inhibitor of CSE, propargylglycine (PPG) (Kabil et
al., 2011), and overexpression of another TSP enzyme, cystathionine β-synthase (CBS) or treatment with H₂S increased median lifespan in *C. elegans* and yeast respectively, highlighting TSP mediated H₂S production as an important mediator of dietary benefits (Hine et al., 2015). Interestingly, a role for H₂S in immune responses is emerging. In T cells, H₂S is required for the differentiation of regulatory T cells by promoting demethylation at the Foxp3 locus by Tet1 and Tet2 (Yang et al., 2015). An anti-inflammatory role for H₂S has been implicated by its action on NF-κB to regulate its transcriptional activity in macrophages (Sen et al., 2012), and its action on antioxidant gene regulation (Yang et al., 2013). These studies highlight that H₂S may generally promote a more anti-inflammatory phenotype in immune cells. In myeloid cells, H₂S can act specifically to inhibit inflammasome activation (Castelblanco et al., 2018; Lin et al., 2018), which may act as an important mechanism to reduce inflammation and initiate tissue remodeling during dietary interventions. However, studies with H₂S have been largely limited to *in vitro* studies using H₂S donors, inhibitors, or whole-body deletions of TSP enzymes, particularly CSE. CBS deficiency in mice leads to neonatal lethality (Gupta et al., 2009), further complicating the investigation of the role of H₂S in immune cells. These complications have led to numerous contradictory reports of the action of H₂S on immune cells, such as the action of H₂S in regulating leukocyte adherence and tissue infiltration or NLRP3 activation (Basic et al., 2017; Spiller et al., 2010; Zanardo et al., 2006). Cell specific and tissue specific genetic deletion may allow for careful dissection of the role of the TSP in modulating immune response in physiological contexts.

Another pathway that is increased during protein and amino acid restriction is integrated stress response mediated autophagy. The integrated stress response allows for
adaptive responses to various stresses such as starvation and ER stress. Stressors are sensed by four different sensors: general control nondepressible 2 (GCN2), protein kinase R (PKR), haem-regulated inhibitor (HRI), and PKR like endoplasmic reticulum kinase (PERK) (Pakos-Zebrucka et al., 2016). GCN2 senses amino acid depletion by sensing the accumulation of uncharged tRNAs (Gallinetti et al., 2013). HRI recognizes heme deficiency, while PKR senses viral infection through activation by double stranded RNA (Pakos-Zebrucka et al., 2016). Finally, PERK is activated by ER stress (Pakos-Zebrucka et al., 2016). Activation of these sensors are integrated by the activation and phosphorylation of eIF2α leading to global translational arrest (Pakos-Zebrucka et al., 2016). One central component of the integrated stress response is autophagy (Kroemer et al., 2010). In addition to GCN2 sensing of uncharged tRNAs, depletion of EAAs leads to interaction of their specific sensors such as SAMTOR (SAM sensor), sestrin 2 (leucine sensor), and CASTOR (arginine sensor) with GATOR1 or GATOR2 to prevent mTORC1 activation and promote autophagy (Kim and Guan, 2019) (Figure 3). Interestingly, autophagy and amino acid sensing also regulates immune function. It is now well established that autophagy inhibits inflammasome activation (Sun et al., 2017). In a model of colitis, PR and leucine restriction were able to reduce inflammation through the inhibition of NLRP3 inflammasome mediated inflammation and reduction of Th17 differentiation. This response was dependent on amino acid sensing through GCN2 (Ravindran et al., 2016). Furthermore, treatment with halofuginone, which activates starvation responses by inhibiting prolyl tRNA charging, also inhibits Th17 cell differentiation (Sundrud et al., 2009). These studies highlight the induction of amino acid starvation responses in immune
cells as potential strategies against inflammation driven pathological states such as obesity, aging, and autoimmunity.

While these different dietary amino acid restrictions may be inducing some common pathways, specific amino acids may target different pathways. Unlike leucine or BCAA restriction diets, MR also induces hepatic FGF21 and lipolytic gene signatures independently of GCN2 (Lees et al., 2017). Instead, MR specific hepatic changes are driven by PERK and glutathione depletion (Wanders et al., 2016). Thus, different mechanisms may drive adaptations to specific amino acid deficiencies in tissues and immune cells (Figure 3). Despite numerous studies investigating the broad metabolic adaptations that occur during PR, how immune cell function is being altered have been lacking. A plethora of early studies have investigated immune defense in the context of protein malnutrition, suggesting alterations in both macrophage and lymphocytic responses to infection. However, these studies were conducted prior to the classification of broad immune subsets and advancement of technological tools and need to be revisited to properly understand how the immune system may be responding during PR without malnutrition in the context of both tissue adaptation and immune defense.

**Integrated Immunometabolic Response to Carbohydrate and Caloric Restriction**

In an event of low glucose availability, such as food restriction, the limited glycogen reserves in the liver and muscle cannot sustain nonessential metabolic demand. Instead, triglycerides undergo fatty acid oxidation, ketogenesis, and ketolysis to support ATP production.
It is known that long-term CR lowers inflammation and protects against thymic lipoatrophy and maintains T cell repertoire during aging (Yang et al., 2009a). Intriguingly, however, CR causes a major redistribution of circulating leukocytes. A two-year multicenter randomized controlled trial where healthy humans achieved approximately 14% CR (CALERIE-II) provided evidence that CR reduces the number of circulating lymphocytes and monocytes without compromising vaccine responses or increasing susceptibility to infections (Meydani et al., 2016). Similarly, studies from Jordan et al found that 19hr of fasting in humans led to reduction of monocytes and dendritic cells in blood but instead increased retention of pro-inflammatory Ly6C$_{hi}$ monocytes in the BM (Jordan et al., 2019). In agreement with prior studies (De Rosa et al., 2015) fasting reduced systemic inflammation in a mouse model of multiple sclerosis. Importantly, monocytes could still respond and control *Listeria monocytogenes* infection during fasting in a PPAR$_{\alpha}$ dependent manner (Jordan et al., 2019). Similarly, negative energy balance mediated reduction in mTOR also led to CXCL13 driven homing of naïve B cells from Peyer’s patches to BM. Moreover, Collins et al found that mice subjected to 50% dietary restriction for 3 weeks caused a loss of peripheral memory CD8 T cells and their surprising accumulation in the BM. Markedly, this redistribution of B and memory T cells led to enhanced protection against secondary bacterial infection (Collins et al., 2019). It is intriguing that during energy deficit, memory T cells do not prefer adipose tissue that is undergoing increased lipolysis with abundant fatty acids as energy substrates, and home instead to BM. CR’s salutary effects on immune system and control of inflammation require further characterization. From the current evidence, switch from glycolysis to fatty acid oxidation appears to be an important immunometabolic checkpoint affected by CR.
For example, production of ketone bodies like β-hydroxybutyrate downstream of fatty acid oxidation, inhibits Nlrp3 inflammasome mediated inflammation and protects against influenza infection by expanding tissue protective γδ T cells in lung and adipose tissue (Goldberg et al 2019). Whether low carbohydrate high fat ketogenic diet that increases fatty acid oxidation and BHB can be harnessed to protect against inflammatory damage, as in Covid-19 immunopathology, requires careful testing. The endogenous immunometabolic mediators that drive CR salutary effects thus offer a promising avenue to regulate inflammation and enhance healthspan.

**Concluding Remarks**

Significant progress over the last decade has firmly established that reciprocal interactions between immune and metabolic systems are required for the host to maintain homeostasis. Immunometabolism, not only encompasses immune cell intrinsic regulation of glycolysis, TCA cycle, pentose phosphate pathway, and fatty acid metabolism to impact inflammation and effector functions, but also entails immune regulation of systemic whole-body metabolism and coupling of positive and negative energy balance to successful immune response. As outlined in this review, the integrated immunometabolic response is in fact vital for regulation of organismal metabolism and host defense. A major influencer of IIMR is the diet. It is evident that metabolic fate of fat, protein and carbohydrates is important for immunologic function. However, how macronutrient intake elicits specific pathways in a host to either induce disease or promote health and longevity is only beginning to be understood. In addition, both immune response and metabolism are regulated by the autonomic nervous system which are not under our conscious control. The neural
regulation of immunometabolism, by which the autonomic nervous system integrates the central nervous system, immune system, and metabolic organs to maintain essential functions and prepare the host for surmounting stressful challenges, is not well understood. Thus, study of Neuro-Immunometabolism may reveal new mechanisms to promote health and understand disease. Identification of immune cells like macrophages in sympathetic nerve niches in adipose tissue represents one mode of neuroimmunometabolic crosstalk that controls inflammation and lipolysis setpoints. These advances have set the stage for the next era of immunometabolism studies where regulation of diet driven metabolic checkpoints are likely to be revealed and potentially implemented in clinical practice to promote health.
Negative energy balance is achieved with energy consumed is less than energy expended. This drives neuroendocrine hormone effectors such as ghrelin, FGF21, and GDF15, and the usage of alternative metabolic fuels such as fatty acids and ketone bodies. These signals get integrated by organs such as the brain, liver, adipose tissue, gut, and skeletal muscle with the immune system to promote metabolic reprogramming that prioritizes fatty acid oxidation over glycolysis. These changes adjust the balance of normal physiological processes such as body temperature, growth, reproduction, and immune response in a way that promotes longevity and health.
Figure 4.3 Obesity and Aging lead to Distinct changes in the immune profile of adipose tissue to drive inflammation and pathology.
Healthy, lean adipose tissue is associated with anti-inflammatory immune subsets, such as CD206 expressing macrophage subsets (Adipose tissue macrophages (ATMs), nerve associated macrophages (NAMs), and vascular associated macrophages (VAMs)), IL-4 expressing eosinophils, IL-10 expressing iNKT cells, B cells, and Tregs, and IL-5 expressing ILC2s. Energy imbalance leads to a disruption of immune and metabolic homeostasis, leading to an infiltration of inflammatory immune cells such as IL-6 and TNFα producing lipid associated macrophages (LAM), elastase producing neutrophils, IgG2C producing B cells, degranulating mast cells, IFNγ producing CD4 T cells, IL-17 producing γδ T cells, and cytotoxic CD8 T cells. Accumulation of danger associated molecular patterns (DAMPs) such as free fatty acids (FA), oxidized LDL (oxLDL), cholesterol crystals, and islet amyloid polypeptides (IAPP) in obesity further drives the activation of NLRP3 inflammasome. These changes in immune mediated inflammation drive pathologies such as insulin resistance, type II diabetes, cardiovascular disease, and cancer, while weakening immune defense against pathogens, leading to higher risk for premature death. Unlike obesity, which is characterized by an accumulation crown like structures (CLS) where immune cells such as T cells and CD9+ macrophages, associated with increased lipid processing and inflammation, accumulate to deal with dying adipocytes, aging sees a reduction of adipose tissue macrophages, which instead increase GDF3 and catecholamine degradation enzymes such as MAOA and COMT to promote lipolysis resistance. Aging also leads to an increase of fat associated lymphoid clusters (FALCs) where B cells and T cells accumulate and contribute to dysregulated adipose homeostasis such as reduced insulin signaling and thermogenesis. While γδ T cells increase with both age and obesity, iNKT cells and Tregs increase with age and decrease with obesity, and while no marked changes in eosinophils are reported in aged adipose, obesity is associated with significant decreases in eosinophil populations. These differences in immune profiles and immune lymphoid structures during aging and obesity suggest that distinct mechanisms contribute to the pathologies associated with each condition.
Figure 4. Protein and essential amino acid restriction induces the transsulfuration pathway and the integrated stress response to regulate immune response.

Induction of the transsulfuration pathway leads to production of byproducts such as hydrogen sulfide, which can reduce NLRP3 inflammasome mediated inflammation and promote and maintain regulatory T cell differentiation. Low amino acid availability leads to the interaction of amino acid sensors such as Sestrin 2 and SAMTOR with GATOR2 and GATOR1 respectively to restrict mTORC1 activation. Furthermore, sensing of uncharged amino acids by GCN2 and lack of glutathione by PERK further inhibits mTORC1 and leads to the activation of eIF2α, stress response genes, and autophagy, inhibiting NLRP3 inflammasome mediated inflammation and Th17 responses.
Chapter 5: Summary and Perspectives

This project began with the aim to identify novel mechanisms that mediated the health benefits during caloric restriction (CR), a pro-longevity intervention. Specifically, we sought to identify novel therapeutic targets to reduce inflammation and improve metabolic health, because many chronic diseases associated with aging and obesity are caused by inflammation-driven metabolic pathology. And while dietary interventions such as CR or protein restriction are successful in reducing inflammation and promoting weight loss and improved metabolic function, they are untested in humans and are extremely difficult to implement in everyday life. We thus investigated transcriptional and metabolic changes in adipose tissue from healthy adults that underwent 2 years of 14% CR (Ravussin et al., 2015), Dixit, unpublished). Through investigation of human caloric restriction, our studies identify that defense of cysteine availability by the transsulfuration pathway is a critical immune-metabolic checkpoint. We find that during caloric restriction, the TSP is induced in response to a depletion of cysteine and its downstream metabolites. Induction of the TSP in turn, can reduce inflammation through the production of H$_2$S. We show that H$_2$S can inhibit the activation of NLRP3 inflammasome, through inhibition of the assembly of NLRP3 inflammasome complex. Furthermore, reduction in sulfur amino acid availability can reduce inflammasome activation by mediating p62-independent autophagy in macrophages. Indeed, the health benefits of methionine restriction (MR), another pro-longevity intervention are blocked by cysteine supplementation, suggesting that limited cysteine availability underlies the benefits of restrictive dietary pro-longevity interventions (Elshorbagy et al., 2011).
Importantly, we generated a model of cysteine deficiency to discover that cysteine availability is required for the defense of core body temperature, and deficiency of cysteine drives unchecked thermogenesis to the point of mortality. Thermogenesis is activated through increased sympathetic signaling that drives lipolysis and UCP1 to generate heat. We discovered that ablation of CSE together with dietary restriction of cysteine in mice causes a complete transformation of the white adipose tissues into a thermogenic brown-like depot. This process involved increased mobilization of adipocyte progenitors and loss of monocyte-derived macrophages. Concurrently, tissue resident macrophages were enriched, with reduced expression of catecholamine degradation enzymes such as COMT, which increases norepinephrine availability to drive adipose tissue browning. While two other groups have previously investigated $Cth^{-/-}$ mice in the context of low cysteine diets (Ishii et al., 2010; Mani et al., 2011), we discovered time that cysteine deficiency mobilizes adipose to generate heat due to increased pressures to defend core body temperature.

This discovery may have therapeutic implications in a few clinical settings. The inhibition of CSE together with restriction of cysteine using specific prescribed diets can serve as a therapeutic strategy to activate thermogenesis as a short-term weight loss intervention in obese patients under a supervised clinical setting. Preliminary experiments suggest that even in the context of high fat diet, cysteine deficiency causes weight loss in $Cth^{-/-}$ mice. While long-term cysteine deficiency is not sustainable due to life-threatening unchecked weight loss, short-term cysteine deficiency through inactivation of the TSP may allow for quick weight loss in specific situations, such as weight loss for surgery. Alternatively, cysteine supplementation may help with unwanted weight loss during specific conditions such as cachexia. The increase in BCAA degradation and threonine in
adipose tissue suggests that skeletal muscle degradation may provide additional amino acid sources for mitochondrial respiration in the adipose tissues. Early studies by Droge and colleagues found that low plasma cystine levels were found in several diseases such as late-stage HIV infection, cancer, sepsis, Crohn’s disease, and major injury and trauma and is associated with skeletal muscle wasting (Droge et al., 1998; Hack et al., 1997). This process has been described as the “Low CG Syndrome” where plasma cysteine, glutamine, and glutathione levels are abnormally low. Supplementation of N-acetyl-cysteine has been a successful therapeutic strategy in the context of HIV (Droge and Holm, 1997; Sekhar et al., 2015). Cysteine supplementation may thus serve as a mechanism to combat cachexia in a number of other diseases. Further investigation on how different metabolic organs such as adipose tissue and skeletal muscle are responding to cysteine deficiency and communicating with each other may allow for the untangling of fat and lean mass loss to provide more precise therapeutic targets in the context of weight loss and cachexia.

In conclusion, we discover that maintenance of cysteine metabolism by the TSP is important for inflammatory and metabolic homeostasis. Further investigation of how adaptations to cysteine deficiency are integrated in different metabolic organs may allow for identification of novel therapeutic strategies.
Chapter 6: Materials and Methods

Human Samples
The participants in this study were part of the CALERIE Phase 2 (Rochon et al., 2011) study which was a multi-center, parallel-group, randomized controlled trial by recruitment of non-obese healthy individuals. 238 adults participated at 3 different locations: Pennington Biomedical Research Center (Baton Rouge, LA), Washington University (St. Louis, MO) and Tufts University (Boston, MA) (NCT00427193). Duke University, (Durham, NC) served as a coordinating center. Participants were randomly assigned to of 25% caloric restriction or ad libitum caloric intake for two years. CR group participants actually reached 14% of CR (Ravussin et al., 2015). Men were between 20 and 50 years old and women were between 20 and 47 years old. Their body mass index (BMI) was between 22.0 and 27.9 kg/m$^2$ at the initial visit. Samples were collected at baseline, 1 year, and 2 years of intervention. Abdominal subcutaneous adipose tissue biopsy was performed on a portion of CR group participants and used for RNA-sequencing and metabolomics in this study. Only 8 participants completed all three time points. All studies were performed under protocol approved by the Pennington institutional review board with informed consent from participants.

Mice
All mice were on the C57BL/6J (B6) genetic background except methionine restriction cohorts, which were on a C57BL/6M background from the NIA. B6 mice were purchased from Jackson Laboratories and maintained in our lab. $Cth^+$ mice (C57BL/6NTac-Cth$^{tm1a(EUCOMM)Hmgulreg}$) were purchased from the European Mouse Mutant Cell Repository. Breeding these mice to Flipase transgenic mice from Jackson Laboratories generated $Cth^{fl/fl}$
mice which were crossed to Adipoq-cre and Albumin-cre, purchased from Jackson Laboratories. Nlrp3<sup>A350VneoR</sup> and Tg(CAG-(cre/Esr1*)5Amc (CAG-ERcre) were purchased from Jackson laboratories and crossed to generate MWS model mice. Ucp1<sup>−/−</sup> mice were purchased from Jackson laboratories and crossed to Cth<sup>−/−</sup> mice. Fgf21<sup>−/−</sup> mice were kindly provided by Dr. Steven Kliewer (UT Southwestern) as described previously (Potthoff et al., 2009), and crossed to Cth<sup>−/−</sup> mice. 17-18 month animals were provided by the NIA. All mice used in this study were housed in specific pathogen-free facilities in ventilated cage racks that deliver HEPA-filtered air to each cage with free access to sterile water through a Hydropac system at Yale School of Medicine. Mice were fed a standard vivarium chow (Harlan 2018s) unless special diet was provided, and housed under 12 h light/dark cycles. All experiments and animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University.

**Diet studies**

For methionine restriction (MR) studies, diets were purchased from Dyets (Appendix 1). C57BL/6M mice were started on control or MR diets at 8wks old or 17-18 months old and were kept on diet for 3 months. For LPS studies, adult C57BL/6J were maintained on control or MR diet for 3 months.

Mice were acutely injected with LPS (Sigma) i.p. at a dose of 2mg/kg for 4 hours. Blood glucose (Breeze) and rectal core body temperature (Physitemp) were measured before and after injection.
*Nlrp3*<sup>A350VneoR</sup> CAG-ER<sub>cre</sub> mice were fed control or MR diet for 1 week prior to i.p. injection of tamoxifen (Sigma). Tamoxifen was injected for 2 consecutive days at 50mg/kg per day while being maintained on diet.

Adipose samples from protein restricted (PR) mice were kindly provided by Dr. Christopher Morrison (Pennington University). Control and PR diets were produced by Research diets, and were designed to be isocaloric by equaling varying protein and carbohydrate, while keeping fat constant. Control diet contained 20% casein by weight, while PR diet contained 5% casein. Mice were maintained on diet ad libitum for 6 months.

For cysteine deficiency and leucine deficiency studies, mice were fed special amino acid diets: control diet, CysF diet, or LeuF diet purchased from Dyets (Appendix 2), for 6 days unless specified otherwise. Prior to amino acid diets, mice were fed standard vivarium chow (Harlan 2018s). Mice were started on amino acid diets between 3-6 months of age. For pair feeding studies, mice were provided with either ad libitum or 2.22-2.27g of diet daily.

**Cell culture**

All steps were performed using sterile technique. To prepare BMDMs, femurs and tibias were collected in RPMI (Life Technologies) + 10% FBS (Omega Scientific). Both ends of the femur were then cut and the femur was flushed with RPMI. The bone marrow was centrifuge at 450 g for 5 min, the supernatant was decanted and red blood cells were lysed using ACK lysis buffer (Quality Biological). After neutralization, bone marrow cells were centrifuged, resuspended in 10 ml of RPMI and placed into one well of a 6 well plate. Non-adherent cells were collected the following morning. The non-adherent cells were
resuspended in media consisting of 10 ml supernatant of non-adherent cells, 14.4 ml L929 conditioned media, 13.6 ml RPMI and MCSF (10ng/ml). An additional 2 ml of fresh media was added 4 d after isolation. Non-adherent cells were collected on day 7 and resuspended at 1x10^6 cells/ml and allowed to adhere overnight.

For M1 polarization, cells were treated with LPS at 1ug/ml + recombinant IFNg (Ebioscience) at 20ng/ml for 24 hours. For M2 polarization, cells were treated with recombinant IL-4 (BD) for 24 hours. For inflammasome activation, cells were treated with ultrapure LPS (Sigma) at 1ug/ml for 4 hours alone or subsequently with 5mM ATP (Sigma) for one hour. For hydrogen sulfide treatment experiments, cells were treated with sodium hydrosulfide (Sigma) at 0-100uM at the time of LPS treatment. For methionine and cysteine restricted experiments, before LPS treatment, cells were washed in PBS and switched to DMEM lacking methionine and cysteine and glutamine. Glutamine () was added at 2.5mM, and L-methionine (Sigma) and L-cysteine (Sigma) were each added at 200uM per well. 3-methyladenine (Sigma) was treated at 5mM at the time of LPS treatment. LysM-cre p62^fl/fl femurs were kindly provided by Dr. Michael Karin, and were received overnight in RPMI. All following steps were done identically as described above. BMDMs from Nlrp3^A350VneoR mice were prepared as described above. The day before inflammasome activation, the cells were treated with 4-hydroxy-tamoxifen (4-OHT) at 0.25mM.

**Potassium efflux assay**

On the day before inflammasome activation, cells were plated 100,000 cells per well of a 96 well plate in 200ul. Conditions were consistent as above. After all cell treatments, media
was changed to fresh media and Asante potassium green 2 (APG4) (Abcam) was added at 5uM and incubated at 37°C for 30min. After 2 washes in PBS, the plate was read in 50ul of PBS according to manufacturer’s directions.

**Thioglycolate**

Mice were injected with a one-time 1ml intraperitoneal injection of 3% thioglycolate medium (BD). 4 days later, peritoneal cells were collected by peritoneal lavage.

**Western blot analysis**

Cell lysates were prepared using RIPA buffer and optionally frozen and stored at -80°C. Samples were left on ice, vortexing every ten min for 30 min. For tissue samples, snap frozen tissues were ground by mortar and pestle in liquid nitrogen and resuspended in RIPA buffer with protease and phosphatase inhibitors. Samples were centrifuged at 14,000g for 15min and the supernatant was collected protein concentration was determined using the DC Protein Assay (Bio-Rad) and transferred to a nitrocellulose membrane. The following antibodies (and source) were used to measure protein expression: IL-1β (Genetex), NLRP3 (Adipogen), β-Actin (Cell Signaling), pHSL p660 (Cell Signaling), ATGL (Cell Signaling), UCP1 (Abcam), CSE (Novus), Caspase-1 (generously provided by Genentech), Tubulin (Sigma), HSL (Cell Signaling), COMT (Biorad), MAOA (Abcam), TH (Cell Signaling); followed by incubation with appropriate HRP-conjugated secondary antibodies (Thermo).
**Gene expression analysis**

Cells or ground tissue (described above) were collected in STAT-60 (). RNA from cells were extracted using Qiagen RNase micro kits following manufacturer’s instructions. For tissue samples, RNA was extracted using Zymo mini kits following manufacturer’s instructions. During RNA extraction, DNA was digested using RNase free DNase set (Qiagen). Synthesis of cDNA was performed using iScript cDNA synthesis kit (Bio-Rad) and real time quantitative PCR (Q-PCR) was conducted using Power SYBR Green detection reagent (Thermo Fischer Scientific) on a Light Cycler 480 II (Roche). Primers used for qPCR are described in Appendix 3.

**Glucose tolerance tests/ Insulin tolerance test**

Mice were fasted 14hr prior to glucose tolerance test. Glucose was given by i.p. injection based on body weight (0.4g/kg). For insulin tolerance test, mice were fasted for 4 hours and insulin was given by i.p. injection based on body weight (0.8U/kg). Blood glucose levels were measured by handheld glucometer (Breeze, Bayer Health Care).

**Multiplex**

Serum was prepared by collecting blood and allowing to clot at room temperature for 2 hours. Samples were then centrifuged for 20 min at 4°C and stored at -80°C until analysis. Inflammatory cytokines, IL-6, MCP-1, IL-1β, and TNFα, were measured in the serum using custom Procartaplex kit (Life Technologies), and measured on a Luminex 200 analyzer.
Flow Cytometry

Intravascular labeling was performed by iv injection of 2.5ug CD45.2-FITC diluted in 100ul PBS. Mice were euthanized exactly 3 minutes after injection for tissue collection. Adipose tissue was digested in HBSS (Life Technologies) + 0.1% collagenase I or II (Worthington Biochemicals). The stromal vascular fraction was collected by centrifugation, and washed and filtered using 100um and 70um strainers. Cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) and then for surface markers including CD45, CD3, B220, CD11b, F4/80, Ly6G, Siglec F, CD163 and all antibodies were purchased from eBioscience or Biolegend. Cells were fixed in 2% PFA. Samples were acquired on a custom LSR II and data was analyzed in FlowJo.

Single-cell RNA sequencing

For stromal vascular fraction, female Cth+/+ and Cth−/− mice were fed CTRL of CysF diet for 4 days. SFAT was collected, with lymph nodes removed, pooled, and digested. For myeloid cells, Cth+/+ and Cth−/− mice were fed CTRL of CysF diet for 3 days. Intravascular labeling was performed by i.v. injection of 2.5ug CD45.2 diluted in PBS for 3 min. SFAT was collected, with lymph nodes removed, pooled, digested, and stained for live/dead, CD11b, CD45, and dump (CD3, B220, Siglec F). Cells were sorted on BD FACSARia, for CD45+ CD11b+ cells that were negative for CD45.2 and the dump channel. Isolated cells were subjected to droplet-based 3’ end massively parallel single-cell RNA sequencing using Chromium Single Cell 3’ Reagent Kits as per manufacturer’s instructions (10x Genomics). The libraries were sequenced using a HiSeq3000 instrument (Illumina). Sample demultiplexing, barcode processing, and single-cell 3’ counting was performed.
using the Cell Ranger Single-Cell Software Suite (10x Genomics). Cellranger count was used to align samples to the reference genome (mm10), quantify reads, and filter reads with a quality score below 30. The Seurat package in R was used for subsequent analysis (Butler et al., 2018). Cells with mitochondrial content greater than 0.05 percent were removed and data was normalized using a scaling factor of 10,000, and nUMI was regressed with a negative binomial model. Principal component analysis was performed using the top 3000 most variable genes and t-SNE analysis was performed with the top 20 PCAs. Clustering was performed using a resolution of 0.4. The highly variable genes were selected using the FindVariableFeatures function with mean greater than 0.0125 or less then 3 and dispersion greater than 0.5. These genes are used in performing the linear dimensionality reduction. Principal component analysis was performed prior to clustering and the first 20 PC’s were used based on the ElbowPlot. Clustering was performed using the FindClusters function which works on K-nearest neighbor (KNN) graph model with the granularity ranging from 0.1-0.9 and selected 0.4 for the downstream clustering. For identifying the biomarkers for each cluster, we have performed differential expression between each cluster to all other clusters identifying positive markers for that cluster. To perform pathway analysis, we compared differential gene expression parameters (0 fold and threshold of at least 10% of cells) between samples. Gene-set enrichment analysis (GSEA) was performed using the FGSEA package in R with the canonical pathways (CP). To understand the trajectory of the adipocyte progenitors, we used Monocle2 to analyze scRNA-seq data of Clusters 0, 1, and 2 (Trapnell et al., 2014).
**Whole tissue RNA sequencing and transcriptome Analysis**

Snap frozen tissues were ground by mortar and pestle in liquid nitrogen and resuspended in STAT-60. RNA was extracted using Zymo mini kits. RNA was sequenced on a HiSeq2500. The quality of raw reads was assessed with FastQC [FastQC]. Raw reads were mapped to the GENCODE vM9 mouse reference genome [GENCODE] using STAR aligner [STAR] with the following options: --outFilterMultimapNmax 15 --outFilterMismatchNmax 6 --outSAMstrandField All --outSAMtype BAM SortedByCoordinate --quantMode TranscriptomeSAM. The quality control of mapped reads was performed using in-house scripts that employ Picard tools [Picard]. The list of rRNA genomic intervals that we used for this quality control was prepared on the basis of UCSC mm10 rRNA annotation file [UCSC] and GENCODE primary assembly annotation for vM9 [GENCODE]. rRNA intervals from these two annotations were combined and merged to obtain the final list of rRNA intervals. These intervals were used for the calculation of the percentage of reads mapped to rRNA genomic loci. Strand specificity of the RNA-Seq experiment was determined using an in-house script, on the basis of Picard [Picard] mapping statistics. Expression quantification was performed using RSEM [RSEM]. For the assessment of expression of mitochondrial genes, we used all genes annotated on the mitochondrial chromosome in the GENCODE vM9 mouse reference genome [GENCODE]. PCA was performed in R. For the PCA, donor effect was removed using the ComBat function from the sva R-package [sva]. Gene differential expression was calculated using DESeq2 [DESeq2]. Pathway analysis was done using fgsea (fast GSEA) R-package [fgsea] with the minimum of 15 and maximum of 500 genes in a pathway and with 1 million of permutations. For the pathway analysis, we used the Canonical Pathways
from the MSigDB C2 pathway set [MSigDB1, MSigDB2], v6.1. The elimination of redundant significantly regulated pathways (adjusted p-value < 0.05) was done using an in-house Python script in the following way. We considered all ordered pairs of pathways, where the first pathway had normalized enrichment score equal to or greater than the second pathway. For each ordered pair of pathways, we analyzed the leading gene sets of these pathways. The leading gene sets were obtained using fgsea [fgsea]. If at least one of the leading gene sets in a pair of pathways had more than 60% of genes in common with the other leading gene set, then we eliminated the second pathway in the pair.

GAM-clustering, identifies modules describing dynamic regulation of metabolism and is based on the previously developed GAM method (Sergushichev et al., 2016). GAM-clustering extends the GAM method by setting the task to find not one but several metabolic modules (connected subnetworks of metabolic network) with the condition that each of these modules should contain as many metabolic genes with high pairwise correlation of their expression as possible. The initial approximation of the final set of modules is carried out by k-medoids clustering of a gene expression matrix for all metabolic genes of a dataset with some arbitrary k (here we used k=32). Each cluster forms a corresponding expression pattern which can be determined as averaged value of its z-normalized gene expression values. The metabolic network used for further analysis is presented as a graph where vertices are metabolites and edges are KEGG database reactions which are mapped with catalyzing them enzymes and corresponding genes. For each particular pattern edges of this graph are scored (weighted) based on their gene expression similarity with this pattern and dissimilarity with other patterns.
For each case of weighted graph, a connected subgraph of maximal weight is found by a signal GMWCS (generalized maximum weight connected subgraph) solver (Ulland et al., 2017) (https://github.com/ctlab/sgmwcs-solver) and is called a metabolic module. This solver uses the IBM ILOG CPLEX library, which efficiently performs many iterations of this method in a reasonable amount of time. Then, each pattern is updated by replacing it with an averaged gene expression of the module’s edges with a positive score. If the pattern is changed, a new score set is calculated and a new iteration is performed. Before moving to the next iteration, small graphs are eliminated from further analysis so that there are no graphs with less than five edges and diameter less than four in the output solution. The algorithm continues until the pattern content stops changing (Gainullina et al., 2020).

Metabolomics and Lipidomics

Metabolite extraction from tissue blocks for metabolome analyses was performed as described previously (Miyazawa et al., 2017). Briefly, one frozen block together with internal standard (IS) compounds (see below) was homogenized in ice-cold methanol (500μl) using a manual homogenizer (Finger Masher (AM79330), Sarstedt), followed by the addition of an equal volume of chloroform and 0.4 times the volume of ultrapure water (LC/MS grade, Wako). The suspension was then centrifuged at 15,000 g for 15 min at 4°C. After centrifugation, the aqueous phase was ultrafiltered using an ultrafiltration tube (Ultrafree MC-PLHCC, Human Metabolome Technologies). The filtrate was concentrated with a vacuum concentrator (SpeedVac, Thermo). The concentrated filtrate was dissolved in 50μl of ultrapure water and used for LC-MS/MS and IC-MS analyses.
We used both internal (added to the tissue before extraction) and external (used to produce calibration curves for each compound) standard compounds for concentration calculation. The detailed method is as follows:

**Internal standard (IS) compounds**

We used 2-morpholinoethanesulfonic acid (MES) and 1,3,5-benzenetricarboxylic acid (trimesate) as ISs for anionic metabolites. These compounds are not present in the tissues; thus, they serve as ideal standards. Loss of endogenous metabolites during sample preparation was corrected by calculating the recovery rate (%) for each sample measurement.

**External standard (ES) compounds**

An external calibration curve was used to calculate the absolute abundance of metabolites. Before sample measurement, we measured the mixture of authentic compounds of target metabolites at three different concentrations in ultrapure water to generate calibration curves. Quantification (amount of metabolites, nmol/mg tissue) was performed by comparing the IS-normalized peak areas against the calibration curves.

**Ion chromatography-tandem mass spectrometry for anionic metabolites**

For metabolome analysis focused on glucose metabolic central pathways, namely glycolysis, TCA-cycle and PPP, those anionic metabolites were measured using an orbitrap-type MS (Q-Exactive focus, Thermo Fisher Scientific, San Jose, CA), connected to an high performance ion-chromatography system (ICS-5000+, Thermo Fisher Scientific) that enables us to perform highly selective and sensitive metabolite quantification owing to the IC-separation and Fourier Transfer MS principle (Hu et al., 2015).
The IC was equipped with an anion electrolytic suppressor (Thermo Scientific Dionex AERS 500) to convert the potassium hydroxide gradient into pure water before the sample enters the mass spectrometer. The separation was performed using a Thermo Scientific Dionex IonPac AS11-HC, 4-μm particle size column. IC flow rate was 0.25 mL/min supplemented post-column with 0.18 mL/min makeup flow of MeOH. The potassium hydroxide gradient conditions for IC separation are as follows: from 1 mM to 100 mM (0–40 min), 100 mM (40–50 min), and 1 mM (50.1–60 min), at a column temperature of 30°C. The Q Exactive focus mass spectrometer was operated under an ESI negative mode for all detections. Full mass scan (m/z 70–900) was used at a resolution of 70,000. The automatic gain control (AGC) target was set at 3 × 10^6 ions, and maximum ion injection time (IT) was 100ms. Source ionization parameters were optimized with the spray voltage at 3 kV and other parameters were as follows: transfer temperature at 320°C, S-Lens level at 50, heater temperature at 300°C, Sheath gas at 36, and Aux gas at 10.

**Liquid chromatography-tandem mass spectrometry for amino acid measurement**

The amount of cationic metabolite including amino-acids in the tissues was quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, a triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source (LCMS-8040, Shimadzu Corporation) was used in the positive and negative-ESI and multiple reaction monitoring (MRM) modes. The samples were resolved on the Discovery HS F5-3 column (2.1 mmI.D. x 150 mmL, 3μm particle, Sigma-Aldrich), using a step gradient with mobile phase A (0.1% formate) and mobile phase B (0.1% acetonitrile) at ratios of 100:0 (0–5 min), 75:25 (5–11 min), 65:35 (11–15 min), 5:95 (15–20 min) and
100:0 (20–25 min), at a flow rate of 0.25 mL/min and a column temperature of 40°C. MRM conditions for each amino acids are listed in Appendix 4.

**Liquid chromatography-mass spectrometry for lipid measurement**

The amount of lipids in the tissues was measured using an orbitrap-type MS (Q-Exactive focus, Thermo Fisher Scientific, San Jose, CA) connected to an high performance liquid chromatography system (UltiMate 3000 RSLC system, Thermo Scientific).

Briefly, the samples were resolved on the Accucore C18 column (2.1 x 150 mm, 2.6 μm, Thermo Scientific), using a step gradient with mobile phase A (10 mM HCOONH4 in 50% ACN (v) + 0.1% HCOOH (v)) and mobile phase B (2 mM HCOONH4 in ACN / IPA / H2O 10:88:2 (v/v/v) + 0.02% HCOOH (v) ) at ratios of 65:35 (0 min), 40:60 (0–11 min), 15:85 (11–12 min), 0:100 (12–21 min), at a flow rate of 0.4 mL/min and a column temperature of 35°C.

The Q Exactive focus mass spectrometer was operated under an ESI positive mode for all detections. Full mass scan (m/z 100–1200) was used at a resolution of 70,000. The automatic gain control (AGC) target was set at 3 × 10⁶ ions, and maximum ion injection time (IT) was 100 ms. Source ionization parameters were optimized with the spray voltage at 3 kV and other parameters were as follows: transfer temperature at 285°C, S-Lens level at 45, heater temperature at 370°C, Sheath gas at 60, and Aux gas at 20.

**Imaging Mass Spectrometry**

Tissues were sectioned at 8-μm thick and thaw-mounted on a conductive indium-tin-oxide (ITO)-coated glass slides (Matsunami Glass Industries, Osaka, Japan). The samples were
measured by a MALDI-Fourier transformation ion cyclotron resonance (FT-ICR) mass spectrometer (soliRX XR 7T, Bruker Daltonics), or a linear ion trap (LIT) mass spectrometer (LTQ XL, Thermo Fisher Scientific). The raster scan pitch was set depending on the experiment from 30 to 150 µm. Data were acquired from m/z 286.65 to 450.00 (for FT-ICR data) in positive ion mode. For LIT data, signals of 5-HT-DPP (m/z 391 > 232) were monitored with a precursor ion isolation width of 1.0 m/z and a normalized collision energy of 45%. Ion images were reconstructed with mSIQuant 2.0.1.1466, ImageQuest 1.1.0 software (Thermo Fischer Scientific) the FT-ICR data, and the LIT data, respectively. After finishing the measurement, the sections were stained by H&E stain to annotate the regions.

MRI-BIRDS temperature mapping

The animals were anesthetized with isoflurane, first at 3% isoflurane in an induction chamber and then at 2-3% during surgery. The animal was laid back on a microwaveable heating pad. Prior to incision, a single dose of bupivacaine was given for analgesia. A 1-2 cm midline incision was made on the neck to expose the jugular vein. Another small incision (<1 cm) was made at the back of the neck. A sterile polyurethane or silicone catheter with a metal guide was inserted from the back of the neck, where the vascular port was fixed to the jugular vein. Prior to implantation the port and the catheter were flushed with heparinized saline (25 IU/ml). The jugular vein was catheterized toward the heart. The skin was closed with surgical sutures after application of triple antibiotic ointment and the vascular port was fixed. The duration of the surgical procedure was 15-20 min. For MR data acquisition, TmDOTMA was purchased from Macrocyclics (Plano, TX, USA).
Temperature mapping with BIRDS was performed on a 9.4T Bruker scanner (Billerica, MA). The respiration rate was monitored during the entire duration of the experiment. A 200mM TmDOTMA− solution was infused at a rate of 60 to 80 µl/h for 1 to 2 hours. The infusion rate was adjusted according to animal physiology. The T2 weighted magnetic resonance (MR) images were acquired with an FOV of 23x23mm², 128x128 matrix, 23 slices of 0.5mm thickness, TR=3s and TE=9ms. The extremely short T1 and T2 relaxation times (<5ms) of the TmDOTMA− methyl group allowed ultrafast temperature mapping with BIRDS using 3D chemical shift imaging (CSI) acquisition with a short TR (10ms) and wide bandwidths (±150ppm). Temperature mapping with BIRDS was started immediately after detection of global MR signal of TmDOTMA− methyl group, at about 1 hour after the start of the infusion. The CSI was acquired using a FOV of 23x15x23mm³, 809 spherical encoding steps, 21min acquisition, and reconstructed to 23x15x23, with a voxel resolution of 1x1x1mm³. Selective excitation of the TmDOTMA− methyl group was achieved using a single band 200µs Shinnar-Le Roux (SLR) RF pulse. The MR spectrum in each voxel was line broadened (200 Hz) and phased (zero-order) in Matlab (MathWorks Inc., MA, USA), and the corresponding temperature Tc was calculated from the chemical shift δCH₃ of the TmDOTMA− methyl group according to

\[ T_c = a_0 + a_1(\delta_{\text{CH}_3} - \delta_0) + a_2(\delta_{\text{CH}_3} - \delta_0)^2 \]

where \( \delta_0 = -103.0 \) ppm and the coefficients \( a_0 = 34.45 \pm 0.01, a_1 = 1.460 \pm 0.003 \) and \( a_2 = 0.0152 \pm 0.0009 \) were calculated from the linear least-squares fit of temperature as a function of chemical shift \( \delta_{\text{CH}_3} \) (Coman et al., 2010).
Temperature Loggers

Animals were anesthetized with isoflurane, first at a rate of 2-3% and maintained at 0.5-2% in oxygen during surgery. Mice were kept on a heating pad throughout surgery. Mice were injected with buprenorphine and bupivacaine as pre-emptive analgesia. A small ventral incision of 1cm was made after clipping hair and disinfection with betadine and 70% ethanol. DST nano-T temperature loggers (Star Oddi) were placed in the peritoneal cavity, and abdominal muscle and skin were sutured closed. Post-surgery, mice were singly housed and provided with Meloixcam for 48 hours. After 7 days, sutures were removed. 10 days after surgery, mice were started on CTRL or CysF diet, and loggers were removed for data collection after euthanization. Loggers were programmed to take temperature readings every 30 minutes.

Metabolic cages

The energy expenditure (EE), respiratory exchange ratio (RER), activity, food intake and water consumption of mice were monitored using the TSE PhenoMaster System (V3.0.3) Indirect Calorimetry System. Each mouse was located in individual chambers for 3 days for acclimation, and switched to experimental diet for 6 days. Each parameter was measured every 30 min. EE and RER were calculated based on the oxygen consumption (O$_2$) and carbon dioxide production (CO$_2$). Mouse activity was detected by infrared sensors, and food intake and water consumption were measured via weight sensors on food and water dispensers located in the cage.
**EchoMRI**

The parameters of body composition were measured in vivo by magnetic resonance imaging (EchoMRI; Echo Medical Systems). The amount of fat mass, lean mass and free water were measured by the analysis. For the analysis, each mouse was placed in an acrylic tube with breathing holes and the tube was inserted in the MRI machine. The analysis per mouse takes approximately 90 sec and automatically calculated numerical results were analyzed.

**Climate chambers**

Mice were acclimated in climate chambers at either 30°C or 22°C, with humidity maintained at 50% under 12 h light/dark cycles. After one week acclimation, mice were switched to custom amino acid diets, either CTRL or CysF diet for 6 days, while maintained in the climate chambers. Mice were handled daily to measure body weight.

**Histology**

Tissues were collected in 10% formalin, embedded in paraffin and sectioned into 5um thick sections. Tissues were stained with hematoxylin and eosin (H&E), or stained for UCP1 (Abcam) and Goat anti-rabbit HRP (DAKO) and developed for color using Abcam DAB substrate kit.

**Quantification and statistical analysis**

Statistical differences between groups were calculated by unpaired 2-tailed t-tests. For comparing groups over time, mice were individually tracked and groups were compared
using paired 2-way ANOVA with Sidak’s correction for multiple comparisons. For all experiments a p-value of $p \leq 0.05$ was considered significant.
## Appendix 1: Chapter 2 Custom Amino Acid Diets

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### Appendix 3: qPCR primers

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tissue macrophages promote myelopoiesis and monocytosis in obesity. Cell Metab 19, 821-835.


