Methylation Controlled J Protein Is A Master Regulator Of Mitochondrial Metabolism

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METHYLATION CONTROLLED J PROTEIN IS A MASTER REGULATOR OF MITOCHONDRIAL METABOLISM

A Dissertation Presented

by

Devin Pierre Champagne

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The Faculty of the Graduate College

of

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ABSTRACT

Methylation controlled J protein (MCJ) is a negative regulator of mitochondrial metabolism that has a substantial impact on overall cell metabolism and function. MCJ is highly expressed by naïve CD8+ T cells, however its role in their immune effector functions was unknown. In this dissertation, it will be demonstrated that MCJ restricts the mitochondrial metabolism of CD8+ T cells, in part by reducing respiratory supercomplex formation. MCJ deficiency enhances the immune effector functions and memory responses of CD8+ T cells in a mitochondrial ATP dependent manner. As a consequence, protection to influenza virus infection is substantially improved. Reduced expression of MCJ therefore promotes viral immunity, however the loss of MCJ is not always beneficial. In cancer, decreased MCJ expression is correlated with ATP binding cassette (ABC) transporter mediated chemotherapy resistance and poor patient responses. This dissertation will also address the role of MCJ in chemoresistance. Increased mitochondrial ATP production due to MCJ deficiency is sufficient to fuel ABC transporter activity, thereby directly promoting chemoresistance. This can be reversed by restoration of MCJ function in chemoresistant cells. Overall, the results presented in this dissertation identify MCJ as a potential therapeutic target, as modulating MCJ expression can significantly affect the severity of viral infections and the responses to chemotherapy.
CITATIONS

Material from this dissertation has been published in the following form:

DEDICATION

To my parents, Tilden and Rachel,

and the Chihuahua that simply did not make any sense, Sissy.
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CHAPTER 1. Comprehensive Literature Review

1. Introduction to metabolism

One of the major characteristics that has been proposed to delineate life from nonlife is metabolism (Koshland, 2002; McKay, 2004), which is the ability to perform the chemical reactions necessary for the production and breakdown of biomolecules, the conversion of nutrients and food sources into usable energy, and the elimination of waste byproducts. In order for an organism to maintain homeostasis, regulatory mechanisms must exist to control the flow of metabolites through the catabolic and anabolic reactions that are performed by cells. To facilitate these metabolic reactions, all mammalian cells ultimately depend on the production of the energy carrier adenosine triphosphate (ATP), of which the majority is synthesized via glycolysis, the tricarboxylic acid cycle (TCA), and oxidative phosphorylation.

1.1. Glycolysis

Glycolysis occurs in the cytosol and functions to break down carbohydrates for the production of ATP and the reducing equivalent NADH (Berg et al., 2002; Voet and Voet, 2011). The most common carbohydrate utilized by cells is glucose, a six-carbon simple sugar that is ubiquitously used as a fuel source and molecular building block. Glucose enters cells through one of 14 known glucose transporters that are differentially expressed throughout the body (Thorens and Mueckler, 2010). Once inside the cell, glucose is readily phosphorylated by the enzyme...
hexokinase to glucose 6-phosphate. The negatively charged phosphate group prevents glucose from exiting the cell and also facilitates entry into glycolysis.

The reactions of glycolysis are broadly characterized into two groups, the preparatory and the payoff phases (Figure 1-1) (Berg et al., 2002; Voet and Voet, 2011). The preparatory phase reactions break down the carbons of glucose into two molecules of glyceraldehyde 3-phosphate and require the hydrolysis of two preformed molecules ATP. In the payoff phase, glyceraldehyde 3-phosphate is ultimately converted into pyruvate. In the process four ATP and two NADH molecules are produced, therefore the overall yield of glycolysis per molecule of glucose metabolized is two molecules each of ATP, NADH, and pyruvate.

Figure 1-1. The reactions of glycolysis
Preparatory phase reactions, red; payoff phase reactions, blue. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F-1,6-BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 1,3-BP, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; HK, hexokinase; PI, phosphoglucone isomerase; PFK-1, phosphofructokinase 1; F-BA, fructose-bisphosphate aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PPH, phosphopyruvate hydratase (enolase); PK, pyruvate kinase.
The activity of glycolysis is regulated by several mechanisms (Berg et al., 2002; Voet and Voet, 2011). The amount of glucose entering cells, and thereby the overall cytosolic concentration, is controlled by the expression and activity of glucose transporters (GLUT) at the cell surface (Thorens and Mueckler, 2010). Reactions that are highly thermodynamically favorable are essentially irreversible, and the enzymes catalyzing them are under reversible allosteric regulation. This is generally due to downstream metabolic intermediates that serve as feedback inhibitors or activators. The rate limiting step of glycolysis is catalyzed by phosphofructokinase (Bosca and Corredor, 1984). Metabolic enzymes are also regulated by posttranslational modifications such that signals from other metabolic pathways or extracellular cues can alter the flux through glycolysis (Wiese and Hitosugi, 2018).

Additionally, transcriptional regulation can limit or increase enzyme abundance in response to major changes that substantially alter metabolic needs (e.g., cell growth and division). In addition, GAPDH can bind the 3’ UTR of some RNAs, thereby regulating protein translation (White and Garcin, 2016). The time required for each of these regulatory mechanisms varies significantly from milliseconds to hours, however all play roles in controlling the flux of intermediates through glycolysis.

While glycolysis is not substantially efficient in terms of net ATP production as the yield is only two ATP per glucose molecule entering the pathway, it is carbon sparing and produces intermediates that can be shuttled to
other metabolic pathways (Berg et al., 2002; Voet and Voet, 2011). The pentose phosphate pathway uses glucose 6-phosphate to generate NADPH and five carbon sugars needed for nucleotide synthesis. Glucose 6-phosphate is also used for the production of glycogen, which predominantly occurs in the liver and skeletal muscle. Glyceraldehyde 3-phosphate is a substrate used for triglyceride and lipid synthesis. The glucose dependent production of the amino acid serine requires 3-phosphoglycerate. In addition, pyruvate serves as a metabolic branchpoint as it can be transaminated to alanine for amino acid synthesis, carboxylated to oxaloacetate for use in the TCA cycle or gluconeogenesis, reduced to lactate (with the corresponding oxidation of NADH) to maintain cellular redox balance, or further oxidized in the mitochondria.

1.2. Mitochondrial respiration

In organisms that can perform aerobic respiration, the complete oxidation of carbon substrates and the production of ATP ultimately occurs via the reactions of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) in the mitochondria.

1.2.1. Tricarboxylic acid cycle

The TCA cycle is a set of cyclic reactions that integrates intermediates from a number of metabolic pathways including the breakdown products of carbohydrates, amino acids, and fatty acids (Figure 1-2) (Berg et al., 2002; Voet and
Voet, 2011). The net yield of one round of the TCA cycle is one GTP (equivalent to one ATP), three NADH, one FADH$_2$, and two CO$_2$ molecules.

Carbons derived from glucose enter the TCA cycle in the form of acetyl-CoA, which is also the final break down product of fatty acid oxidation (Berg et al., 2002; Voet and Voet, 2011). Fatty acids are a significant source of energy and consist of an aliphatic chain of varying length bound to a fully oxidized carbon in
the form of a carboxylic acid. Saturated fatty acids contain no double bonds between the carbon atoms of the chain (*i.e.*, they are saturated with hydrogen atoms), while unsaturated fatty acids contain one or more double bonds in either a *cis* or *trans* configuration.

The breakdown of fatty acids occurs mainly in the mitochondrial matrix via the process of β-oxidation. Fatty acids are also metabolized to a lesser degree in peroxisomes (Poirier *et al.*, 2006). Similar to glycolysis, β-oxidation is tightly regulated by different mechanisms (Eaton, 2002; Schulz, 1994). The majority of fatty acids in the body are stored in adipocytes, which, along with the liver, control their availability to other cells. Skeletal muscle also serves as a depot for fatty acid storage (Berg *et al.*, 2002; Voet and Voet, 2011). Allosteric regulation plays a major role in regulating β-oxidation, with a notable example being the potent inhibition of the carnitine shuttle by malonyl-CoA (Foster, 2012). Additionally, the overall abundance of mitochondria varies between cell types based on metabolic needs, which limits the overall capacity for β-oxidation (and mitochondrial respiration in general) (D'Erchia *et al.*, 2015).

Amino acids are also a significant fuel source used by mammalian cells (Berg *et al.*, 2002; Voet and Voet, 2011). These biomolecules consist of a central carbon atom bound to an amine (-NH₂), a carboxylic acid (-COOH), and a variable side chain (R group). The side chains found in eukaryotes differ in length, charge, and polarity, all of which provide different chemical properties that are exploited
by cells. In addition, the amine functional group is a weak base that can be protonated at cytosolic pH to produce a positively charged ammonium group (\(-\text{NH}_3^+\)). Likewise, the carboxylic acid group can be deprotonated to become a negatively charged carboxylate (\(-\text{COO}^-\)). Amino acids are therefore zwitterions that exist in a pH-dependent equilibrium between all three possible charge states (neutral, negative, and positive). The functional groups of some side chains can also be neutral or charged, which adds further chemical complexity.

Excess amino acids that are not used for protein synthesis or other biological processes (e.g., neurotransmitter synthesis) are metabolized into intermediates that can directly enter the TCA cycle (Figure 1-3). While most amino acid catalysis pathways result in the formation of acetyl-CoA, succinyl-CoA, fumarate, oxaloacetate, and α-ketoglutarate are also produced. In rapidly dividing cells (e.g., cancer cells and proliferating lymphocytes), the breakdown of the amino acid glutamine via glutaminolysis is a major energy production pathway (Figure 1-4) (Newsholme, 2001; Yang et al., 2017). In addition, several amino acids can be readily converted to others through the action of transaminases such as the conversion of glutamate to alanine or aspartate (Figure 1-4).
Figure 1-3. Amino acid catalysis
Like glycolysis, the TCA cycle integrates metabolites from other metabolic pathways and as such, functions as a metabolic hub within the cell (Berg et al., 2002; Voet and Voet, 2011). A notable export is citrate, which can be shuttled out of mitochondria for fatty acid synthesis. Additionally, oxaloacetate can be used in a number of pathways including amino acid and fatty acid synthesis. Imports include fumarate, which is produced by the urea cycle, and as noted above, the final products of amino acid catabolism.

The TCA cycle is regulated by allosteric regulation in response to cellular energy charge (Berg et al., 2002; Voet and Voet, 2011). As such, ATP and NADH are negative regulators, while ADP is a positive regulator. In addition, NAD⁺ and FAD are key substrates for several reactions that are readily available during times of low energy charge. The production of α-ketoglutarate is the irreversible rate

Figure 1-4. The reactions of glutaminolysis

GDH, glutamate dehydrogenase; GPT, glutamate pyruvate transaminase; GOT, glutamate oxaloacetate transaminase.
limiting step of the TCA cycle. Unlike glycolysis, the TCA cycle is not carbon sparing as two carbons are lost as CO₂ during each cycle, however the NADH and FADH₂ produced are used in the synthesis of substantial amounts of ATP via oxidative phosphorylation.

1.2.2. Oxidative phosphorylation

Oxidative phosphorylation (OXPHOS) is facilitated by the large, multi-subunit protein complexes of electron transport chain (ETC), which are integrated into the inner mitochondrial membrane (Berg et al., 2002; Voet and Voet, 2011). Through a series of coordinated redox reactions, the ETC ultimately transfers electrons from NADH and FADH₂ to oxygen, which is the final electron acceptor in mammals. In the process, protons are pumped across in the inner mitochondrial membrane into the inner membrane space (IMS) to facilitate the synthesis of ATP.

Figure 1-5. Reactions and complexes of the electron transport chain
Adapted from Sazanov (2015).
Complex I (NADH : ubiquinone oxidoreductase) is the largest of the ETC complexes at ~970 kDa and transfers the electrons from NADH to Coenzyme Q$_{10}$ (CoQ). In the process, four protons are translocated from the matrix to the IMS. The quaternary structure of Complex I is L-shaped and consists of 45 individual subunits in mammals (Sazanov, 2015). It is organized as a core of 14 subunits that are conserved from bacteria surrounded by a shell of 31 supernumerary subunits. A notable example is NDUFv1, which serves as the NADH binding site (Deng et al., 1990).

While the structure of Complex I has been studied for decades, it generally the least understood as only partial structures were known until the recent publication of the nearly complete ovine Complex I at high resolution (3.9 Å) (Fiedorczuk et al., 2016). Complex I has been shown to exist in two distinct conformations – the catalytically active (A) form and the inactive dormant (D) form (Babot et al., 2014). It is still unclear, however, what regulates the transition between the A and D forms as well as the specific mechanism(s) connecting proton translocation and electron transfer.

Since Complex I catalyzes the first set of ETC reactions, deficiencies can lead to severe dysfunction in mitochondrial metabolism and cause significant pathology (Scheffler, 2015). Tissues that are most sensitive to Complex I dysregulation are the liver, kidney, muscle (both skeletal and cardiac), and those of the nervous system. As such, patients experience a myriad of symptoms such as lactic acidosis, myalgia, ataxia, epilepsy, and vision problems (Scheffler, 2015).
In addition to overall deficiencies in mitochondrial respiration, defects in or dysregulation of Complex I activity result in increased levels of mitochondrial reactive oxygen species (ROS) (Vinogradov and Grivennikova, 2016; Wirth et al., 2016). Excess ROS production can lead to a breakdown in cellular redox potential and is a major source of oxidative stress (Schieber and Chandel, 2014). In disease states such as ischemia reperfusion injury, Complex I plays a predominant role in ROS production via reverse electron transport (RET) (Chouchani et al., 2014; Scialo et al., 2017).

Complex II (succinate dehydrogenase) is the smallest ETC complex at ~120 kDa and is comprised of four subunits (Sun et al., 2005). Complex II reduces CoQ via the oxidation of FADH$_2$. Unlike the other members of the ETC, Complex II does not translocate protons into the IMS. Additionally, it catalyzes the conversion of succinate to fumarate and thus plays a direct role in the TCA cycle. Deficiencies in Complex II can also lead to mitochondrial disorders and have been implicated in disease pathology (Jain-Ghai et al., 2013).

The lipid soluble CoQ formed by Complexes I and II travels through the inner mitochondrial membrane to Complex III (Coenzyme Q : cytochrome c oxidoreductase). Complex III is an ~240 kDa dimer of two identical monomers that are each comprised of 11 subunits each and catalyzes the transfer of electrons from CoQ to cytochrome c (Iwata et al., 1998). Each electron transferred to cytochrome c results in one proton being translocated into the IMS. Complex III is also a major
site of mitochondrial ROS production, and deficiencies are common causes of mitochondrial disorders (Benit et al., 2009).

Reduced cytochrome c migrates to Complex IV (cytochrome c oxidase), which catalyzes the final redox reactions of the ETC. Complex IV is a homodimer and is ~400 kDa in size (Tsukihara et al., 1996). There are 13 subunits in each monomer. Complex IV transfers two electrons from cytochrome c to oxygen, producing water. In the process, two protons are translocated into the IMS. Similar to the other complexes, deficiencies in Complex IV activity can result in ROS production and mitochondrial disorders (Rak et al., 2016).

The protons transferred to the IMS cannot freely diffuse back into the matrix. As such, an electrochemical gradient is produced across the inner membrane as the IMS and matrix become positively and negatively charged, respectively. According to the chemiosmotic theory (Mitchell, 1961), the potential energy of this gradient – known as the proton motive force – is used by ATP Synthase (also known as Complex V) to facilitate the oxidative phosphorylation of ADP to ATP.

1.2.3. Respiratory supercomplexes

The macromolecular organization of the electron transport chain has been an intense area of research for decades. Two models of the supramolecular organization have been proposed that are effectively direct opposites of one another – the solid and fluid state models (Figure 1-6). The solid state model posits
that the respiratory complexes associate to form large aggregates that coordinate the reactions of the ETC (Keilin and Hartree, 1947). The aggregates are proposed to contain pathways that direct the movement of CoQ and cytochrome c between the individual complexes. In contrast, the fluid state model posits that individual complexes freely diffuse throughout the inner mitochondrial membrane and that CoQ and cytochrome c are not restrained to specific paths (Hackenbrock et al., 1986).

The fluid model was generally accepted until analyses of mitochondrial extracts using blue native gel electrophoresis (BNE) showed that the complexes of the ETC can organize into supramolecular structures known as respiratory supercomplexes (Schagger and Pfeiffer, 2000). It was originally thought that supercomplexes were simply artifacts of the mild detergent solubilization employed by BNE, however their presence has been confirmed using other experimental methods (Acin-Perez et al., 2008; Dudkina et al., 2005). In addition, crystal structures of mammalian respiratory supercomplexes were recently solved (Gu et al., 2016; Letts et al., 2016).
There is still debate in the literature over the specific purpose(s) served by supercomplexes (Milenkovic et al., 2017). Several proposed benefits to cells have been suggested including decreased production of ROS, regulation of ETC activity, and prevention of protein aggregation in the mitochondrial matrix (Milenkovic et al., 2017). Despite a lack of definitive evidence for these proposals, there have been several reports showing a significant correlation between supercomplex abundance and increased mitochondrial respiration. Additionally, increased ROS production has been detected after chemical disruption of supercomplexes containing Complexes I and III (Maranzana et al., 2013). Future studies are needed to elucidate the mechanistic role of respiratory supercomplexes in the regulation of mitochondrial metabolism.
1.3. Urea cycle

In addition to the production of usable energy, mitochondria are vital to the removal of ammonia from cells (Berg et al., 2002; Voet and Voet, 2011). Ammonia is a toxic byproduct of amino acid breakdown that is ultimately excreted as urea in ureotelic organisms. The conversion of ammonia to urea is facilitated by the urea cycle, which primarily occurs in the liver and kidneys and is regulated by substrate availability. The urea cycle is a highly energy demanding process as it requires the hydrolysis of three ATP molecules, one of which is fully hydrolyzed to AMP. This cyclic set of five reactions starts in the mitochondria and also has cytosolic components (Figure 1-7).

Like other pathways, the urea cycle produces intermediates that can be shuttled to other pathways (Berg et al., 2002; Voet and Voet, 2011). Citrulline is used in the synthesis of polyamines, while arginine has multiple fates including protein synthesis, production of the signaling and immune effector molecule nitric oxide (NO), and the synthesis of other amino acids. Additionally, fumarate can be oxidized in the TCA cycle. The final step of the cycle yields urea, which is ultimately excreted in urine.
2. Introduction to cancer biology

Cancer is a disease characterized by abnormal cell growth that can disrupt the normal functions of the healthy tissues and cells of the body. In addition, the process of metastasis induces the spread of these altered cells from their site of development to other regions of the body. Cancer is currently the second leading cause of death in the U.S., and approximately of 40% of Americans will experience a cancer diagnosis during their lifetime according to the National Cancer Institute. While nearly all tissues and cells of the body can become cancerous, certain tissues are more susceptible to transformation (Table 1-1).
Table 1-1. Incidence and death rates of the most common cancers in the U.S.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Incidence</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>123.6</td>
<td>20.9</td>
</tr>
<tr>
<td>Prostate</td>
<td>114.9</td>
<td>19.5</td>
</tr>
<tr>
<td>Lung</td>
<td>61.5</td>
<td>43.4</td>
</tr>
<tr>
<td>Colorectal</td>
<td>39.8</td>
<td>14.5</td>
</tr>
<tr>
<td>Uterine</td>
<td>25.9</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Rates are per 100,000 individuals according to the American Cancer Society.

Cancer diagnoses are graded based on disease progression and are staged according to criteria defined by the American Joint Committee on Cancer. The scale used varies by cancer type, however they are all based on the extent to which healthy tissue has been affected. The breast cancer system starts with Stage 0, where a benign growth is localized and not yet considered cancerous. Tumors that have invaded healthy breast tissue or local lymph nodes are considered cancerous and graded as Stage I, II, or III depending on the extent of spread. Stage IV breast cancer has metastasized to other parts of the body, represents the most progressed form of the disease, and is often the most difficult to treat. Other examples of grading schemes are the Ann Arbor staging system for lymphomas and the Gleason Score for prostate cancer.

While treatment of the initial manifestation of low grade cancer is often effective, patients can relapse as the treatment may not clear all cancerous cells. While all cancer cells generally have an increased ability to proliferate compared to their nontransformed counterparts, certain cells within a tumor have an
enhanced self-renewal capacity. These are known as cancer stem cells and have the ability to differentiate into all of the cells that comprise a tumor. Cancer stem cells are often highly resistant to treatment and can be left behind while the bulk of the tumor is eliminated. As such, they are thought to be a major cause of relapse and are also implicated in metastasis (Carnero et al., 2016; Li et al., 2015; Owens and Naylor, 2013; Schwarz-Cruz et al., 2016).

2.1. Cancer treatment strategies

Several treatments for cancer have been devised, however the specific regimen used varies for each patient due to numerous factors. These include the type of cancer, location within the body, size of the tumor(s), and the age and medical history of the patient. The most commonly used treatment is chemotherapy. This treatment consists of the administration of one or more chemotherapeutic agents, many of which are natural products originally discovered in bacteria and plants. While these drugs are toxic to all cells of the body, their mechanisms of action are designed to target rapidly dividing cells. As such, several classes of chemotherapy agents are used in the clinic that effectively target different mechanisms that regulate the cell cycle.

The most effective overall in terms of the number of cancers that respond are the anthracyclines, which are derivatives of compounds discovered in Streptomyces bacteria (e.g., daunorubicin, doxorubicin). Anthracyclines work by intercalating into DNA, thereby inhibiting the activity of topoisomerase II and the
synthesis of new DNA molecules. Nucleotide analogs (e.g., fluorouracil) also inhibit DNA synthesis either directly or by interfering with nucleotide production. Platinum based drugs (e.g., cisplatin) covalently crosslink DNA to prevent synthesis and repair of DNA damage. Chemotherapeutic agents can also interfere with the cytoskeletal machinery, thereby restricting cell division. Examples are the vinca alkaloids (e.g., vincristine) that were originally discovered in the rosy periwinkle (*Catharanthus roseus*) and interact with tubulin to inhibit microtubule polymerization. In contrast, taxanes (e.g., paclitaxel) prevent depolymerization by stabilizing microtubules. Taxanes originate from trees of the *Taxus* genus (yews). Other classes of chemotherapeutic agents include inhibitors of kinases (e.g., erlotinib), histone deacetylases (e.g., vorinostat), and transcription (e.g., actinomycin D).

While many cancers initially respond to treatment, the development of chemoresistance is a major cause of poor patient outcomes. There are several mechanisms of chemoresistance including modifications to the drug target, changes in the metabolism or cellular distribution of the drug, and increased DNA repair capacity, however the most common mechanism is through activity of ATP binding cassette (ABC) transporters (Fodale *et al.*, 2011; Kathawala *et al.*, 2015).

These efflux pumps normally function to export xenobiotics from cells, however in the case of cancer cells they remove chemotherapeutic agents. While ABC transporters came into existence well before the use of chemotherapy as they are found in all domains of life, chemotherapeutic agents are often biological
products and consequently are substrates for multiple ABC transporters (Dlugosz and Janecka, 2016; Pan et al., 2016; Ween et al., 2015). Due to this, chemoresistance often manifests as multidrug resistance, a phenomenon that is characterized by resistance to multiple drugs simultaneously (Gillet and Gottesman, 2010). Multidrug resistance often renders several classes of chemotherapeutic agents ineffective, thereby significantly limiting treatment options.

ABC transporters comprise a large family of diverse membrane bound efflux pumps that use the energy of ATP hydrolysis to actively transport substrates across membranes (Ambudkar et al., 2003). There are currently 48 known in humans, and the family is subdivided into seven groups based on structure (ABCA, B, C, D, E, F, and G) (Dean et al., 2001). ABC transporters are generally dimers that consist of two nucleotide binding domains and several membrane spanning regions that form a channel in the membrane (Figure 1-8) (Linton and Higgins, 2007). Up to two ATP molecules are needed for efflux of one substrate molecule, therefore the activity of ABC transporters is highly energy demanding (Linton and Higgins, 2007; Patzlaff et al., 2003; Poolman et al., 2005). The role of metabolism in the function of ABC transporters is a later focus of this dissertation (CHAPTER 3).
Figure 1-8. Structure of the murine ABC transporter ABCB1
Protein Data Bank ID: 3G5U (Aller et al., 2009).

The first family member discovered was ABCB1, which is also known as P-glycoprotein 1 (P-gp) and multidrug resistance protein 1 (MRP1), while other notable examples commonly involved in chemoresistance are ABCG2 and ABCC1 (Allikmets et al., 1998; Cole et al., 1992; Doyle et al., 1998; Miyake et al., 1999; Roninson et al., 1984; Ueda et al., 1987). Despite the correlation of ABC transporter expression and the development of chemoresistance, there are currently no approved therapies directly targeting these efflux pumps. All three generations of inhibitors that have been tested in clinical trials have failed to show efficacy, with the majority of failures due to off target effects and toxicity (Robey et al., 2018). In addition to these pharmacological effects, it has been shown that genetic variation in ABC transporters, specifically shown for ABCB1, can affect the outcome of
treatment (Chaturvedi et al., 2013; Reed and Parissenti, 2011; Tulsyan et al., 2016). Therefore, novel therapies for inhibiting ABC transporter function represent a significant medical need in the treatment of chemoresistance. Later in this dissertation it will be shown that reducing mitochondrial respiration in cancer cells can effectively reverse ABC transporter mediated chemoresistance, a finding that may lead to the development of novel metabolism-based therapies (CHAPTER 3).

Surgery is also used in the treatment of cancer. While many solid tumors can be physically removed, not all cancers are treatable by this method due to the extensive normal tissue damage that would occur during their removal (e.g., brain cancers). Surgery can also be combined with chemotherapy to increase efficacy. Chemotherapy administered prior to surgery is known as neoadjuvant therapy and has the goal of shrinking a tumor for more efficient removal and to allow for less normal tissue damage. Administration after surgery is known as adjuvant therapy and targets cancer cells that were not removed or those that may have not been detected during screening assays. The primary goal of adjuvant therapy is to lessen the risk of relapse.

Radiation therapy (also known as radiotherapy) is also used to treat cancer and has the goal of inducing sufficient DNA damage such that cell death occurs. Rapidly dividing cells are the most sensitive to radiation treatment, however normal tissue is also affected. External beam radiation is used for many types of cancer and is a localized treatment that directs high energy beams of photons,
protons, or electrons to the site of a tumor. In contrast, internal radiation is the administration of radioactive nuclides into the body. This can occur via systemic administration or as brachytherapy where a sealed radiation source is implanted near the tumor.

While chemotherapy, radiotherapy, and surgery have been used and refined for decades, there is still a need for new types of therapy as many patients do not respond to these treatments. The latest advances in cancer treatment are based on immunotherapy, which employs the tools of the immune system to treat cancer. One strategy is through the use of monoclonal antibodies that target cells of the immune system (Apetoh et al., 2015; Wargo et al., 2015). As described later (§3.2), mechanisms exist that negatively regulate immune responses. These can be exploited by cancer cells, thereby downmodulating immune responses and protecting tumor cells from clearance. These regulatory mechanisms are known as checkpoint inhibitors, and a notable example is programmed cell death protein 1 (PD-1) ligand (PD-1L), which blocks T cell immunity by engaging PD-1 on T cells (Sharpe and Pauken, 2018). Blocking antibodies (e.g., pembrolizumab) can effectively prevent this interaction and have been shown to effectively improve treatment outcomes. In addition, bispecific antibodies have been generated that target antigens on cancer cells and immune cells simultaneously, increasing the likelihood of their interaction (e.g., catumaxomab) (Brinkmann and Kontermann, 2017; Kontermann and Brinkmann, 2015).
In addition, immune cells from patients can be genetically altered \textit{ex vivo} to improve their cancer immunity function and then administered back to the patient. A notable example is the addition of a chimeric antigen receptor to T cells, which was recently approved for the treatment of certain types of leukemia. The engineered cells, known as CAR-T cells, are induced to express a TCR/antibody chimera that allows for specific targeting of cancer cells (Kochenderfer \textit{et al.}, 2010). Cellular immunotherapy is currently an intense area of research and numerous clinical trials are ongoing.

2.2. \textbf{Tumor metabolism}

There are six acquired capabilities of cancer that have been described as the “hallmarks” of cancer (Hanahan and Weinberg, 2000). These characteristics are common to all cancers, and their acquisition is facilitated by the inherent genomic instability and genetic diversity of cancer cells (Figure 1-9). As such, these hallmarks have been targets of intense research for decades. This paradigm was recently revisited, and alterations to cellular energetics was proposed as an “emerging hallmark” of cancer (Hanahan and Weinberg, 2011). It has since been demonstrated that dysregulated metabolism is not just a hallmark of cancer, but it is a key driver of development and progression.
The metabolism of cells has been studied for over a century, and discoveries of its role in cancer were pioneered by Otto Warburg in the early 1920s (Warburg et al., 1927). Under normal oxygen conditions (normoxia), mammalian cells typically convert pyruvate derived from glycolysis to acetyl-CoA for further oxidation in mitochondria. However, the reduction of pyruvate to lactate and the corresponding oxidation of NADH to NAD$^+$ can also occur under normoxic conditions and is often detected in rapidly dividing cells. Warburg noted that even in conditions where oxygen concentrations were sufficient for oxidation to occur, glucose was still fermented to lactate (Warburg et al., 1927). This metabolic state was later termed aerobic glycolysis and is generally known as the Warburg effect.
The altered metabolism of cancer cells serves to promote anabolic processes (Liberti and Locasale, 2016; Vander Heiden et al., 2009). While glycolysis is generally considered a catabolic pathway, it produces metabolic intermediates that support the biosynthesis needed for cell growth and proliferation. A number of signaling and transcription factors have been shown to induce aerobic glycolysis in cancer (Figure 1-10). A notable example is the well-known tumor suppressor p53, which is commonly mutated in cancer cells (Ozaki and Nakagawara, 2011). While most famous for its role in DNA repair, p53 also negatively regulates flux through glycolysis (Zhang et al., 2014). Activation of phosphatidylinositol-3 kinase (PI3K) also plays a major role in upregulating glycolysis through regulation of the downstream modulators protein kinase B (Akt) and the mammalian target of rapamycin complex (mTORC) (DeBerardinis and Chandel, 2016). The combined effects of these and other regulators leads to an overall change in cancer cell metabolism that supports biogenesis.
Figure 1-10. Signaling pathways regulating cancer metabolism.
Adapted from DeBerardinis and Chandel (2016).

The abnormal accumulation of some metabolic intermediates can have a significant effect on the metabolism and growth of tumor cells. Termed oncometabolites, these intermediates can alter DNA and histone methylation in ways that promote tumor growth and progression (Sciacovelli and Frezza, 2016; Yang et al., 2013). A notable example is D-2-hydroxyglutarate, which has been shown to have substantial impacts on the epigenetic landscape of cancer cells (Figure 1-11). In addition, common products of mitochondrial respiration (e.g., acetyl-CoA, α-ketoglutarate) have also been shown to affect epigenetic markers of
histones, thereby altering chromatin structure and gene transcription (Ward and Thompson, 2012).

Figure 1-11. Biological changes induced by D-2-hydroxyglurate
Adapted from Yang et al. (2013).

Several changes in mitochondrial metabolism in cancer have been shown to promote cancer progression (Wallace, 2012; Zong et al., 2016). Mutations in key enzymes of the TCA cycle, including succinate dehydrogenase (Complex II), lead to increased levels of intermediates (e.g., succinate, fumarate) that serve as feedback inhibitors and promote aerobic glycolysis (Kurelac et al., 2011). Activation of the MYC pathway promotes glutaminolysis, which provides a supplemental carbon source to the TCA cycle (a process known as anaplerosis) in order to produce citrate and support fatty acid synthesis (DeBerardinis et al., 2008).
In addition, it has been shown that reduced Ca\textsuperscript{2+} flux into mitochondria occurs in cancer, which has been proposed to play a role in preventing apoptosis (Wallace, 2012). These examples, along with others not mentioned, all promote tumorigenesis and cancer progression via alterations to mitochondrial function.

Additionally, the metabolism of cancer cells serves not only to promote tumor development, but it can also modulate immune responses. Due to the high utilization of glucose, cancer cell metabolism results in “deserts” of low glucose in the tumor microenvironment that effectively starve tumor infiltrating lymphocytes (Lyssiotis and Kimmelman, 2017). Furthermore, waste products of cancer metabolism can alter immune responses as it has been shown that lactate excreted by cancer cells negatively affects the glycolytic activity of T cells, which suppresses cytotoxic T cell function (Fischer et al., 2007).

3. Overview of the immune system

An important characteristic of life is the ability to discern between what components constitute the body and those of other organisms (self versus nonself). The need to protect the self from nonself threats led to the evolution of the immune system, which is a complex suite of tissues, cells, and molecules that defend an organism from disease. The human immune system is capable of detecting and eradicating a wide variety of pathogens, including many different types of viruses, bacteria, parasites, and fungi. It is also capable of neutralizing internal threats, such as eliminating cancerous cells and tumors.
The complex responses of the immune system are heavily regulated to prevent autoimmunity, which occurs when the immune system attacks the normal cells and tissues of the body. In addition, allergic responses to normally harmless substances can result in severe, life-threatening conditions (e.g., anaphylaxis). Thus, a balance must be maintained such that immune responses occur while damaging effects to the body are limited.

The human immune system can be broadly separated into two major arms, the innate and adaptive immune systems. The immune system can also be further divided into humoral immunity, which is due to the immune effector functions of proteins and other soluble components (e.g., the complement system and antibodies), and cell mediated immunity, which is immunity due to the actions of leukocytes (Figure 1-12).
3.1. Innate immunity

The innate immune system is comprised of physical and chemical barriers, proteins such as the complement system and inflammatory mediators, and innate immune cells. Innate immunity is fast-acting and is often the first to encounter and respond to an invader. In addition to pathogens, it is also activated by endogenous signals from damaged or stressed cells.

Several barriers exist that separate or differentiate the body into different compartments, and innate immunity is crucial for their protection (Owen et al., 2013). Physical barriers are present where the body encounters the environment. These barriers are generally lined with epithelial cells that are protected by a layer of mucus, which is a thick secretion of glycoproteins, antimicrobial peptides,
antibodies, and other factors. This is known as a mucosal layer and protects areas such as the linings of the lungs and gastrointestinal tract. As living systems require constant transport of nutrients and waste products, none of these barriers are completely impermeable and are therefore armed with multiple defenses from pathogens.

Chemical barriers are another line of defense against invading pathogens. These exist in several forms including broadly acting antibiotics in the form of antimicrobial peptides (e.g., β-defensins) and enzymes (e.g., lysozyme). Additionally, the pH of the stomach is a chemical barrier that acts against ingested pathogens. The specific defenses present depend on the nature of the barrier and vary by location in the body.

Innate immune responses to pathogens are generally initiated by the engagement of pattern recognition receptors (Brubaker et al., 2015; Takeuchi and Akira, 2010). These innate receptors recognize conserved molecular patterns known as pathogen associated molecular patterns. A classic example from bacteria is lipopolysaccharide (LPS), which activates Toll-like receptor 4 (TLR4) (Park and Lee, 2013). Pattern recognition receptors also detect damage associated molecular patterns, which are endogenous cues of cellular stress and tissue damage (Schaefer, 2014). The release of mitochondria DNA is one such example and is recognized by TLR9 (Kumagai et al., 2008). A major effect of signals downstream of TLR engagement is the activation of the inflammasome, which is a multi-
subunit protein scaffold that facilitates the production and release of inflammatory mediators (e.g., IL-1β) (Broz and Dixit, 2016).

The induction of an acute inflammatory response is the first line of defense when the innate immune system is activated (Owen et al., 2013). Acute inflammation is characterized by five classic symptoms - heat, pain, redness, swelling, and loss of function. It can be caused by responses to pathogens, but it can also manifest after an injury that results in tissue damage. These responses are initiated by the release of inflammatory mediators that activate downstream signaling cascades. Proinflammatory cytokines such as IL-1, IL-6, and TNF are key components of the inflammatory cascade. A subset of cytokines known as chemokines are also released and function to recruit other immune cells to the site of infection or injury. Whether from pathogens or tissue damage, the responses initiated are facilitated by innate immune cells.

The most abundant cells of the innate immune system are macrophages, neutrophils, natural killer cells, and dendritic cells. When a pathogen enters the body, it is typically first engaged by tissue resident macrophages (Davies et al., 2013). These cells serve as “vacuum cleaners” for the body as they endocytose and break down invading microbes and debris in a process known as phagocytosis. Macrophages also release cytokines and other mediators that direct and further activate immune responses (e.g., IL-1β, TNF). Additionally, macrophages can serve as antigen presenting cells to directly activate the adaptive immune system.
Macrophages are found throughout the body and exist in two major phenotypes – classic (M1) and alternative (M2) macrophages (Shapouri-Moghaddam et al., 2018). M1 macrophages are proinflammatory cells that secrete inflammatory cytokines (e.g., IL-1β, IFNγ). These macrophages are highly phagocytic and effectively clear bacteria and cell debris. In contrast, M2 macrophages are anti-inflammatory and serve roles in dampening immune responses and in wound healing. Most tissue resident macrophages are of the M2 phenotype, however the molecular mechanisms that induce their differentiation are not clear.

Neutrophils are also phagocytic cells, but they generally reside in the blood, bone marrow, or vascular bed until activated (Kolaczkowska and Kubes, 2013). Neutrophils enter the tissue at the site of inflammation via a process called chemotaxis in response to chemokines released during the inflammatory response (e.g., IL-8). In addition to phagocytosis, neutrophils eliminate pathogens by releasing granules filled with antimicrobial peptides and enzymes (e.g., defensins, lysozyme) in a process known as degranulation. They can also release neutrophil extracellular traps (NETs), which are primarily composed of DNA and effectively bind and trap pathogens (Papayannopoulos, 2018). Activation and release of NETs occurs by NETosis, which is a specialized form of programmed cell death (Zawrotniak and Rapala-Kozik, 2013).

Natural killer cells are cytotoxic lymphocytes that are critical for immunity to intracellular bacteria and viruses (Vivier et al., 2008). Unlike extracellular
bacteria and parasites, intracellular pathogens are not easily engaged by immune cells. Therefore, the most effective method of clearance is to induce death of the infected cell. NK cells recognize changes induced by infection – the so called state of “altered self” – and induce programmed cell death in the target cell. One mechanism employed is through the detection of decreased cell surface expression of major histocompatibility complex (MHC) molecules by infected cells, which is often induced by viral infections. Nearly all nucleated cells express MHC molecules, and NK cells are sensitive to differences in their expression. NK cells are also crucial for the elimination of transformed cells and tumors. NK cells induce cell death by releasing granules containing death mediators (e.g., perforin, granzymes). NK cells also secrete cytokines that further direct the immune system (e.g., recruitment of macrophages to phagocytose dead cell debris).

Dendritic cells are phagocytic antigen presenting cells that reside in tissues along with tissue resident macrophages (Mildner and Jung, 2014; Sato and Fujita, 2007). They function to processes pathogens and present antigens to the adaptive immune system. As such, they serve as messengers of the innate immune system that activate adaptive immune responses. Dendritic cells migrate from the site of infection to the lymph nodes, where they present whole and processed antigen to the cells of the adaptive immune system. Dendritic cells also secrete cytokines that direct and further activate immune responses (e.g., TNF, IL-10, IL-12).

Other less common cells of the innate immune system are eosinophils, basophils, and mast cells (Rosenberg et al., 2013; Siracusa et al., 2010; Wernersson
and Pejler, 2014). These, along with neutrophils, are known as granulocytes due to the abundance of granules containing antimicrobial factors in their cytoplasm. Unlike neutrophils, however, eosinophils, basophils, and mast cells are involved in allergic responses and are important for the clearance of parasites. Due to the decreased parasite burdens found in the developed world, these cells are mainly associated with excessive allergic responses and inflammatory diseases (e.g., asthma) and are the key mediators of extreme allergic response symptoms (e.g., anaphylaxis) (Fulkerson and Rothenberg, 2013; Sharma and Bayry, 2015; Voehringer, 2013).

3.1.1. Complement system

In addition to the cell mediated immunity described above, the innate immune system also has humoral components. A notable example is the complement system, which is comprised of serum proteins that are activated in a catalytic cascade that ultimately leads to the breakdown of the membrane integrity of invading pathogens (Ricklin et al., 2010). Complement components can also induce the opsonization of pathogens, thereby enhancing innate immune cell function. Complement activation also releases proinflammatory mediators that direct and recruit other components of the immune system. Complement components are synthesized in the liver as inactive precursors that can be activated by three distinct pathways, all of which lead to the formation of the membrane attack complex (MAC) on the surface of an invading pathogen. The
MAC leads to the release of cytosolic components and eventual death of the invading cell.

3.2. Adaptive immunity

While the innate immune system is generally effective against most threats, the receptors used to detect pathogens are static and limited in diversity. Due to the constant evolution of pathogens, they can adapt such that detection by the innate immune system is avoided or rendered less effective. To combat this, jawed vertebrates evolved the adaptive immune system. Adaptive immune responses are mediated by T and B cells and are highly specific due to the diverse nature of their antigen receptors. T cell receptors (TCRs) and B cell receptors (BCRs) are produced by a process known as V(D)J recombination that through genetic rearrangement forms enormous numbers of unique receptors (Figure 1-13) (Schatz and Ji, 2011). In this way, the adaptive immune system can adapt to changes in pathogens and becomes more specific as immune responses progress.
3.2.1. T cells

T cells are the facilitators of cell-mediated immunity within the adaptive immune system. They develop from common lymphoid precursors derived from the bone marrow, and the majority mature in the thymus (Owen et al., 2013). There are several subsets of T cells, however all are characterized by the presence of a T cell receptor (TCR) on the cell surface. TCRs recognize peptide antigens presented on MHC molecules and are highly diverse in nature. There are two major types of T cells, which are defined by the expression of the glycoproteins CD4 or CD8. These cell surface molecules enhance the binding of the TCR to MHC molecules and also functionally differentiate T cells into the major subsets. CD4+ T cells are generally known as helper T cells as they direct the immune responses of other cells, and their TCR recognizes peptides presented by MHC class II molecules. CD8+ T cells are distinguished by cytotoxic activity and are restricted to peptides presented by MHC class I molecules.
TCRs are heterodimers formed by the association of two peptide chains (Reinherz et al., 1999). The most common type of T cell bears a TCR formed by the association of α and β chains. These are known as αβ T cells and can effectively respond to all pathogens. The other type of TCR is a combination of γ and δ chains, which marks the less understood γδ T cells that are often found in mucosal linings (e.g., intestinal barriers) (Chien et al., 2014; Vantourout and Hayday, 2013).

The development of T cells begins in the bone marrow and is completed in the thymus (Figure 1-14). Common lymphoid progenitor cells destined to become T cells migrate from the bone marrow to the thymus where they differentiate into double negative (DN) thymocytes, defined by a lack of both CD4 and CD8 expression. During the DN stages of development thymocytes proliferate, lose the potential to become B cells, and rearrange the TCRβ chain genes. They then transition to the double positive (DP) stage, characterized by expression of both CD4 and CD8 and formation of the TCRα chain.

The binding of the complete TCR to self peptide expressing MHC molecules is then tested in a process known as positive selection (Klein et al., 2009). If the TCR does not bind any MHC molecules, the thymocyte will die by apoptosis. Proper engagement of an MHC molecule will activate a signaling cascade through the TCR that induces lineage commitment and the transition to the single positive (SP) stage. Mature T cells only express CD8 or CD4, and this depends on whether their TCR engaged MHC class I or II molecules, respectively. Thymocytes also undergo
negative selection in the SP stage, a process which is vital to preventing autoimmunity (Klein et al., 2014). If a TCR strongly engages the MHC molecule, it has a high chance of being autoreactive (specific for self peptides). Therefore, thymocytes that receive a strong signal through the TCR are eliminated by apoptosis.

Figure 1-14. Stages of T cell development
Adapted from Owen et al. (2013).
MHC molecules are cell surface proteins that are essential for adaptive immunity (Owen et al., 2013). MHC molecules are broadly characterized into two major classes. MHC class I are expressed by nearly all cells (including platelets), however red blood cells are a notable exception. MHC class I molecules present self-derived peptides and mainly function in immunity to intracellular pathogens (e.g., viruses) and transformed cells. MHC class II are expressed by professional antigen presenting cells (APCs; comprised of dendritic cells, macrophages, and B cells) and present peptides derived from processed antigens. Professional APCs can also present processed antigen on MHC class I via a process known as cross presentation.

After maturation in the thymus, T cells enter the circulation as naïve cells. In this state, they are relatively quiescent and exhibit low levels of DNA transcription and protein translation. They can be found in the blood and in tissues, however most are found in the secondary lymphoid organs (e.g., lymph nodes, spleen). Due to the enormous energy demands of an immune response, T cells remain in the naïve state until activated.

T cell activation requires engagement of the TCR with cognate peptide presented by an MHC molecule, however the TCR itself does not contain a signaling component. Signal transduction requires the T cell coreceptor CD3 and the ζ chain (CD247) (Gaud et al., 2018; Smith-Garvin et al., 2009). Complete activation of naïve T cells also requires signaling from co-stimulatory molecules (Gaud et al., 2018; Smith-Garvin et al., 2009). The is primarily due to CD28, however
other molecules such as inducible costimulatory (ICOS) can serve this function (Chen and Flies, 2013). In addition, there are regulatory molecules such as cytotoxic T lymphocyte associated protein 4 (CTLA4) and programmed cell death protein 1 (PD-1) and that negatively regulate T cell activation (Chen and Flies, 2013).

**Figure 1-15. Regulation of T cell activation**
Adapted from Chen and Flies (2013).

### 3.2.1.1. CD4+ T cells

When naive CD4+ T cells become activated, they differentiate into helper T cells (Owen *et al.*, 2013). These cells direct the immune responses of other leukocytes and play a central role in the progression, strength, restriction, and
conclusion of immune responses. Helper T cells perform this function by producing cytokines that act on other immune cells and by fully activating B cells to produce antibodies and induce class switching. Helper T cells are activated by professional antigen presenting cells, and they differentiate into several types with specialized effector functions depending on the cytokine environment and nature of the immune response.

Figure 1-16. Helper T cell subsets
Adapted from O'Shea and Paul (2010).

The majority of activated CD4+ T cells are classified at type 1 helper (T\(_{\text{H}1}\)) or T\(_{\text{H}2}\) effector cells (Mosmann et al., 1986; Romagnani, 1991). Immunity driven by T\(_{\text{H}1}\) cells is most effective against intracellular pathogens. These cells are
characterized by high production of IFNγ and expression of the transcription factors STAT4 and T-bet (Farrar et al., 2002; Luckheeram et al., 2012; Romagnani, 1994). In contrast, TH2 cells are most effective against extracellular pathogens (Walker and McKenzie, 2018). They are induced by IL-4 and IL-6 signaling and predominantly produce cytokines involved in allergic responses (e.g., IL-4, IL-5, IL-13) (Luckheeram et al., 2012; Romagnani, 1994). TH2 cells activate a number of innate immune cells including eosinophils, basophils, and mast cells as well as IgE and IgG antibody producing B cells (Annunziato et al., 2015).

Follicular B helper T (T\textsubscript{FH}) cells are a subset of helper T cells that are localized to the periphery of B cell follicles in secondary lymphoid organs (Crotty, 2014). T\textsubscript{FH} cells mediate the generation of germinal centers, in which B cells proliferate, differentiate, and undergo antibody class switching and affinity maturation. T\textsubscript{FH} cells are therefore crucial to the production of antibodies that effectively target pathogens. The cytokine IL-21 and the immune checkpoint protein ICOS induce T\textsubscript{FH} cell differentiation (Luckheeram et al., 2012).

Another subset is comprised of T helper 17 (TH17) cells, which are proinflammatory cells that produce significant amounts of IL-17 (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Harrington et al., 2005). These cells are important mediators of immune responses at mucosal sites and are enriched in the linings of the lungs and intestinal tract. The differentiation of TH17 cells is induced by transforming growth factor β (TGFβ) and IL-6 signaling and the
transcription factors STAT3 and RORγt (Luckheeram et al., 2012; Yang et al., 2007). Excessive inflammation caused by T_{H}17 cells has been shown to exacerbate diseases (e.g., psoriasis) (Tesmer et al., 2008), which may be due (at least in part) to the signals that induce T_{H}17 differentiation competing with those that induce regulatory T (T_{reg}) cells.

T_{reg} cells are immunosuppressive T cells that are important for the maintenance of tolerance and the prevention of autoimmunity. The transcription factor forkhead box P3 (FOXP3) directs the differentiation of T_{reg} cells, expression of which begins in the thymus during the later stages of T cells development (Luckheeram et al., 2012; Ohkura et al., 2013). The TCR of T_{reg} cells is often specific to self peptides, however activation of these cells leads to dampened immune responses (Josefowicz et al., 2012). T_{reg} cells restrict immune responses by producing anti-inflammatory cytokines (e.g., IL-10, IL-35) and by releasing cytolytic mediators (e.g., granzyme B) that induce apoptosis of effector immune cells (Josefowicz et al., 2012). T_{reg} cells also alter the cytokine environment by rapidly absorbing cytokines released by other cells (Pandiyan et al., 2007). Defects in the number and/or function of T_{reg} cells have been implicated in the pathology of several diseases (e.g., cancer, autoimmunity), however the exact mechanisms involved are unclear.

In addition to these commonly known subsets, other T helper cell subsets have been reported that are less understood. A subset characterized by high production of IL-9 was recently described and termed T helper 9 (T_{H}9) cells
(Kaplan et al., 2015). This subset currently lacks specific biomarkers, but Th9 cells are thought to be involved in allergy responses. Similarly, T helper 22 (Th22) cells are characterized by substantial production of IL-22. Originally thought to related to Th17 cells, Th22 cells have also been shown to produce IL-13 and granzymes and are implicated in the pathology of asthma and atopic dermatitis (Plank et al., 2017). In addition, functionally unique CD4+ T cells with cytotoxic activity have been described (Takeuchi and Saito, 2017), contrasting with the dogma of immunology that only CD8+ T cells have cytolytic activity.

3.2.1.2. CD8+ T cells

The other major class of effector T cells are cytotoxic T (Tc) cells, which develop from naïve CD8+ T cells (Owen et al., 2013). Similar to NK cells, Tc cells provide immunity to intracellular pathogens and eliminate transformed cells through the production and release of cytolytic granules. A key difference from NK cells, however, is that Tc cells are specifically activated as their TCR is restricted to a unique peptide presented on an MHC class I molecule.

The main function of Tc cells is to induce cell death. Engagement of their TCR with an infected or transformed cell triggers the release of the components of cytotoxic granules (mainly perforin and granzymes), which induce programmed cell death in the target cell. Tc cells can also induce cell death through expression of other effector proteins such as first apoptosis signal receptor (Fas) ligand (FasL), which binds to Fas expressed on target cells (Owen et al., 2013).
Another major function of Tc is to produce cytokines, including significant amounts of IFNγ. There are currently three defined subtypes of cytotoxic T cells based on their cytokine secretion profile (Annunziato et al., 2015; Kondo et al., 2009; Mosmann et al., 1995). T cytotoxic 1 (Tc1) cells are the most common type of effector cytotoxic cell and produce significant amounts of IFNγ (Mosmann et al., 1995). The transcription factors T-bet and Eomes are critical for both their production of IFNγ and cytolytic activity (Glimcher et al., 2004). Similar to Th2 cells, T cytotoxic 2 (Tc2) cells produce IL-4, IL-5, and IL-13 and are involved in immunity to parasites and in allergy responses (Mosmann et al., 1995). Lastly, T cytotoxic 17 (Tc17) cells are proinflammatory cells characterized by production of IL-17 (Kondo et al., 2009). Activation of STAT3 and signaling through RORγt are required for their differentiation (Annunziato et al., 2015).

In addition to these subsets, a functionally unique type of Tc cells was recently described. In response to IL-6, a subset of Tc cells can produce IL-21 during an influenza infection, which is sufficient to support B cell antibody production and class switching (Yang et al., 2016). These “helper” cytotoxic T cells, along with the “cytotoxic” helper T cells described previously (§3.2.1.1) are examples of the immense and as yet understood complexity of the immune system.
3.2.2. **B cells**

B cells function to produce and secrete antibodies (Owen *et al.*, 2013). B cells mainly reside in the lymph nodes and spleen and are activated through direct interaction with antigens from pathogens, which engage the B cell receptor on the cell membrane. B cells also function as antigen presenting cells, and, along with dendritic cells and activated macrophages, are known as professional antigen presenting cells as they express MHC class II molecules and can effectively activate CD4$^+$ T cells. B cells also secrete cytokines and chemokines when activated. Like T cells, B cells also develop from common lymphoid progenitor cells, however they develop and mature in the bone marrow.

B cells are characterized by the cell surface expression of the B cell receptor (BCR), which, like the TCR, is formed through the process of V(D)J recombination (Schatz and Ji, 2011). Naïve B cells are activated by binding of the cognate ligand to the BCR. This leads to endocytosis of the antigen, which is then processed and presented to helper T cells via MHC class II molecules. B cells that receive T cell help undergo a rapid proliferation and begin secreting antibodies, which are soluble versions of the BCR.

There are five major types of antibodies that can be produced by B cells – immunoglobulin M (IgM), IgD, IgA, IgG, and IgE (Owen *et al.*, 2013). The BCR of naïve B cells is in the form of IgM or IgD. To produce the other types of antibodies, germinal center B cells undergo a process known as class switching (Stavnezer *et al.*, 2008). The specific type of antibody induced depends on the cytokine
environment and the type of pathogen that initiated the immune response. Additionally, antibody specificity for antigen increases over time as B cells introduce mutations in antibody genes via somatic hypermutation (Teng and Papavasiliou, 2007). B cells that produce higher affinity antibodies preferentially receive help from T\textsubscript{FH} cells, which leads to their selection over time. The combined effects of somatic hypermutation and T\textsubscript{FH} cell selection are known as affinity maturation.

### 3.3. Immunologic memory

After the stimulus that initially activated the immune response is eliminated, the cells of the immune system undergo a process known as contraction during which the majority of the activated leukocytes die (Marrack \textit{et al.}, 2010). Unlike innate immune cells, however, some activated T and B cells differentiate into long lived memory cells (Owen \textit{et al.}, 2013). These cells serve as sentinels in the event of another infection with the same pathogen and can persist for decades. Due to the individual nature of the pathogens and other threats that each organism experiences, the memory cell repertoire of every individual is unique. Unlike a primary immune response, activation of memory cells occurs quickly, is less dependent on costimulatory molecules, and leads to rapid protection from secondary infections.

Both CD\textsuperscript{4+} and CD\textsuperscript{8+} memory T cells form and have been classified into three subsets (Mahnke \textit{et al.}, 2013; Mueller \textit{et al.}, 2013; Sallusto \textit{et al.}, 1999). Central
memory T (T\textsubscript{CM}) cells circulate between the blood and secondary lymphoid organs (\textit{e.g.}, spleen, lymph nodes). T\textsubscript{CM} cells exhibit self-renewal, have a large proliferative capacity, can differentiate into effector cells, and produce significant amounts of IL-2. Effector memory T (T\textsubscript{EM}) cells can be found migrating between the blood and nonlymphoid peripheral tissues. T\textsubscript{EM} cells have a lower proliferative capacity compared to T\textsubscript{CM} cells but are potent cytotoxic cells that can rapidly produce substantial amounts of effector cytokines (\textit{e.g.}, IFN\textsubscript{γ}). Tissue resident memory (T\textsubscript{RM}) cells do not circulate and therefore permanently remain in the tissue in which they develop (Gebhardt \textit{et al.}, 2009; Masopust \textit{et al.}, 2010). These cells serve as immediate responders to invading pathogens and are especially effective against recurrent, localized challenges from the same pathogen.

In contrast to these “classically” derived memory cells that develop after antigen exposure, a subset of memory cells that are antigen naïve have been recently described. These cells, known as virtual memory T (T\textsubscript{VM}) cells (White \textit{et al.}, 2017) have been shown to comprise a substantial portion of the pool of all CD8\textsuperscript{+} T cells in naïve mice (15 – 20 %) (Dobber \textit{et al.}, 1992; Le Campion \textit{et al.}, 2002). Instead of foreign antigen, it is thought that cytokine signaling leads to their development (Haluszczak \textit{et al.}, 2009). Future studies are needed to characterize this unique memory cell subset.
3.4. Immunometabolism

In recent years, the metabolism of immune cells has become an intense area of research. Unlike the majority of cells in the body, those of the immune system experience rapid and drastic changes in nutrient availability and oxygen concentrations during the normal course of their function. Accordingly, the metabolic state of immune cells has been shown to directly correlate with immune effector functions (Figure 1-17).

Figure 1-17. Metabolism of T cells
Adapted from Buck et al. (2015).
Naïve and certain memory cells (e.g., T<sub>CM</sub> cells) are commonly found in relatively resource rich compartments such as the blood, spleen, and lymph nodes (McNamee <i>et al.</i>, 2013). In contrast, activated effector cells that traffic to the nutrient and oxygen poor environments of infected and/or inflamed tissues experience extracellular signals and cues are exceedingly different from those of nutrient replete compartments (Eltzschig and Carmeliet, 2011). As such, the metabolic demands of effector cells are significantly different from those of resting cells. In order to survive and function in these vastly different niches, cells of the immune system have the ability to substantially reprogram their metabolism (Buck <i>et al.</i>, 2015; Pearce <i>et al.</i>, 2013).

Naïve and resting memory T cells are small and relatively quiescent, however they require ATP for the cytoskeletal rearrangements necessary to navigate the blood and lymphatic vessels. To maintain this state, they primarily utilize oxidative phosphorylation fueled by glucose and fatty acids to produce ATP (van der Windt and Pearce, 2012; Wang <i>et al.</i>, 2011). In contrast, effector T cells are large, highly proliferative, and secrete substantial amounts of cytokines and other effector molecules. Since glycolytic pathways are more supportive of biosynthesis, effector T cells rely on aerobic glycolysis (Figure 1-18) (Pearce <i>et al.</i>, 2013).
Signaling pathways activated via engagement of the TCR, costimulatory molecules, and cytokines initiate the upregulation of glucose and amino acid transporters (Carr et al., 2010; Frauwirth et al., 2002; Sinclair et al., 2013) and promote the metabolic switch from OXPHOS to aerobic metabolism (Frauwirth et al., 2002). For example, CD28 ligation leads to increased PI3K activity, which activates Akt and mTORC. The combined effect of these integrated promotes the differentiation and proliferation of effector T cells (Buck et al., 2015; Delgoffe et al., 2011; Pollizzi et al., 2015; Pollizzi and Powell, 2015). In addition, metabolic changes induced by these pathways during activation have lasting effects as memory cell development requires CD28 signaling during the initial stages of naïve T cell activation (Klein Geltink et al., 2017).
Consistent with the switch to aerobic glycolysis, β-oxidation of fatty acids is downregulated during T cell activation (Wang et al., 2011). While effector cells primarily utilize aerobic glycolysis, OXPHOS is still utilized by these cells (Wang et al., 2011). However, the paradigm of effector T cells primarily using aerobic glycolysis is not universal as T_{reg} cells have been shown to depend on β-oxidation and OXPHOS (Michalek et al., 2011). Consistent with this, mTORC signaling represses FoxP3+ T_{reg} cell differentiation, while the conventional effector subsets (T_{H1}, T_{H2}, T_{H17}) are promoted (Delgoffe et al., 2009; Kopf et al., 2007). In addition, while the metabolism of T_{FH} cells is currently unclear, the transcription factor Bcl6 (which defines the lineage) suppresses glycolysis (Oestreich et al., 2014), indicating that these cells may also rely more on OXPHOS.

As effector cells undergo contraction and become memory T cells, their metabolism undergoes additional reprogramming. Like naïve cells, memory T cells primarily rely on OXPHOS for their metabolic needs. However, they have increased mitochondrial mass, which instills these cells with a significantly higher spare respiratory capacity (van der Windt et al., 2012). The cytokine IL-15, which is critical to the differentiation of memory T cells, is important for the generation of this phenotype (van der Windt et al., 2012). It has also been shown that memory T cells heavily rely on the oxidation of fatty acids, which they produce and store in membrane bound compartments in the cytosol (Araki et al., 2009; O'Sullivan et al., 2014; Pearce et al., 2009; van der Windt and Pearce, 2012).
The morphology of mitochondrial networks has also been shown to vary between T cell activation states, and the patterns are consistent with their metabolic phenotypes. In effector T cells, mitochondria exhibit a punctate, fissed morphology, while those of memory T cells appear as a fused and connected network (Buck et al., 2016). Fused mitochondrial networks are thought to promote mitochondrial function, including the generation of respiratory supercomplexes (Cogliati et al., 2013; Mishra et al., 2014).

Metabolic changes have also been shown to affect cells of the innate immune system (O’Neill and Pearce, 2016). Like activated T cells, M1 macrophages heavily rely on aerobic glycolysis. They also exhibit a “broken” TCA cycle, whereby succinate and citrate accumulate, and this ultimately facilitates the effective secretion of IL-1β (Mills et al., 2017). In contrast, the TCA cycle is fully functional in M2 macrophages as they primarily utilize OXPHOS for ATP synthesis. In addition, a unique requirement of glycogen as a fuel source for dendritic cell activation and effector function was recently described (Thwe and Amiel, 2018; Thwe et al., 2017).

Taken together, these studies indicate that the metabolic state of immune cells is adapted to promote effector functions, and more detailed analyses of the metabolic requirements of specific effector functions are currently areas of intense research. Later in this dissertation, the effects of increased mitochondrial respiration on the effector functions of CD8+ T cells in the context of viral immunity will be discussed in detail (CHAPTER 2).
4. Methylation controlled J protein

4.1. Discovery

Methylation controlled J protein (MCJ) was originally discovered as a gene expressed in normal ovarian epithelial cells, but little to no expression was detected in several ovarian cancer cell lines (Shridhar et al., 2001). MCJ is a small protein of ~150 amino acids in length that is encoded by the DNAJC15 gene (Kampinga et al., 2009). In humans, the DNAJC15 gene locus spans approximately 86,000 base pairs on chromosome 13 and encodes a 2792 base pair transcript containing 6 exons and 5 introns (Shridhar et al., 2001). The MCJ protein contains a short N-terminal sequence (35 amino acids) followed by a single transmembrane domain (Hatle et al., 2007; Shridhar et al., 2001). The C-terminus contains a canonical J domain, defining MCJ as a member of the large DNAJ family of protein co-chaperones (Hatle et al., 2007; Shridhar et al., 2001).

4.2. Classification

DNAJ family members (also called J proteins) are homologous to heat shock protein 40 (Hsp40) of Escherichia coli. There are currently 49 identified human DNAJ proteins that all contain a highly conserved J domain, but their specific function is determined by non-conserved domains (e.g., the N-terminus of MCJ) (Qiu et al., 2006). The current nomenclature divides DNAJ proteins into three subfamilies based on the location of the J domain and the presence of other...
conserved protein motifs (Kampinga et al., 2009). Type A members contain a J domain in the N-terminus, a glycine/phenylalanine rich region, a cysteine rich region, and a C-terminus that varies in structure. Type B members also contain an N-terminal J domain and a glycine/phenylalanine rich region, but they lack a cysteine rich region. Type C members are the most numerous and are defined by only having a J domain, which can be present in either the N- or C-terminus. MCJ contains a J domain in the C-terminus but does not have a glycine/phenylalanine or cysteine rich region, therefore it is a type C DNAJ protein (Hatle et al., 2007; Shridhar et al., 2001).

4.3. Evolutionary history

MCJ is proposed to have evolved after a gene duplication event involving the mitochondrial import inner membrane translocase subunit 14 (TIM14) gene of yeast (Hatle et al., 2007), a vital component of the inner membrane translocase in mitochondria (Mokranjac et al., 2003). TIM14 is also a DNAJC family member and is designated as DNAJC19. In humans and other vertebrates, MCJ and DNAJC19 are both paralogs of yeast TIM14 that are found in mitochondria. Both have a highly conserved J domain in the C-terminus and a predicted transmembrane domain, however MCJ also contains a unique N-terminal sequence (Hatle et al., 2007) (Figure 1-19).
Figure 1-19: Comparison of MCJ and DNAJC19
Non-conversed amino acid residues of DNAJC19 isoforms relative to MCJ are indicated by bold lettering and dashes.

To date, there has only been one phylogenetic analysis of the origin of MCJ (Hatle et al., 2007). It suggests that MCJ coalesced prior to the divergence of ecdysozoans and vertebrates, however MCJ homologs are only detectable in extant vertebrate lineages (Hatle et al., 2007). The absence of homologs in other animal clades indicates that it was likely lost during the course of their further divergence from vertebrates over time (Hatle et al., 2007). Interestingly, the amino acid sequence of MCJ is conserved among a diverse number of vertebrate populations (Table 1-2), indicating that unknown selective pressure(s) may be present. Future studies are needed to clarify the origin and evolutionary history of MCJ.
## Table 1-2. Conservation of MCJ across vertebrate populations

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<tr>
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<th>Common Name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
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<td>Mouse</td>
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<td>Chimpanzee</td>
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</tr>
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<td>Orangutan</td>
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<td>Dog</td>
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</tr>
<tr>
<td><em>Felis catus</em></td>
<td>Cat</td>
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Dashes indicate missing amino acid residues relative to other sequences.
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4.4. Regulation of DNAJC15 expression

The expression of MCJ mRNA and protein has been detected in a variety of cell types. In primary tissues, high expression of MCJ has been shown in several metabolically active tissues (e.g., heart, liver), while other active tissues (e.g., brain, skeletal muscle) show substantially less expression (Hatle et al., 2013). MCJ expression also significantly varies by cell type, including those that are quite similar. For example, naïve CD8+ T cells highly express MCJ, while CD4+ T cells do not express MCJ (Hatle et al., 2013). MCJ has also been detected in a number of cancer cell types as described below. The mechanisms that promote MCJ expression in most tissues and cells are largely unknown, however DNA methylation is a known mechanism of silencing expression.

4.4.1. DNA methylation

The first report of MCJ identified it as a gene that is negatively regulated by DNA methylation (Shridhar et al., 2001). Treatment of ovarian cancer cells with a methyltransferase inhibitor resulted in the expression of MCJ mRNA in a dose dependent manner, therefore the gene was named methylation controlled J protein (Shridhar et al., 2001). The effect of methyltransferase inhibitors resulting in increased MCJ expression has also been seen in several cancer cell lines including gastric cancer (Mikata et al., 2006), melanoma (Muthusamy et al., 2006), and by a different group in ovarian cancer (Menendez et al., 2007), which indicates that DNA methylation is likely a common regulatory mechanism. Correlations
between increased methylation of DNAJC15 and low to no MCJ expression have
been reported in brain cancer (Lindsey et al., 2006), breast cancer (Boettcher et al.,
2010), and non-transformed human peripheral blood mononuclear cells (PBMCs)
(Zimmermann et al., 2016).

Interestingly, differences have been found for the specific region of the
DNAJC15 gene that must be methylated in order to silence expression. In ovarian
cancer cells, reduced MCJ expression correlated with increased methylation of a
CpG island in exon 1, however expression was independent of the methylation
status of the promoter (Strathdee et al., 2004). A similar pattern was found in brain
cancer and melanoma, where methylation within the exon 1 CpG island strongly
correlated with lower MCJ expression (Lindsey et al., 2006; Muthusamy et al.,
2006). However, an analysis of transcriptomic data of tumors from a large number
of breast cancer patients showed a strong correlation of increased DNA
methylation at three sites in the proximal promoter region with decreased MCJ
expression (Fernandez-Cabezudo et al., 2016). Additionally, reduced acetylation of
histone markers in both the promoter and exon 1 has been associated with
increased methylation of the exon 1 CpG island in ovarian cancer, indicating that
alterations to overall chromatin structure likely play a role in the regulation of MCJ
expression (Strathdee et al., 2004). Therefore, cell and/or tissue specific differences
in the methylation status of DNAJC15 contribute to the regulation of MCJ
expression, at least in cancer cells.
It is possible that heterogeneity in DNA methylation within tissues also leads to differential MCJ expression. Variability in MCJ expression among the tumor cells of breast cancer patients has been reported, however the mechanism responsible for this heterogeneity was not addressed (Fernandez-Cabezudo et al., 2016). Compared to normal kidney tissue, Wilm’s tumors from the same patient show hypermethylation of DNAJC15 (Ehrlich et al., 2002). Differences in the degree of DNA methylation between grades of ovarian cancer have been shown (Houshdaran et al., 2010). Validation of this proposal and whether the regulation of MCJ expression by DNA methylation can be extended to nontransformed tissues are topics for future studies.

DNAJC15 methylation has also been shown to vary among individuals and exhibits a pattern of heritability. An analysis of twins with Dutch ancestry showed that the DNA methylation pattern of DNAJC15 is significantly heritable (Boks et al., 2009). This was also seen in a cohort of individuals of Mexican American descent (Carless et al., 2013). Lower DNAJC15 methylation rates have been reported in the breast tumors of African American versus non-African American patients (Conway et al., 2015). In addition, variable expression of MCJ alleles between European and African populations has been attributed to a single nucleotide polymorphism in exon 1 (Tian et al., 2018). Therefore, the regulation of MCJ expression at the gene level can vary by cell and tissue type as well as genetic background.
4.4.2. Signaling molecules and transcription factors

Little is known of regulatory factors that control MCJ expression as there have been only a few studies on the signaling molecules and pathways involved. The cytokine IL-6 has been shown to downregulate MCJ expression in breast and liver cancer cell lines, however the mechanistic connection IL-6 signaling and MCJ expression is currently unknown (Hatle et al., 2007; Navasa, Martin-Ruiz, et al., 2015). IL-6 has been shown to upregulate DNA methyltransferase 1 (DMNT1) activity (Li et al., 2012), therefore alterations in DNA methylation may be involved. In addition, the cytokine IFN-γ reduces MCJ expression in macrophages through a mechanism dependent on the transcription factor Ikaros family zinc finger protein 1 (Ikaros) (Navasa, Martin-Ruiz, et al., 2015). IFN-γ signaling activates casein kinase 2 (CK2), which regulates Ikaros activity via phosphorylation (Navasa, Martin-Ruiz, et al., 2015). The activation of this pathway may be specific to macrophages, however, as no changes in MCJ were detected in a liver cancer cell line or primary CD8+ T cells after incubation with IFN-γ (Navasa, Martin-Ruiz, et al., 2015).

Ikaros has been shown to regulates the development of several types of immune cells, including macrophages, T cells, and B cells (Westman et al., 2002). Two potential binding sites for Ikaros have been identified that are upstream of the transcriptional start site in the mouse MCJ promoter (Navasa, Martin-Ruiz, et al., 2015). Binding of Ikaros to the site proximal to the promoter was confirmed in macrophages (Navasa, Martin-Ruiz, et al., 2015) and as shown later in this thesis,
in CD8$^+$ T cells (CHAPTER 2). Ikaros directed downregulation of MCJ was also shown to be independent of DNA methylation (Navasa, Martin-Ruiz, et al., 2015).

4.5. Localization to mitochondria

Initial studies investigating MCJ expression were performed using an overexpression system, which led to the conclusion that MCJ localized to the Golgi apparatus and other nonidentifiable vesicles (Hatle et al., 2007). It was later determined that overexpression of MCJ causes mitochondrial dysfunction and swelling that substantially alters mitochondrial morphology (Hatle et al., 2013). Several reports have since established that endogenously derived MCJ localizes to the mitochondria (Calvo et al., 2016; Hatle et al., 2013; Navasa, Martin, et al., 2015; Pagliarini et al., 2008; Schusdziarra et al., 2013).

The first indication that MCJ is in a mitochondrial compartment was a large-scale mitochondrial proteome analysis that detected MCJ in the mitochondria of several different tissues (MitoCarta) (Pagliarini et al., 2008). A follow up to this report (MitoCarta2.0) confirmed these results (Calvo et al., 2016). This finding was also shown in biochemical analyses of murine MCJ in the heart and CD8$^+$ T cells (Hatle et al., 2013). The localization of MCJ is not affected by cellular transformation as it has also detected in the mitochondria of several cancer cell lines (Hatle et al., 2013; Navasa, Martin, et al., 2015; Schusdziarra et al., 2013).

An *in silico* analysis of the MCJ amino acid sequence indicated that it does not contain any currently known mitochondrial targeting sequences (Schusdziarra
et al., 2013). MCJ is, however, predicted to contain a transmembrane domain, indicating that it is an integral membrane protein (Hatle et al., 2007; Schusdziarra et al., 2013). Within mitochondria, MCJ localizes to the inner mitochondrial membrane (Hatle et al., 2013; Schusdziarra et al., 2013; Sinha et al., 2014), which has been shown in both mouse (heart and CD8+ T cells) and human cells (HeLa and HEK 293T cells) (Hatle et al., 2013; Schusdziarra et al., 2013; Sinha et al., 2014). MCJ contains a single putative transmembrane domain (Hatle et al., 2007; Schusdziarra et al., 2013), indicating that the N- and C-termini face opposite sides of the inner mitochondrial membrane, however the precise orientation of the termini is currently unclear as conflicting results have been published in the literature.

Using a Hidden Markov model that predicts protein topology, the N-terminus of MCJ was predicted to be in an intracellular compartment, while the C-terminus was predicted to be in an extracellular compartment (Hatle et al., 2007). Therefore based on the results of this prediction algorithm, MCJ is a single pass transmembrane protein with its N-terminus in the inner membrane space and C-terminus in the matrix (Hatle et al., 2007).

A biochemical analysis of mitochondria and mitoplasts did not agree with this prediction (Schusdziarra et al., 2013). Mitoplasts are generated by removing the outer mitochondrial membrane, while the inner membrane is left intact. Proteinase treatment induced the loss of the N-terminus of MCJ in mitoplasts, indicating it resides in the inner membrane space (Schusdziarra et al., 2013). A major limitation, however, is that untreated mitoplasts were not shown or
indicated, therefore it cannot be excluded that MCJ loss may have occurred during mitoplast generation.

In contrast, other studies have reported results that agree with the above topology prediction. Phage display analysis using the N-terminal sequence of MCJ as bait indicated that MCJ likely interacts with the Complex I subunit NDUFv1 (Hatle et al., 2013), which is found in the matrix. Additionally, later in this dissertation (CHAPTER 3) it will be shown that a mimetic of MCJ that is targeted to the matrix can restore the function of MCJ in deficient cells. Future studies will clarify these apparent discrepancies in the orientation of MCJ in the inner mitochondrial membrane.

4.6. Function

As stated above, MCJ is member of the DNAJ family of co-chaperones that is found in mitochondria. All members of this family contain a canonical DNAJ domain, however their specific function(s) are mediated by non-conserved regions. For MCJ, the functional domain is thought to be in the N-terminus as it has no significant homology to any other known eukaryotic protein.

4.6.1. Negative regulation of mitochondrial metabolism

Biochemical analyses have shown that MCJ is a negative regulator of Complex I (Hatle et al., 2013). As a consequence of increased Complex I activity in the absence of MCJ, cells experience significantly higher mitochondrial membrane potential and ATP production rates (Barbier-Torres et al., 2017; Champagne et al.,
A concomitant increase in mitochondrial oxygen consumption after MCJ loss has also been shown (Barbier-Torres et al., 2017; Champagne et al., 2016; Navasa, Martin, et al., 2015). These effects of MCJ deficiency have been shown in a number of tissues and cell types, including the heart, liver, macrophages, T cells, and breast cancer cells (Barbier-Torres et al., 2017; Hatle et al., 2013; Navasa, Martin, et al., 2015). Later in this dissertation, the negative regulation of mitochondrial metabolism in CD8+ T cells (CHAPTER 2) and other types of cancer (CHAPTER 3) will be described.

As described previously (§1.2.3), the complexes of the ETC can associate into supramolecular structures known as respiratory supercomplexes. MCJ deficiency increases the abundance of supercomplexes in the heart, indicating that it is refractory to their formation (Hatle et al., 2013). Additionally, biochemical analyses have revealed that MCJ only associates with Complex I in its monomeric form and that it does not bind Complex I in supercomplexes (Hatle et al., 2013). This dissertation will also describe the regulation of supercomplexes by MCJ in CD8+ T cells (CHAPTER 2).

4.6.2. Regulation of liver metabolism

MCJ is highly expressed in the liver of both mice and humans, however no obvious differences are observed between the livers of wild-type and MCJ deficient mice under normal physiological conditions (Hatle et al., 2013). This indicates that MCJ is not essential for normal liver function. In contrast, substantial
differences are seen in response to fasting conditions. During a fast, the liver releases glucose derived from its stores of glycogen for use as an energy source by other cells of the body. If glycogen becomes limited, adipocytes begin to release fatty acids. Hepatocytes then import these released fatty acids for repackaging and release in a form usable by other cells (Berg et al., 2002; Voet and Voet, 2011). In extreme conditions, such as prolonged fasting, fatty acids build up beyond the capacity for efficient repackaging producing a condition known as steatosis (fatty liver).

Using a mouse model of steatosis, it was shown that MCJ deficiency prevents steatosis as hepatocytes have an increased capacity to metabolize fatty acids (Hatle et al., 2013). Loss of MCJ also reduced the levels of free fatty acids and triglycerides in the serum, indicating a systemic overall improvement (Hatle et al., 2013). In humans, nonalcoholic fatty liver disease (NAFLD) is a spectrum disorder of the liver (Rinella, 2015). Mild cases present as chronic steatosis, while liver inflammation and fibrosis are symptoms of progressed disease. While MCJ significantly affects the development of steatosis in mice, it remains to be determined whether MCJ plays a role in NAFLD.

A number of therapeutic drugs have off-target effects on mitochondria, with a notable example being acetaminophen. Overdose is currently the primary cause of acute liver failure (AFL) and a major cause of drug induced liver injury (DILI). Furthermore, a significant proportion of liver transplants are secondary to acetaminophen overdose induced AFL. Hepatotoxicity due to acetaminophen

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overdose is complex and not fully understood (McGill and Jaeschke, 2013; Yoon et al., 2016), however it is correlated with impaired mitochondrial respiration and increased oxidative stress due to the production of reactive intermediates (notably NAPQI) and ROS. N-acetylcysteine (NAC), the only available treatment, mitigates liver damage by supplementing the cellular antioxidant pool, however treatment is only effective within the first 8 – 10 hours of the overdose. Thus, there is a significant need for other therapies for acetaminophen poisoning.

In a mouse model of acetaminophen overdose, MCJ deficiency lead to lower levels of hepatocyte death, liver damage, serum transaminase levels, and liver inflammation (Barbier-Torres et al., 2017). This protective effect was correlated to lower ROS production, reduced oxidative stress, and increased Complex I activity (Barbier-Torres et al., 2017). Importantly, treatment with an siRNA targeting MCJ up to 24 hours post drug exposure significantly attenuated liver toxicity and inflammation (Barbier-Torres et al., 2017), indicating that MCJ is a viable therapeutic target for drug induced liver toxicity. This was further supported by the detection of increased levels of MCJ in the liver of patients experiencing DILI relative to healthy controls (Barbier-Torres et al., 2017). Additionally, MCJ expression was highest in DILI patients overdosing on drugs known to be mitotoxic compared to overdoses due to drugs that are not classified as hazardous to mitochondria (Barbier-Torres et al., 2017).
4.6.3. Regulation of immune cell metabolism

CD8+ T cells play a significant role in protection from intracellular pathogens. Compared to naïve CD4+ T cells, CD8+ T cells abundantly express MCJ (Hatle et al., 2013). As expected, this leads to lower mitochondrial membrane potential and Complex I activity in these cells (Hatle et al., 2013). The role of MCJ in CD8+ T cell immune effector responses is a later focus of this dissertation (CHAPTER 2).

MCJ is also expressed by macrophages and has a profound effect on their immune effector functions. Loss of MCJ in macrophages decreases production of TNF after engagement of multiple TLR ligands, including LPS (Navasa, Martin, et al., 2015). Interestingly, the decrease in TNF secretion was due to reduced activity of the metalloprotease tumor necrosis factor α converting enzyme (TACE) rather than decreased Tnf gene expression or TNF protein production (Navasa, Martin, et al., 2015). Using a mouse model of fulminant acute liver failure, it was shown that reduced TNF production in the absence of MCJ is protective (Navasa, Martin, et al., 2015). MCJ loss in macrophages is therefore beneficial to protection from inflammatory insults.

4.6.4. Role in chemoresistance

The loss of MCJ expression, however, is not always beneficial. As described previously, increased methylation of the DNAJC15 gene and/or reduced MCJ expression has been shown in a number of different cancers (§4.4), however only
a few studies have shown functional consequences of these differences. The first report of MCJ showed that restoration of its expression induced sensitivity to commonly used chemotherapeutic agents in ovarian cancer cell lines, indicating that loss of MCJ had conferred resistance to these drugs (Shridhar et al., 2001). This correlation has also been seen in breast cancer as a chemoresistant derivative of MCF7 cells exhibited increased DNAJC15 methylation and lower MCJ expression relative to the parental cell line (Boettcher et al., 2010). The ability to prevent doxorubicin accumulation, thereby mediating chemoresistance, was also increased in MCJ deficient breast cancer cells (Hatle et al., 2007). These findings were supported using mouse models as the tumor cells of MCJ deficient mouse mammary tumor virus (MMTV-PyMT) mice were also highly resistant to chemotherapy (Fernandez-Cabezudo et al., 2016).

A retrospective study of breast cancer patients indicated that the levels of MCJ expression in tumors was predictive of relapse free survival after chemotherapy treatment (Fernandez-Cabezudo et al., 2016). Furthermore, a prospective analysis of breast cancer patients revealed that MCJ expression correlated with increased responses to neoadjuvant therapy (Fernandez-Cabezudo et al., 2016). In addition, higher levels of DNAJC15 methylation correlated with poor responses to chemotherapy and reduced long term survival in ovarian cancer patients (Strathdee et al., 2005). While the exact mechanism(s) are unclear, loss of MCJ correlates with increased expression of ATP binding cassette (ABC) transporters (Hatle et al., 2007). The mechanistic connection between the
regulation of mitochondrial metabolism by MCJ and ABC transporter mediated chemoresistance is addressed later in this dissertation (CHAPTER 3).

4.6.5. Other potential functions

MCJ has been reported to affect the import of mitochondrial proteins (Schusdziarra et al., 2013; Sinha et al., 2014). The import of nuclear encoded proteins into mitochondria is facilitated by the translocase of the inner membrane (TIM), formed by the association of the TIM22 complex at the inner membrane and TIM23 complex in the matrix (Dudek et al., 2013). Protein transport requires the function of DNAJC19 in yeast (Mokranjac et al., 2003), therefore a potential role for MCJ was investigated as they share similar evolutionary origins as described previously (§4.3). MCJ was shown to interact with and stimulate regulators of the TIM23 complex in cell free assays, however the loss of MCJ was dispensable for import in whole cells (Schusdziarra et al., 2013; Sinha et al., 2014). MCJ is therefore nonessential for mitochondrial protein import in intact cells.

MCJ has also been reported to influence the activity of the mitochondrial permeability transition pore (MPTP) (Sinha and D'Silva, 2014). Mitochondria have a significant role in the induction of apoptosis via the release of factors through the MPTP complex, of which expression and increased activity are promoted by cell death stimuli (Bonora and Pinton, 2014). While the molecular components and regulators of the MPTP complex are still a matter of debate, peptidylprolyl isomerase D (also called cyclophilin D or CypD) is a known essential activator
(Baines and Gutierrez-Aguilar, 2018). MCJ have been shown to directly interact with CypD and recruits it to the MPTP complex, which thereby enhances MPTP activity in response to cisplatin treatment (Schusdziarra et al., 2013). However, these studies were performed using overexpression systems that, as described previously (§4.5), can dysregulate the localization of MCJ and mitochondrial morphology. Thus, further studies on the potential role of MCJ in the MPTP complex are needed.

5. Specific aims of this dissertation

5.1. Aim 1

_Determine the role of MCJ in CD8+ T cell immune responses._ MCJ is a negative regulator of Complex I activity that is abundantly expressed by CD8+ T cells relative to other leukocytes (Hatle et al., 2013; Navasa, Martin, et al., 2015). As such, loss of MCJ expression in these cells leads to increased mitochondrial respiration, including higher membrane potential (Hatle et al., 2013). However, the impact of these changes on the immune function of CD8+ T cells was unknown. The goal of this aim was to determine how the regulation of mitochondria by MCJ influences the development, activation, proliferation, and effector functions of CD8+ T cells in the context of viral immunity.
5.2. Aim 2

*Determine the role of MCJ in maintenance or induction of chemoresistance.*

Decreased MCJ expression in cancer cells is correlated with a chemoresistant phenotype and poor patient responses to chemotherapy (Fernandez-Cabezudo *et al.*, 2016; Hatle *et al.*, 2007; Strathdee *et al.*, 2005). This has been attributed to increased ABC transporter expression in the absence of MCJ (Hatle *et al.*, 2007), however the mechanistic connection to chemoresistance was unclear. The goal of this aim was to address how increased mitochondrial respiration due to MCJ deficiency in cancer leads to chemoresistance.

6. References


CHAPTER 2. Fine-Tuning of CD8+ T cell Mitochondrial Metabolism by the Respiratory Chain Repressor MCJ Dictates Protection to Influenza Virus

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1. Summary

Mitochondrial respiration is regulated in CD8+ T cells during the transition from naive to effector and memory cells, but mechanisms controlling this process have not been defined. Here we show that MCJ (methylation-controlled J protein) acted as an endogenous break for mitochondrial respiration in CD8+ T cells by interfering with the formation of electron transport chain respiratory supercomplexes. Metabolic profiling revealed enhanced mitochondrial metabolism in MCJ-deficient CD8+ T cells. Increased oxidative phosphorylation and subcellular ATP accumulation caused by MCJ deficiency selectively increased the secretion, but not expression, of interferon-γ. MCJ also adapted effector CD8+ T cell metabolism during the contraction phase. Consequently, memory CD8+ T cells lacking MCJ provided superior protection against influenza virus infection. Thus, MCJ offers a mechanism for fine-tuning CD8+ T cell mitochondrial metabolism as an alternative to modulating mitochondrial mass, an energetically expensive process. MCJ could be a therapeutic target to enhance CD8+ T cell responses.

2. Introduction

Metabolism is emerging as a major factor that regulates the function and differentiation of immune cells and influences the course of an immune response (Pearce et al., 2013; van der Windt and Pearce, 2012; Wang and Green, 2012). Naive, effector, and memory T cell subsets have distinct metabolic profiles to provide the
energy and bioenergetic precursors required for cell growth and expansion. Naive cells use glucose and free fatty acids (FFA) as sources of ATP through mitochondrial oxidative phosphorylation (OXPHOS) (van der Windt et al., 2012; Wang et al., 2011). After activation, CD8+ T cells undergo a metabolic reprogramming and switch to glycolysis as a source of ATP. Effector T cells can also use glutamine to generate ATP through glutaminolysis, which can further fuel OXPHOS (Carr et al., 2010; Wang et al., 2011). Proliferation of effector CD8+ T cells appears to be more dependent on glucose than effector CD4+ T cells (Frauwirth et al., 2002; Macintyre et al., 2011). In contrast, production of some cytokines by effector CD8+ T cells is not affected by a strong inhibition of glycolysis (Cham et al., 2008), and cytotoxic activity can occur in the absence of glucose (MacDonald and Koch, 1977).

Effector CD8+ T cells further reprogram metabolism during memory cell generation in response to antigen and cytokine withdrawal. Memory CD8+ T cells primarily use FFA oxidation in mitochondria as the main energy pathway (Araki et al., 2009; Pearce et al., 2009; van der Windt et al., 2012). Additionally, memory CD8+ T cells manifest a greater increase in both OXPHOS and aerobic glycolysis following activation compared with naive cells, and the induction of glycolysis is dependent on mitochondrial ATP (van der Windt et al., 2013). Importantly, intervention of metabolism with metformin (AMPK activator) or rapamycin (mTOR inhibitor) to promote FFA oxidation enhances the generation of memory CD8+ T cells and protection against viral infection (Araki et al., 2009; Pearce et al., 2009; van der Windt et al., 2013).
A recent study revealed that memory CD8+ T cells have developed their own intrinsic pathways to mobilize fatty acids for oxidation (Pearce et al., 2009). Considering this highly dynamic metabolic reprogramming, CD8+ T cells likely utilize specific checkpoints to regulate these transitions and their effector functions. However, while a number of studies have addressed the effect of different metabolic substrates that fuel the mitochondrial electron transport chain (ETC), little is known about endogenous mechanisms that control mitochondrial respiration and, thereby, the immune response.

Methylation controlled J protein (MCJ), a protein encoded by the DNAJC15 gene, is a member of the DnaJ family of chaperones. MCJ is a small protein with features that distinguish it from other DnaJ family members. While most DnaJ family members are soluble proteins, MCJ contains a transmembrane domain and has a unique N-terminal domain that shares no significant sequence similarity with any other known protein. MCJ was first reported in ovarian cancer cells as a gene negatively regulated by methylation (Shridhar et al., 2001; Strathdee et al., 2004). Loss of MCJ is associated with chemoresistance of human breast and ovarian cancer cell lines (Hatle et al., 2007; Shridhar et al., 2001; Strathdee et al., 2005). We recently showed that MCJ is abundantly expressed primarily in tissues with a highly active mitochondrial metabolism, including heart and liver (Hatle et al., 2013). Within the immune system, MCJ is highly expressed in CD8+ T cells, but not in CD4+ T and B cells (Hatle et al., 2013), and less in macrophages (Navasa, Martin, et al., 2015). Importantly, MCJ localizes to the inner membrane of
mitochondria (Hatle et al., 2013; Schusdziarra et al., 2013) and acts as a negative regulator of the ETC. MCJ deficiency in vivo results in increased complex I activity and mitochondrial membrane potential (MMP) without affecting mitochondrial mass (Hatle et al., 2013). Endogenous MCJ in primary tissues associates with complex I and acts as a natural inhibitor, making MCJ one of the first described endogenous negative regulators of complex I. The activity of complex I is enhanced by assembly in “respirasomes,” which are mitochondrial ETC supercomplexes containing complexes I, III, and IV (Acin-Perez et al., 2008). Supercomplexes facilitate efficient transfer of electrons to enhance complex I activity and minimize electron “leak” that results in ROS production (Moreno-Lastres et al., 2012). We have shown that MCJ interferes with the formation of these supercomplexes in heart (Hatle et al., 2013), a mechanism to inhibit complex I activity and MMP.

Although MCJ is abundantly present in CD8+ T cells, its role in regulating mitochondrial metabolism and function of these cells is unknown. Here, we show that MCJ acts a negative regulator of mitochondrial respiration in CD8+ T cells. MCJ deficiency did not affect proliferation of naive CD8+ T cells upon activation, nor activation marker or cytokine gene expression. However, increased OXPHOS in MCJ-deficient CD8+ T cells enhanced the secretion of cytokines and sustained the metabolic state of effector CD8+ T cells during the contraction phase. MCJ-deficient memory CD8+ T cells had greater protective capacity against influenza
virus infection. Therefore, MCJ is emerging as an important negative regulator of mitochondrial activity of CD8\(^+\) T cells.

3. Results

3.1. Loss of MCJ Promotes Respiratory Supercomplexes and Mitochondrial Metabolism in Naive CD8\(^+\) T cells

To investigate the role of MCJ in CD8\(^+\) T cell development and function, we used MCJ-deficient mice previously described to have no obvious phenotypic alterations under physiological conditions (Hatle et al., 2013). Although CD8\(^+\) T cells freshly isolated from MCJ-deficient mice display higher mitochondrial membrane potential (MMP) (Supplementary Figure 2-1A), there was no difference in the percentage (Supplementary Figure 2-1B) or number (data not shown) of CD8\(^+\) or CD4\(^+\) T cells in the spleen and lymph nodes (LN) of WT and MCJ-deficient mice. No difference in the expression of activation markers such as CD44 was observed (Supplementary Figure 2-1C). The percentage (Supplementary Figure 2-1B) and number (data not shown) of single-positive, double-positive (DP), or double-negative (DN) populations in the thymus were also comparable. Thus, MCJ deficiency does not affect the development of CD8\(^+\) and CD4\(^+\) T cells in the thymus or homeostasis in the periphery.

MMP is the driver for oxidative phosphorylation (OXPHOS), generation of ATP, and oxygen consumption. To investigate the impact that increased MMP in MCJ-deficient CD8\(^+\) T cells has on mitochondrial respiration, we examined the
oxygen consumption rate (OCR) in freshly isolated CD8+ T cells using the Seahorse MitoStress assay. Correlating with the increased MMP, OCR was also elevated in MCJ-deficient CD8+ T cells compared with WT CD8+ T cells (Figure 2-1A). In contrast, the extracellular acidification rate (ECAR), a parameter for glycolysis, was not affected in MCJ-deficient CD8+ T cells (Figure 2-1B). Mitochondrial ATP production, determined by subtracting the OCR in the presence of oligomycin (Complex V/ATP Synthase inhibitor) from the OCR at baseline, was also higher in MCJ-deficient CD8+ T cells (Figure 2-1C). Thus, MCJ is a negative regulator of mitochondrial respiration in CD8+ T cells.

To identify the impact of MCJ on the overall metabolism of naive CD8+ T cells, we performed nonbiased high-throughput metabolic profiling. Metabolome analysis of CD8+ T cells freshly isolated from WT and MCJ-deficient mice showed a large number of metabolic intermediates from different pathways equally present in both (Supplementary Table 2-1). However, there was a significant increase in the amounts of several amino acids in MCJ-deficient CD8+ T cells (Figure 2-1D). Most of the amino acids that were significantly elevated belonged to the essential amino acid group (Figure 2-1D). In contrast, only tyrosine in the conditionally essential amino acid group and asparagine in the non-essential group were significantly increased (Figure 2-1D).

The preferential accumulation of amino acids that need to be imported suggested a greater amino acid transport occurred in the absence of MCJ. Since amino acid transport is highly dependent on ATP, increased mitochondrial
OXPHOS due to MCJ deficiency could be responsible for this transport. To investigate this, we incubated freshly isolated CD8+ T cells in culture medium. After 16 hours, the metabolic profile of the culture supernatants was analyzed to determine the amino acid consumption. The amounts of amino acids were significantly reduced in the culture supernatant of MCJ-deficient CD8+ T cells (Figure 2-1E). In addition, metabolic flux analyses with $^{13}$C- and $^{15}$N-labeled amino acids showed increased uptake of extracellular $[^{13}$C, $^{15}$N]-glutamine (Figure 2-1D). Thus, the increased mitochondrial respiration resulting from MCJ deficiency in naive CD8+ T cells in vivo promotes amino acid uptake.

The other metabolite significantly increased in MCJ-deficient CD8+ T cells was succinate (Figure 2-1F and Supplementary Table 2-1), which is oxidized to fumarate by succinate dehydrogenase (ETC complex II) in the TCA cycle. However, the amounts of fumarate and the other components of the TCA cycle were not different (Figure 2-1F). These results suggested that elevated succinate in MCJ-deficient CD8+ T cells was the result of impaired complex II activity. Indeed, reduced complex II activity was found in freshly isolated MCJ-deficient CD8+ T cells as measured by OCR in response to succinate using a Seahorse Extracellular Flux analyzer (Figure 2-1G). In addition, metabolic flux analyses of freshly isolated CD8+ T cells incubated with $^{13}$C- and $^{15}$N-labeled amino acids revealed an increase in newly synthesized succinate, but not fumarate (the product of complex II), in MCJ-deficient CD8+ T cells (Figure 2-1H). Thus, complex II appears to be uncoupled from the rest of the ETC in MCJ-deficient CD8+ T cells.
We have shown that the absence of MCJ in heart favors the accumulation of mitochondrial respiratory supercomplexes (Hatle et al., 2013), composed of complexes I, III, and IV, but not complex II. To investigate whether supercomplexes were increased in naive MCJ-deficient CD8+ T cells, we generated mitochondrial extracts with digitonin to preserve supercomplexes and resolved them by blue native electrophoresis (BNE) as previously described (Yang et al., 2015). The supercomplex region was excised, resolved by SDS-PAGE, and analyzed by Western blot for subunits of complexes I (NDUFA9) and IV (CoxIV). The amount of supercomplexes were higher in MCJ-deficient CD8+ T cells (Figure 2-II). Monomeric complex IV analyzed as a control and was not different (Figure 2-II). To further support the accumulation of supercomplexes in MCJ-deficient CD8+ T cells, we resolved mitochondrial extracts by BNE, followed by immunoblot analysis for subunits of complexes I (NDUFA9) and III (Core1). Increased NDUFA9 amounts were present in the supercomplex region in MCJ-deficient CD8+ T cells, while the amounts of NDUFA9 in the monomeric complex I region were comparable between MCJ-deficient and WT cells (Figure 2-IJ). Similar results were obtained for Core1 (Figure 2-IJ). Thus, loss of MCJ promotes the formation of respiratory supercomplexes in naive CD8+ T cells. Uncoupling complex III from complex II could compromise complex II activity and cause the observed accumulation of succinate. Together, these results indicate that the function of MCJ in naive CD8+ T cells is to restrict mitochondrial metabolism, and a deficiency in MCJ alters normal mitochondrial metabolism.
Figure 2-1. MCJ Restrains Mitochondrial Respiration in Naive CD8+ T Cells
(A) Baseline OCR, (B) ECAR, and (C) OCR linked to mitochondrial ATP production (baseline OCR minus OCR in the presence of oligomycin) of freshly isolated cells as determined by MitoStress (A and C) or Glycolysis Stress assays (B). (D and F) Metabolic profiles of cells were determined by UPLC-MS analysis. Relative amounts of (D) amino acids and (F) TCA cycle metabolites. (E) Equal numbers of cells were incubated in culture medium for 16 hours, and metabolic profiles of the culture supernatants were determined. (G) Complex II activity was determined by examining OCR at baseline and in the response to succinate with rotenone (Succ + Rote) and malonate. (H) Cells were cultured with $^{13}$C- and $^{15}$N-labeled amino acids, and metabolic flux was determined by UPLC-MS analysis. $^{13}$C-succinate and $^{13}$C-fumarate peak areas shown. (I and J) Mitochondrial extracts were resolved by BNE. (I) Bands corresponding to supercomplexes (SC) or monomeric complex IV were excised, resolved by SDS-PAGE, and examined by Western blot analysis for NDUFA9 (complex I) and CoxIV (complex IV). (I) Proteins separated by BNE were examined by Western blot analysis for NDUFA9 and Core1 (complex III). Bands corresponding to supercomplexes (SC) and monomeric complexes I (CI) and III (CIII) regions of the Western blot shown. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. Results are representative of 2 - 3 experiments. See also Supplementary Figure 2-1 and Supplementary Table 2-1.

3.2. MCJ Deficiency Does Not Affect Proliferation, but Enhances the Secretion of Cytokines in Activated CD8$^+$ T cells

We investigated whether the enhanced mitochondrial metabolism found in MCJ-deficient CD8$^+$ T cells could alter proliferation. Freshly isolated CD8$^+$ T cells were stained with CFSE and activated with anti-CD3 and anti-CD28 antibodies, and proliferation was analyzed by flow cytometry. No differences were observed in the frequency of proliferating cells or number of cell divisions (Figure 2-2A), the survival of cells after 2 days of activation (Figure 2-2B), or expression of the cell surface activation markers CD69 and CD25 (Figure 2-2C). In contrast, greater IFN-γ (Figure 2-2D) and IL-2 (Figure 2-2E) production was detected in activated MCJ-deficient CD8$^+$ T cells as determined by ELISA. Thus, loss of MCJ does not interfere with activation or expansion of CD8$^+$ T cells, but results in a greater production of cytokines.
Production of cytokines by CD8+ T cells upon activation is primarily regulated at the level of gene expression either by transcription or mRNA stability. However, analysis of cytokine mRNA by quantitative RT-PCR showed no difference in the amounts of IFN-γ and IL-2 mRNA between activated MCJ-deficient and WT CD8+ T cells (Figure 2-2F). Thus, MCJ had no effect on cytokine gene expression in CD8+ T cells. A recent study reported that aerobic glycolysis promotes IFN-γ production at the translational level in effector CD4+ T cells (Chang et al., 2013). However, intracellular staining analysis for IFN-γ showed no difference between WT and MCJ-deficient CD8+ T cells (Figure 2-2G). Similarly, there was no difference in IL-2 intracellular staining (Figure 2-2G). Thus, the increased amounts of cytokines in MCJ-deficient CD8+ T cell supernatants were not due to increased gene or protein expression.

Secretion of cytokines is another mechanism that regulates the overall amount of cytokines being produced, although little is known about the pathways involved. To investigate whether the increased IFN-γ in the supernatants of MCJ-deficient CD8+ T cells was caused by enhanced secretion, we activated CD8+ T cells with anti-CD3 and anti-CD28 antibodies for 2 days, washed, and then incubated equal numbers of cells in medium alone without any additional stimuli for different periods of time. IFN-γ in the supernatants from MCJ-deficient CD8+ T cells after 2 hours was higher than WT CD8+ T cells (Figure 2-2H). IFN-γ in MCJ-deficient CD8+ T cell supernatants continued increasing for at least 4 hours and
then remained constant, while the amount secreted by WT CD8+ T cells did not increase (Figure 2-2H). Treatment with cycloheximide (CHX), an inhibitor of protein synthesis, did not affect the IFN-\(\gamma\) produced during this period (Supplementary Figure 2-2A), although CHX prevented new protein synthesis triggered by the activation of naive CD8+ T cells (Supplementary Figure 2-2B). These results further support the conclusion that increased protein translation was most likely not the primary cause of enhanced IFN-\(\gamma\) production by MCJ-deficient CD8+ T cells. IL-2 and GM-CSF were also elevated in the supernatant of MCJ-deficient CD8+ T cells after 4 hours (Supplementary Figure 2-2C), indicating that the effect of MCJ on secretion is not restricted to IFN-\(\gamma\). To further confirm the enhanced capacity of secretion by MCJ-deficient CD8+ T cells, we performed ELISpot assays for IFN-\(\gamma\). The number of spots with a larger area (high IFN-\(\gamma\) producers) was increased in MCJ-deficient CD8+ T cells (Figure 2-2I). Enhanced secretion should result in lower accumulation of intracellular IFN-\(\gamma\) if no additional synthesis takes place. Intracellular staining for IFN-\(\gamma\) after activated cells were washed and incubated in medium alone showed lower IFN-\(\gamma\) in MCJ-deficient CD8+ T cells after 1 hour (Supplementary Figure 2-2D). Together these results indicate that MCJ deficiency augments cytokine production primarily by promoting the cytokine secretion capacity of effector CD8+ T cells.
Figure 2-2. MCJ Deficiency Does Not Affect Effector CD8+ T Cell Proliferation but Increases the Secretion of Cytokines

WT (blue) and MCJ-deficient CD8+ T cells (MCJ def, red) were activated for 2 days with anti-CD3 and anti-CD28 antibodies. (A) Proliferation determined by CFSE staining. Grey histograms show unstimulated cells. (B) Cell survival determined by Live Dead staining. (C) Cell surface expression of CD69 and CD25. Grey histograms show unstimulated cells. (D and E) Culture supernatants of activated cells were examined for (D) IFN-γ and (E) IL-2 by ELISA. (F) IL-2 and IFN-γ mRNA expression determined by qRT-PCR. (G) Intracellular staining for IFN-γ and IL-2. Grey histograms show unstimulated cells. (H) Activated cells were washed and replated at equal numbers in medium alone. IFN-γ in the culture supernatants over time was determined by ELISA. (I) Number of IFN-γ producing cells determined by ELISpot assay. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. Results are representative of 2 – 3 experiments. See also Supplementary Figure 2-2.
3.3. Localized Mitochondrial Production of ATP in the Absence of MCJ Promotes Cytokine Secretion

We used an unbiased metabolomics approach to investigate which metabolic pathways were regulated by MCJ in effector CD8\(^+\) T cells. Metabolic profiling was obtained from equal numbers of WT and MCJ-deficient CD8\(^+\) T cells after 2 days of activation and 4 hours of resting. Although no obvious phenotypic differences in terms of size or activation markers could be detected between the cell types, MCJ deficiency caused well-defined metabolic changes (Supplementary Table 2-2). Similar to naive CD8\(^+\) T cells, the absence of MCJ in activated CD8\(^+\) T cells resulted in increased amounts of amino acids, but this increase was not restricted to essential amino acids (Figure 2-3A). In addition, a number of intermediate metabolites of the TCA and urea cycle pathways, two of the main mitochondrial pathways, were among the most elevated components in activated MCJ-deficient CD8\(^+\) T cells relative to WT CD8\(^+\) T cells (Supplementary Table 2-2). Similar to naive CD8\(^+\) T cells, succinate was also drastically increased in activated MCJ-deficient CD8\(^+\) T cells, but fumarate, malate, and 2-hydroxyglutarate were also higher (Figure 2-3B). Citrulline and arginosuccinate, components of the urea cycle, were also increased (Figure 2-3C). Although there was no difference in proliferation, nucleotide pathways were also upregulated in the absence of MCJ (Figure 2-3D). Nucleotide synthesis is primarily cytosolic, however mitochondrial metabolic pathways feed into these pathways, as purine salvage reactions consume aspartate to produce fumarate and recover AMP upon deamination to
IMP. Thus, an unbiased metabolic screening revealed increased mitochondrial activity in MCJ-deficient effector CD8+ T cells.

Effector CD8+ T cells are primarily glycolytic and use ATP from glycolysis instead of mitochondrial OXPHOS as the major source of energy. Because the absence of MCJ enhances overall mitochondrial activity, we examined intracellular ATP. Higher amounts of total ATP were present in MCJ-deficient CD8+ T cells that were activated for 2 days and rested for 4 hours (Figure 2-4A). To determine whether this increased production of ATP resulted from increased mitochondrial respiration, we performed Seahorse MitoStress analysis. OCR was higher in activated MCJ-deficient CD8+ T cells, as shown by the effect of oligomycin on baseline OCR (Figure 2-4B). These data indicate that the rate of ATP synthesis by mitochondria was increased in the absence of MCJ. In contrast, analysis of ECAR in response to glucose showed no difference between the two cell types (Figure 2-4C). Thus, loss of MCJ promotes mitochondrial respiration without altering the glycolytic rate of effector CD8+ T cells.

The normal rate of glycolysis as determined by ECAR analysis correlated with the normal rate of proliferation and gene expression observed in MCJ-deficient CD8+ T cells. While glycolysis-derived ATP represents the predominant source of energy and is sufficient for these processes in activated cells, mitochondrial derived ATP could be essential for other CD8+ T cell functions such as cytokine secretion. Due to the dynamic characteristics of mitochondria, this organelle could provide a subcellular microenvironment rich in ATP without the
need to raise total cytosolic ATP. We investigated the presence of ATP-rich microdomains within activated CD8+ T cells using a fluorescent probe used to identify ATP and ADP intracellular accumulation. Confocal microscopy of live cells showed only a few punctate ATP probe accumulations in activated WT CD8+ T cells, while ATP puncta were abundant and prominent in MCJ-deficient CD8+ T cells (Figure 2-4D). The subcellular ATP accumulation in MCJ-deficient CD8+ T cells represented the mitochondrial derived ATP pool since treatment with oligomycin prevented their formation (Supplementary Figure 2-3A). Moreover, costaining with the ATP probe and Mitotracker, a mitochondrial marker, revealed colocalization of the ATP puncta with mitochondria in activated MCJ-deficient CD8+ T cells (Figure 2-4E). Thus, MCJ deficiency facilitates the formation of ATP-rich microdomains within activated CD8+ T cells.

To address whether the increased secretion of IFN-γ found in MCJ-deficient CD8+ T cells was mediated by increased mitochondrial ATP production, we activated CD8+ T cells for 2 days and treated them with oligomycin during the last 4 hours of activation. Cells were then washed and incubated at equal numbers in medium alone for 4 hours. IFN-γ in the supernatants was determined by ELISA. Inhibition of mitochondrial ATP synthesis by oligomycin suppressed the enhanced secretion of IFN-γ by MCJ-deficient CD8+ T cells (Figure 2-4F). In contrast, oligomycin did not reduce intracellular IFN-γ (Supplementary Figure 2-3B). Thus, MCJ acts as an endogenous negative regulator of mitochondrial
respiration, restricting the production of mitochondrial ATP and secretion of cytokines such as IFN-γ.

Figure 2-3. MCJ Deficiency Enhances Mitochondrial Metabolism in Effector CD8⁺ T Cells

(A – D) WT (blue) and MCJ-deficient (MCJ def, red) CD8⁺ T cells were activated for 2 days with anti-CD3 and anti-CD28 antibodies, and metabolic profiles were determined by UPLC-MS. Relative peak areas of (A) amino acids and metabolites of the (B) TCA cycle, (C) urea cycle, and (D) nucleotide pathways shown. *p < 0.05 by unpaired t test. Avg ± SD shown (n = 3 mice). See also Supplementary Table 2-2.
Figure 2-4 Increased Oxidative Phosphorylation in MCJ-Deficient Effector CD8\(^+\) T Cells Facilitates IFN-\(\gamma\) Secretion

WT (blue) and MCJ-deficient CD8\(^+\) T cells (MCJ def, red) were activated with anti-CD3 and anti-CD28 antibodies for 2 days. (A) ATP concentration in cells rested in medium without stimuli for 4 hours. (B) OCR of cells rested for 12 hours at baseline and in response to oligomycin (O), FCCP (F), and rotenone with antimycin (R+A) by Seahorse MitoStress assay. (C) ECAR in cells after addition of glucose by Seahorse Glycolysis Stress assay. (D) Live cells stained with ATP probe (green) were visualized by confocal microscopy. Right panels show a magnification of the cells indicated by the white arrows. (E) Live cells costained with ATP probe (green) and Mitotracker (red) were visualized by confocal microscopy. (F) Cells were incubated with oligomycin during the last 4 hours of activation and then rested for 4 hours. IFN-\(\gamma\) in the supernatants was determined by ELISA. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. Scale bar represents 10 nm. Results are representative of 2–3 experiments. See also Supplementary Figure 2-3.

3.4. MCJ Attenuates Mitochondrial Metabolism during the Contraction Phase of Effector CD8\(^+\) T cells In Vivo

CD8\(^+\) T cells reprogram their mitochondrial metabolism during the differentiation from naive to effector and from effector to memory stages. Memory CD8\(^+\) T cells also have greater mitochondrial mass and maximum respiratory
capacity (van der Windt et al., 2012). Because MCJ acts as an internal break for mitochondrial function in tissues with a high content of active mitochondria, we investigated MCJ expression during the contraction phase of effector CD8+ T cells. To examine MCJ expression in individual cells, we used MCJ-deficient mice because they contain a β-galactosidase reporter gene inserted in the MCJ locus, while WT CD8+ T cells served as negative controls (Supplementary Figure 2-3C). We performed β-galactosidase activity assays by flow cytometry analysis in CD8+ T cells that were freshly isolated, activated (effector), and rested in medium alone after activation (rested effector) to mimic the contraction phase of effector cells and development of memory cells. The frequency of MCJ expressing cells was dramatically decreased in effector cells relative to naive cells (Figure 2-5A). We recently showed that Ikaros, a transcriptional repressor known to attenuate gene expression (John and Ward, 2011), binds the DNAJC15 gene (encodes MCJ) promoter in macrophages to silence MCJ expression (Navasa, Martin-Ruiz, et al., 2015). Because Ikaros has been previously reported in CD8+ T cells (O’Brien et al., 2014), we performed chromatin immunoprecipitation analysis (ChIP) to determine whether Ikaros also binds the DNAJC15 promoter in CD8+ T cells. Relative to naive CD8+ T cells, greater Ikaros binding to the DNAJC15 promoter was found in activated CD8+ T cells (Figure 2-5B). Thus, MCJ is present in naive CD8+ T cells as an additional checkpoint to restrict potential effector function.
The frequency of CD8$^+$ T cells expressing MCJ increased again when effector cells were rested in the presence of medium without additional stimuli (Figure 2-5A). During the contraction phase, effector CD8$^+$ T cells modulate their metabolism, become smaller and less active, and most die except for a few that survive to become memory cells (D’Cruz et al., 2009). We examined whether the observed reacquisition of MCJ in resting effector CD8$^+$ T cells contributed to the attenuation of metabolism or cell survival in vitro during the contraction phase. CD8$^+$ T cells were activated for 2 days, washed, and then incubated in medium alone (without addition of cytokines). After 24 hours of resting, most WT and MCJ-deficient CD8$^+$ T cells remained alive (Figure 2-5C). However, after 48 hours only a few WT CD8$^+$ T cells remained alive, whereas many MCJ-deficient CD8$^+$ T cells were still viable even at 72 hours (Figure 2-5C). In addition, the surviving MCJ-deficient CD8$^+$ T cells maintain the “blastic cell stage” (large) reminiscent of effector cells (data not shown). Analysis of MMP in live cells after 48 hours of resting showed that a high proportion of WT CD8$^+$ T cells had low MMP (Figure 2-5D), similar to naive CD8$^+$ T cells (Supplementary Figure 2-1A). In contrast, a large fraction of rested effector MCJ-deficient CD8$^+$ T cells displayed high MMP (Figure 2-5D). To determine whether the increased survival in rested MCJ-deficient CD8$^+$ T cells in vitro was due to their intrinsic metabolic state (high MMP) or due to increased IL-2 production that can promote cell expansion, a blocking anti-IL-2 antibody was added during the resting period. No differences were detected in the survival of MCJ-deficient CD8$^+$ T cells after blocking IL-2
(Supplementary Figure 2-3D). Furthermore, the addition of small amounts of recombinant IL-2 during the resting period resulted in increased expansion of both WT and MCJ-deficient CD8+ T cells (Supplementary Figure 2-3E). Thus, reacquisition of MCJ in rested effector CD8+ T cells contributes to the reduction of mitochondrial activity, restoration of a quiescent metabolic state and fitness impairment of effector CD8+ T cells during the contraction phase in vitro.

To address the role of MCJ in the contraction phase of antigen specific effector CD8+ T cells in vivo, we crossed MCJ-deficient mice with OT-I TCR transgenic mice, which express a TCR that recognizes ovalbumin. MCJ deficiency did not affect the development of OT-I CD8+ T cells (Supplementary Figure 2-4A). Similar to polyclonal CD8+ T cells, naive MCJ-deficient OT-I CD8+ T cells also displayed high MMP relative to WT CD8+ T cells (Figure 2-5E). CD8+ T cells were purified from OT-I and MCJ-deficient OT-I mice, activated for 2 days in vitro, and then further expanded for 3 days. An equal number of cells from each genotype were combined and cotransferred into the same WT recipient mice (Figure 2-5F). After 2 weeks, donor cells present in LN and spleen of host mice were examined by flow cytometry using CD90.1 and CD90.2 markers (Supplementary Figure 2-4B and C). Phenotypic characterization of the cells based on CD44 and CD62L (memory and homing markers) as well as KLRG1 and CD127 (markers that define subsets of long lived effector and memory T cells (Jameson and Masopust, 2009; Kaech and Wherry, 2007; Sarkar et al., 2008)) did not show differences between WT and MCJ-deficient CD8+ OT-I T cells (Supplementary Figure 2-4B and C). The
overall percentage of cell recovery in both donors was comparable (data not shown). However, as determined by forward scatter (Figure 2-5G), only a few WT OT-I CD8+ T cells showed a large blastic phenotype (Figure 2-5H). In contrast, a greater fraction of MCJ-deficient OT-I CD8+ T cells displayed the large blastic phenotype (Figure 2-5H). Thus, in vivo MCJ does not seem to contribute to cell survival but does participate in the attenuation of metabolism during the transition from effector to rested effector cells.
Figure 2-5. MCJ Deficiency Sustains the Metabolic Activity of Effector CD8\(^+\) T Cells during the Contraction Phase

WT (blue) and MCJ-deficient CD8\(^+\) T cells (MCJ def, red). (A) Frequency of \(\beta\)-galactosidase\(^+\) MCJ-deficient CD8\(^+\) T cells that were naive, activated for 2 days with anti-CD3 and anti-CD28 antibodies, or activated for 2 days and then rested in medium without stimuli for 48 hours (2 d + 48 h) or 72 hours (2 d + 72 h) as determined by flow cytometry analysis. (B) Ikaros binding to the DNAJC15 gene (encodes MCJ) promoter in naive and activated (2 days) cells by ChIP assay. Fold increase over rabbit IgG immunoprecipitates relative to input shown. (C) Cells were activated for 2 days, washed, and rested in medium. The number of live cells recovered relative to the initial number is shown. (D) MMP was examined by TMRE staining in cells activated for 2 days and rested for 48 hours. (E) MMP in freshly isolated OT-I and MCJ-deficient OT-I CD8\(^+\) T cells. (F - H) OT-I (CD90.1\(^+\)) and MCJ-deficient OT-I (CD90.1\(^+\) CD90.2\(^+\)) CD8\(^+\) T cells were activated for 2 days and then expanded with IL-2 for 3 days. Equal numbers of each cell type were then adoptively transferred into WT (CD90.2\(^+\)) recipient mice. LN and spleen of
recipients were harvested after 2 weeks and analyzed for donor cells. (G and H) Forward scatter of CD8+ OT-I T cells as determined by flow cytometry analysis. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. Results are representative of 2 – 3 experiments. See also Supplementary Figure 2-3 and Supplementary Figure 2-4.

3.5. MCJ Deficiency Enhances the Antiviral Protective Activity of Memory CD8+ T cells

Memory CD8+ T cells are believed to play an important role in protection against influenza viral infection (La Gruta and Turner, 2014). Recent studies indicate that increased mitochondrial respiration in CD8+ T cells results in increased memory activity (van der Windt et al., 2013). We investigated the role of MCJ in protective memory CD8+ T cell responses using an influenza virus infection model. WT and MCJ-deficient mice were intranasally infected with a sublethal dose of influenza A/Puerto Rico/8/34 (PR8) H1N1 virus. As expected, based on the predominant role of innate immunity in primary infection with influenza virus, no significant differences were observed in weight loss and recovery or survival between infected WT and MCJ-deficient mice during primary infection (Figure 2-6A). Analysis of influenza NP-tetramer+ CD8+ T cells 2 weeks post-infection also showed a similar frequency between infected WT and MCJ-deficient mice (Figure 2-6B), indicating that the loss of MCJ did not affect the expansion of effector CD8+ T cells in vivo, consistent with the in vitro studies. However, ex vivo analysis of IFN-γ production by CD8+ T cells showed higher IFN-γ in the supernatants of MCJ-deficient CD8+ T cells (Figure 2-6C). Thus, MCJ
deficiency results in increased IFN-γ production by effector CD8+ T cells in vivo during virus infection.

We then addressed whether the lack of MCJ could affect memory CD8+ T cell development. Analysis of influenza NP-tetramer+ CD8+ T cells 3 and 5 weeks post-infection showed a lower percentage relative to 2 weeks post-infection as expected, but there was no difference in the frequency between WT and MCJ-deficient mice (Figure 2-6B and Supplementary Figure 2-5A). This further supported the finding that lack of MCJ does not affect cell survival during the generation of memory CD8+ T cells from effector cells in vivo. Phenotypic analysis of NP-tetramer+ CD8+ T cells for CD44, CD62L, KLRG1, and CD127 showed no substantial difference between WT and MCJ-deficient CD8+ T cells (Supplementary Figure 2-5B and data not shown). Lower CD27 expression was found in the MCJ-deficient CD8+ T cell memory population (Supplementary Figure 2-5B). CD27low memory cells mediate rapid protective immunity against acute infection and manifest high cytolytic activity (Olson et al., 2013).

To investigate the protective capacity of memory MCJ-deficient CD8+ T cells, we performed adoptive transfer of equal numbers WT and MCJ-deficient CD8+ T cells obtained 5 weeks post-infection separately into WT recipient mice. Recipient mice were then infected with a lethal dose of PR8 virus. As expected, recipient mice that received cells from infected WT mice were not protected, did not recover their weight, and died between 8 and 9 days post lethal infection (Figure 2-6E and F). In contrast, most recipient mice that received cells from
infected MCJ-deficient mice were protected and survived (Figure 2-6E). Mice receiving MCJ-deficient CD8+ T cells lost weight initially, indicating that they had been infected, but they recovered to a healthy state (Figure 2-6F). Thus, the absence of MCJ in memory CD8+ T cells confers greater protective capacity against influenza virus infection.

Death caused by some of the highly pathogenic influenza viruses (such as H5N1 influenza) is often associated with a strong systemic immune response and cytokine storm. To rule out that the death of the recipients of WT CD8+ T cells was caused by an exuberant immune response, we examined cytokine production 6 days post-infection with the lethal dose. The concentration of inflammatory cytokines (IL-6, KC) and chemokines (CXCL10) in bronchoalveolar lavage fluid (Figure 2-6G) and serum (Supplementary Figure 2-6A) were comparable. These results indicate that the protection found in mice that received MCJ-deficient CD8+ T cells was not due to an attenuated immune response relative to mice that received WT CD8+ T cells.

To investigate whether the protective capacity of memory MCJ-deficient CD8+ T cells was due to an improved effector function to clear virus, we examined PR8 virus titers in the lung of recipient mice 6 days post-infection with the lethal dose. Higher virus titers were present in host mice that received WT CD8+ T cells (Figure 2-6H), indicating that memory MCJ-deficient CD8+ T cells were more efficient in clearing influenza virus. To examine the effector function of memory MCJ-deficient CD8+ T cells, we isolated CD8+ T cells from the mediastinal LN
(MLN) 6 days after the lethal infection and determined ex vivo IFN-\(\gamma\) production by culturing cells in medium without stimuli. IFN-\(\gamma\) was higher in MCJ-deficient CD8\(^+\) T cells (Figure 2-6l); however, the NP-tetramer\(^+\) cell frequency was not different (Supplementary Figure 2-6B). Ex vivo IFN-\(\gamma\) production by MCJ-deficient CD8\(^+\) T cells isolated from the lung was also increased (Supplementary Figure 2-6C), as was the number of IFN-\(\gamma\) secreting cells as determined by ELISpot assay (Figure 2-6l). However, the frequency of NP-tetramer\(^+\) CD8\(^+\) T cells in the lung was comparable (Supplementary Figure 2-6D), showing a great secretory capacity of memory MCJ-deficient CD8\(^+\) T cells.

Secretion of granules by exocytosis is dependent on both Ca\(^{2+}\) and ATP. We investigated whether increased mitochondrial ATP production in MCJ-deficient CD8\(^+\) T cells could also facilitate an increase in exocytosis of cytotoxic granules present in effector cells by CD107a mobilization assay. CD8\(^+\) T cells were isolated from the MLN 6 days after lethal infection and incubated in medium containing monensin with or without ionomycin to provide the Ca\(^{2+}\) signal. The frequency of CD107a\(^+\) MCJ-deficient CD8\(^+\) T cells treated with ionomycin was significantly higher than WT CD8\(^+\) T cells (Figure 2-6K), indicating that MCJ-deficient CD8\(^+\) T cells have a greater capacity to function as cytotoxic cells. Thus, increased mitochondrial respiration caused by the loss of MCJ in CD8\(^+\) T cells results in increased antiviral protective activity during memory responses by enhancing the secretion both of effector cytokines as well as cytotoxic granules.
Figure 2-6. Loss of MCJ Confers Viral Protective Activity to Memory CD8\(^+\) T Cells

WT (blue) and MCJ-deficient CD8\(^+\) T cells (MCJ def, red). (A – D) Mice were infected with a sublethal dose of PR8 virus (primary infection). (A) Percent weight loss over time. (B) Percentage of NP-tetramer\(^+\) cells in the spleen 2 weeks post infection. (C) Ex vivo IFN-\(\gamma\) production of cells isolated from the spleen and MLN 2 weeks post infection by ELISA. (D) Percentage of NP-tetramer\(^+\) cells from the spleen and MLN of mice 5 weeks post infection. (E – K) Mice were infected with a sublethal dose of PR8 virus (primary infections). CD8\(^+\) T cells were isolated 5 weeks post infection and adoptively transferred into naive WT recipients, which were then infected with a lethal dose of PR8 virus. (E) Survival and (F) percent weight loss over time. (G–K) Recipients were sacrificed 6 days post lethal infection. (G) BALF cytokines and chemokines. (H) Lung PR8 virus titer by quantitative RT-PCR. (I) Ex vivo production of IFN-\(\gamma\) by MLN CD8\(^+\) T cells by ELISA. (J) IFN-\(\gamma\) secretion by lung CD8\(^+\) T cells by ELISpot assay. (K) CD107a mobilization assay of MLN CD8\(^+\) T cells cultured with (Iono) or without (Med) ionomycin. *\(p < 0.05\) by log-rank test for Kaplan-Meier survival curve and by unpaired \(t\) test for all others. Avg ± SD (\(n \geq 3\)) shown. Results are representative of 2 – 3 experiments. See also Supplementary Figure 2-5 and Supplementary Figure 2-6.
4. Discussion

Mitochondria play a key role in balancing cellular metabolism primarily as the site for OXPHOS through the ETC and as a source of ATP. Recently, it has become clear that tight regulation of mitochondrial metabolism occurs during the reprogramming of CD8+ T cells. A number of molecules have been shown to be required for maximum efficiency of the ETC and OXPHOS. However, very little is known about negative regulatory mechanisms that restrict mitochondrial respiration. We have recently identified MCJ as one of the first known negative regulators of complex I activity through its effect on the formation of respiratory supercomplexes (Hatle et al., 2013). Our earlier studies demonstrated that the absence of MCJ prevents the development of steatosis by accelerating fatty acid metabolism within the liver (Hatle et al., 2013). Here we have shown that MCJ restrains mitochondrial respiration in CD8+ T cells. In the absence of this natural break, CD8+ T cells have enhanced OXPHOS leading to increased secretion of cytokines by effector CD8+ T cells. In addition, MCJ deficiency interferes with the metabolic adaptation during the contraction phase of effector CD8+ T cells and results in greater antiviral protective activity of memory CD8+ T cells.

Effector and memory CD8+ T cells need ATP for effector functions in addition to cell growth and expansion. While CD8+ T cells primarily use glycolysis instead of OXPHOS for proliferation (Frauwirth et al., 2002; Macintyre et al., 2011) other sources might provide the ATP required for processes with high ATP consumption. Production of some cytokines by effector CD8+ T cells is not affected
by strong inhibition of glycolysis (Cham et al., 2008), and cytotoxic activity can take place in the absence of glucose (MacDonald and Koch, 1977). Considering the dynamic aspect of mitochondria as organelles that can rapidly relocate in the cytosol, it is quite possible that mitochondria can create a microenvironment that is highly rich in ATP in specific locations without elevating overall cytosolic ATP. Mitochondria have been shown to relocate to the edge of lamellipodia and are critical for providing the energy for migration of cells (Morlino et al., 2014). Our studies revealed the presence of microenvironments where ATP and ADP accumulate in CD8⁺ T cells located in the proximity of mitochondria. Here we have shown that increased mitochondrial respiration in CD8⁺ T cells lacking MCJ had no effect on CD8⁺ T cell proliferation or cytokine gene expression, but it enhanced the secretion of cytokines such as IFN-γ. Little is known about the mechanisms of cytokine secretion in T cells; however, secretion of intracellular components often is dependent on ATP (Jena, 2013; Monteleone et al., 2015) and the source of this ATP, whether mitochondrial or cytosolic, has yet to be determined. Secretion of IFN-γ by CD8⁺ T cells in the absence of MCJ is dependent on ATP derived from mitochondria. Thus, through OXPHOS mitochondria can regulate effector function of CD8⁺ T cells independently of cell expansion.

The presence of respiratory supercomplexes in mammalian cells has been demonstrated in tissues such as heart. Respirasomes bring the individual complexes together to facilitate the efficient transfer of electrons between
complexes while preventing electron leak and, thereby, production of ROS. Here we revealed the presence of supercomplexes in naive CD8\(^+\) T cells and increased supercomplex formation in the absence of MCJ. Naive CD8\(^+\) T cells primarily use mitochondria and OXPHOS relative to activated CD8\(^+\) T cells; however, mitochondrial ROS are almost undetectable (Hatle et al., 2013). It is possible that the presence of supercomplexes prevents the formation of ROS and enhances survival of naive cells. Although increased MMP is normally associated with increased ROS, in MCJ-deficient CD8\(^+\) T cells MMP is higher but ROS is not (Hatle et al., 2013). This is most likely due to abundance of supercomplexes in these cells.

To date, there is no clear evidence that complex II (succinate dehydrogenase) is also recruited to supercomplexes. Because complex III receives electrons from both complex I and complex II, the recruitment of complex III to supercomplexes might cause an uncoupling of complex III from complex II. Therefore, the attenuation of complex II activity in the absence of MCJ could be a mechanism to prevent electron leakage since its corresponding acceptor, complex III, is sequestered, explaining the accumulation of succinate in MCJ-deficient CD8\(^+\) T cells.

Memory CD8\(^+\) T cells utilize mitochondrial respiration for both their generation and effector function. However, instead of using glucose to feed mitochondrial respiration through pyruvate, memory CD8\(^+\) T cells perform \(\beta\)-oxidation of fatty acids. Recently, it has been shown that these cells utilize lipolysis to generate their own fuel (O'Sullivan et al., 2014). Pathways leading to increased mitochondrial respiration are associated with a superior memory CD8\(^+\)
T cell response. In our study, we have identified MCJ as an endogenous negative regulator of OXPHOS in CD8+ T cells. Lack of MCJ sustained the active metabolism of effector CD8+ T cells during the contraction phase and resulted in greater effector memory CD8+ T cell responses to influenza virus. Although MCJ deficiency had an impact in the metabolic adaptation during the contraction phase of effector CD8+ T cells, it did not seem to have a substantial effect on overall survival of those effector cells in vivo. MCJ deficiency appeared to provide a survival advantage to effector cells when these cells were rested in medium alone without additional cytokines in vitro. This is most likely due to the fact that during in vitro resting effector WT CD8+ T cells undergo cytokine withdrawal. We also show here that WT memory CD8+ T cells alone failed to provide protection against a lethal dose of influenza virus. Strikingly, MCJ-deficient memory CD8+ T cells were highly protective against lethal infection with influenza. Using the LCMV infection model, it has been shown that CD4+ T cells can rescue exhausted CD8+ T cells during chronic viral infection (Aubert et al., 2011). We also observed that a small frequency of CD4+ T cells was sufficient for WT memory CD8+ T cells to provide protection against influenza virus infection (data not shown). Because MCJ deficiency results in enhanced CD8+ T cell responses, it is not evolutionarily clear why MCJ is expressed in CD8+ T cells. While CD8+ T cells are key for protection, an exaggerated cytotoxic CD8+ T cell response could cause non-specific tissue damage. We propose that MCJ was acquired in CD8+ T cells as a strategy to
restrain their metabolism and prevent a prolonged effector function that could be harmful.

5. Experimental Procedures

5.1. Mice

Mouse strains used were C57BL/6J (WT), MCJ-deficient C57BL/6 (Hatle et al., 2013), OT-I TCR transgenic, and MCJ-deficient OT-I. All mice were maintained at the University of Vermont animal care facility and used under procedures approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC).

5.2. Cell Preparation and Culture

Cells were purified by negative selection or positive selection using the MACS Cell Separation System (Miltenyi). Cytokine production was determined by ELISA. Detailed protocols are described in Supplemental Experimental Procedures.

5.3. Extracellular Flux Analysis

Oxygen consumption (OCR) and extracellular acidification rates (ECAR) were analyzed using a XF24 Extracellular Flux analyzer (Seahorse Bioscience). Detailed protocols are described in Supplemental Experimental Procedures.
5.4. Intracellular ATP Concentration

ATP concentration was determined using the ATPlite Luminescence Assay System and a TD-20/20 Luminometer. Detailed protocols are described in Supplemental Experimental Procedures.

5.5. Flow Cytometry Analyses

Flow cytometry analyses were performed using an LSRII Flow Cytometer (BD Biosciences). Cell proliferation, survival, and MMP were determined using CFSE, Live Dead Cell Viability Assay, and TMRE (Molecular Probes). β-galactosidase activity was determined using the FACS Fluorescent Blue lacZ β-Galactosidase Detection Kit (Marker Gene Technologies). Intracellular cytokine staining was performed without brefeldin A or monensin. CD107a mobilization assay was performed using monensin and an anti-CD107a PE antibody (Biolegend) with or without ionomycin. Detailed protocols are described in Supplemental Experimental Procedures.

5.6. Chromatin Immunoprecipitation

ChIP assays were performed using the SimpleChip Enzymatic Chromatin IP Kit (Cell Signaling) with anti-Ikaros or rabbit IgG. Detailed protocols are described in Supplemental Experimental Procedures.

5.7. Influenza Infection and Analyses

Mice were infected intranasally with a sublethal dose of PR8 virus. CD8⁺ T cells were isolated after 5 weeks, equal numbers of NP-tetramer⁺ cells were
transferred to naive WT mice. Recipients were infected with a lethal dose and sacrificed 6 days later. CD8\(^+\) T cells from the MLN and lung were examined for ex vivo IFN-\(\gamma\) production by ELISA and/or ELISpot assay and for effector and memory cell surface markers, NP-tetramer\(^+\) cell frequency, and/or CD107a mobilization by flow cytometry. BALF and serum were analyzed by Luminex assay (Millipore). Lung was examined for PR8 virus titer by quantitative RT-PCR for acid polymerase. Detailed protocols are described in Supplemental Experimental Procedures.

5.8. Confocal Microscopy

Cells were examined by confocal microscopy using ATP probe and/or Mitotracker and a Zeiss LSM 510 Meta Confocal Laser Scanning microscope (Carl Zeiss Microscopy). Detailed protocols are described in Supplemental Experimental Procedures.

5.9. Statistical Analyses

Statistical significance was determined by \(t\) test or long-rank test as indicated. Bars represent average \(\pm\) SD or SEM as indicated. \(p < 0.05\) was considered statistically significant.

6. References


7. Supplementary Information

7.1. Supplementary Figures

Supplementary Figure 2-1. MCJ does not affect T cell development in the thymus or homeostasis in the peripheral immune system but increases amino acid import

WT, blue; MCJ-deficient (MCJ def), red. (A) Mitochondrial membrane potential (MMP) of freshly isolated CD8+ T cells from the spleen and LN was determined by TMRE staining and flow cytometry analysis. (B) CD4 and CD8 expression by thymocytes, splenocytes, and lymph node (LN) cells was examined by flow cytometry analyses. Numbers indicate the percentage of cells in each gate. (C) CD44 expression by CD4+ and CD8+ T cells from the spleen and LN was determined by flow cytometry analysis. (D) Freshly isolated CD8+ T cells were cultured in the presence of [13C- and [15N-labeled amino acids for the indicated times, and [13C, [15N]-glutamine was determined by UPLC-MS analysis. *p < 0.05 by paired t test. Avg ± SEM (n = 3) shown. See also Figure 2-1 and Figure 2-5.
Supplementary Figure 2-2. MCJ deficiency increases the secretion, but not production, of cytokines by CD8⁺ T cells

(A) MCJ-deficient CD8⁺ T cells were activated for 2 day with anti-CD3 and anti-CD28 antibodies. Cells were then washed and rested in medium (Med) or in the presence of cycloheximide (CHX, 5 µg/ml) for 12 hours. IFN-γ in the culture supernatants was determined by ELISA. (B) Total protein content in 5 x 10⁶ freshly isolated CD8⁺ T cells prior to (unstim) and 20 hours after activation with anti-CD3 and anti-CD28 antibodies in the absence (-) or presence (CHX) of cycloheximide. (C and D) WT (blue) and MCJ-deficient (MCJ def, red) CD8⁺ T cells were activated for 2 days with anti-CD3 and anti-CD28 antibodies. (C) Cells were then washed and incubated without stimuli for 4 hours. IL-2 and GM-CSF in the culture supernatants were then determined by ELISA. (D) Cells were then washed and rested for 1 hour, and intracellular IFN-γ was determined by intracellular cytokine staining and flow cytometry analysis. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. See also Figure 2-2.
Supplementary Figure 2-3. MCJ deficiency in CD8⁺ T cells increases mitochondrial ATP production, IFN-γ secretion, and cell survival

(A) Confocal microscopy images in combination with bright field of live MCJ-deficient CD8⁺ T cells activated for 2 days with anti-CD3 and anti-CD28 antibodies followed by incubation with or without oligomycin for 2 hours. Left, cells cultured in medium alone (unstained). Middle, cells in medium with ATP probe. Right, cells incubated with oligomycin and ATP probe. (B) MCJ-deficient CD8⁺ T cells were activated for 2 days, and oligomycin was added during the last hour of activation. Cells were then washed and replated with oligomycin for 4 hours. Intracellular IFN-γ was determined by intracellular cytokine staining and flow cytometry analysis. Red, medium alone; green, oligomycin. The percentage of cells in each gate is indicated. (C) Freshly isolated WT (blue) and MCJ-deficient (red) CD8⁺ T cells were analyzed for β-galactosidase activity by flow cytometry. The
percentage of β-galactosidase+ MCJ-deficient cells is indicated. WT cells are shown as a negative control. (D and E) WT (blue) and MCJ-deficient (MCJ def, red) CD8+ T cells were activated for 2 days, washed, and replated at equal numbers in medium without stimulation. After 24 hours, culture supernatants were supplemented with (D) anti-IL-2 antibody or (E) recombinant IL-2. The number of live cells was determined 24 hours later (48 hours after replating) by Trypan blue exclusion. Bars indicate cell survival relative to the initial number of cells. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. See also Figure 2-4 and Figure 2-5.

Supplementary Figure 2-4. MCJ-deficient CD8+ OT-I T cells maintain their large blastic size during the contraction phase in vivo
OT-I, blue; OT-I MCJ-deficient (MCJ def), red. (A) CD4 and CD8 expression by splenocytes and lymph node (LN) cells was examined by flow cytometry analyses. Numbers indicate the percentage of cells in each gate. (B and C) OT-I and OT-I MCJ-deficient CD8+ T cells were activated with anti-CD3 and anti-CD28 antibodies for 2 days and then expanded with recombinant IL-2 for an additional 3 days. Equal numbers of OT-I (CD90.1+) and OT-I MCJ-deficient (CD90.1+ CD90.2+) CD8+ T cells were combined and adoptively transferred to C57BL/6 (CD90.2+) recipient mice (n = 4). LN and spleen were harvested after 2 weeks and analyzed by flow cytometry. (B) Dot plots showing FSC and SSC of total spleen and LN cells from recipients (upper panel) and CD90.1 and CD90.2 expression used to discriminate donor and recipient cells (lower panel). (C) OT-I and OT-I MCJ-deficient CD8+ Va2+ (OT-I TCR) T cells gated as shown in (B) were examined for FSC and surface expression of CD44, CD62L, KLRG1, and CD127. Numbers indicate the percentage of cells in each gate. Note the increased fraction of OT-I MCJ-deficient CD8+ T cells that have a large, blastic size (higher FSC). See also Figure 2-5.

Supplementary Figure 2-5. MCJ deficiency does not increase the number of influenza-specific CD8+ T cells or have a significant effect on memory cell populations

(A and B) WT and MCJ-deficient (MCJ def) mice (n = 5) were infected with a sublethal dose of influenza virus and allowed to recover from this primary
infection. (A) The number of NP-tetramer+ CD8+ cells in the spleen was determined 3 weeks postinfection by flow cytometry analysis. (B) Five weeks post primary infection, CD8+ T cells were isolated from the spleen and draining MLN and pooled for adoptive transfer into naïve WT (C57BL/6) recipient mice. Shown is the phenotype of the donor CD8+ T cells analyzed for CD8, NP-tetramer, KLRG1, CD127, CD62L, CD27, and CD44 surface expression by flow cytometry analysis. *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. See also Figure 2-6.

Supplementary Figure 2-6. Effector function of influenza-specific memory CD8+ T cells

WT recipient mice were lethally infected with influenza after receiving either WT or MCJ-deficient (MCJ def) CD8+ T cells from donor mice that had recovered from a sublethal primary infection with influenza. Recipients were sacrificed 6 days post lethal infection. (A) Cytokines in the serum were analyzed by Luminex assay. (B) Influenza specific CD8+ T cells in the spleen were determined by staining with NP-tetramer and flow cytometry analysis. (C) CD8+ T cells from the lung were incubated in medium without stimulation for 11 hours, and IFN-γ in the culture supernatant was determined by ELISA. (D) Influenza specific CD8+ T cells in the lung were determined as in (B). *p < 0.05 by unpaired t test. Avg ± SD (n ≥ 3) shown. See also Figure 2-6.
7.2. Supplementary Tables

Supplementary Table 2-1. Metabolome Analysis of Naive CD8⁺ T Cells

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*Median value of 3 independent biological replicates. †Fold increase of median peak area calculated as KO/WT. ‡Determined by unpaired t test (n = 3).
Supplementary Table 2-1 (continued)

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Supplementary Table 2-2. Metabolome Analysis of Activated CD8+ T Cells

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*Median value of 3 independent biological replicates. †Fold increase of median peak area calculated as KO/WT. ‡Determined by unpaired t test (n = 3).
### Supplementary Table 2-2 (continued)

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<td>7.71E+06</td>
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<tr>
<td></td>
<td>creatinine</td>
<td>6.31E+05</td>
<td>5.60E+05</td>
<td>0.89</td>
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<tr>
<td><strong>Sulfur metabolism</strong></td>
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<tr>
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<tr>
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<td>1.20E+05</td>
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<tr>
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<td>Cys-Gly</td>
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<td>9.92E+05</td>
<td>1.01</td>
</tr>
</tbody>
</table>
7.3. Supplemental Experimental Procedures

7.3.1. Mice

All mice were bred and maintained under sterile conditions at the University of Vermont animal care facility and used under procedures approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC). Mouse strains used were WT C57BL/6J (Jackson Laboratories), MCJ-deficient C57BL/6 (Hatle et al., 2013), OT-I TCR transgenic (Hogquist et al., 1994), and MCJ-deficient OT-I.

7.3.2. Cell preparation and culture

CD8+ T cells were purified from spleen and lymph nodes (LN) by negative selection as previously described (Farley et al., 2006) and by positive selection using the MACS Cell Separation System (Miltenyi) as recommended by the manufacturer. The purity of isolated cells was confirmed by flow cytometry. Cells were activated in vitro using plate bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (1 µg/mL) antibodies (BioXcell). For analysis of cytokine secretion during resting, cells were activated for 2 days, washed, and incubated at equal numbers in medium alone without additional stimuli for 4 - 12 hours. Culture supernatants of activated and rested cells were analyzed for IFN-γ, IL-2, and/or GM-CSF by ELISA. Cells were also incubated with or without oligomycin (20 µM, Sigma) or cycloheximide (5 µg/ml, Sigma). For cell survival analysis, activated cells were washed and incubated at equal numbers in medium without further stimulation.
or the presence of exogenous cytokines. In some cases, the culture medium was supplemented with a blocking anti-IL-2 antibody or recombinant IL-2 after 24 hours. Live cells were determined by Trypan blue exclusion at 24, 48, and 72 hours after replating.

7.3.3. Mitochondrial respiration and extracellular acidification

Oxygen consumption rates (OCR) of CD8+ T cells were analyzed under basal conditions and in response to sequential injections of oligomycin, FCCP, and rotenone with antimycin A (1 µM each) using the Seahorse MitoStress Test Kit. Extracellular acidification rates (ECAR) were analyzed under basal conditions and in response to glucose (10 mM) using the Seahorse Glycolysis Stress Test Kit. OCR linked to mitochondrial ATP production (ATP linked OCR) was determined by subtracting OCR of CD8+ cells reached after treatment with oligomycin from OCR obtained at baseline. Complex II linked respiration was determined using a modified version of a previously described method (Salabei et al., 2014). Briefly, XF24 cell culture microplates were coated with Cell-Tak (50 uL at 22.4 µg/mL, Corning) and freshly isolated CD8+ T cells were plated in MAS-BSA assay solution (220 mM mannitol, 70 mM sucrose, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, 0.2% fatty acid free BSA) containing XF Plasma Membrane Permeabilizer (PMP, 1 nM, Seahorse Bioscience) and ADP (4 mM, Sigma). Oxygen consumption rates were measured at baseline and in response to sequential injections of succinate (10 mM, Sigma) with rotenone (2 µM, Seahorse Bioscience)
and malonate (500 µM, Sigma). All extracellular flux analyses were performed using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as recommended by the manufacturer.

7.3.4. Mass spectrometry based metabolomics

For metabolomics analyses, CD8\(^+\) T cells from WT and MCJ KO mice were purified and either left unstimulated or activated for 2 days prior to mass spectrometry analysis. For culture supernatants, unstimulated CD8\(^+\) T cells were incubated in medium without stimulation for 16 hours. For heavy isotope and flux analyses, unstimulated cells were incubated in medium supplemented with Cell Free Amino Acid Mixture - \(^{13}\)C, \(^{15}\)N (2 mM, Sigma) for 5 minutes or 5 hours. Metabolomics and flux analyses were performed as previously reported (D'Alessandro et al., 2015). Briefly, 2 x 10\(^6\) cells and 20 µl of cell media were extracted in 1 ml and 980 µl of cold lysis and extraction buffer (methanol : acetonitrile : water at 5 : 3 : 2), respectively. After discarding protein pellets, 10 µl of water and methanol soluble fractions were run through a Kinetex C18 1.7 µm, 100 x 2.1 mm reversed phase column (phase A: water, 0.1 % formic acid; phase B: acetonitrile, 0.1 % formic acid; Phenomenex) via an ultra high performance chromatographic system (UHPLC, Ultimate 3000, ThermoFisher). UHPLC was coupled in line with a high resolution quadrupole Orbitrap instrument run in both polarity modes (QExactive, ThermoFisher) at 140,000 resolution (at 200 m/z). Metabolite assignment, heavy isotopologue distributions and peak integration for
relative quantitation were performed using Maven software (Princeton) against
the KEGG pathway database and an in house validated standard library (>650
compounds, Sigma, IROATech). Integrated peak areas were exported to Excel
(Microsoft) and elaborated for statistical analysis ($t$ test, ANOVA) and hierarchical
clustering analysis (HCA) through Prism (GraphPad Software) and GENE-E
(Broad Institute), respectively.

7.3.5. Mitochondrial respiratory supercomplexes

Mitochondrial fractions were obtained by differential centrifugation,
solubilized with 2 % digitonin (Sigma), and resolved by electrophoresis using
NativePAGE Novex 4 – 16 % Bis-Tris protein gels (Invitrogen) as previously
described (Hatle et al., 2013; Yang et al., 2015). Bands corresponding to respiratory
supercomplexes and monomeric Complex IV were excised from the Blue Native
gel, eluted, resolved by SDS-PAGE, and analyzed by Western blot for NDUFA9
and CoxIV (MitoScience). Proteins were also transferred directly from the Blue
Native gel to PVDF membrane for Western blot analysis for NDUFA9 and Core1
(MitoScience).

7.3.6. Intracellular ATP Concentration

Intracellular ATP concentration in $10^4$ CD8$^+$ T cells was determined using
the ATPlite Luminescence Assay System (PerkinElmer) and a TD-20/20
Luminometer (Turner BioSystems) as recommended by the manufacturers.
7.3.7. Flow cytometry analyses

For cell proliferation analysis, cells were stained with CFSE (5 µM, Molecular Probes), activated for 24 or 48 hours, and examined by flow cytometry analysis to determine proliferation. Control (unstimulated) cells were stained and incubated in medium alone for 16 hours before analysis. For cell survival analysis, cells were stained using the Live Dead Cell Viability Assay (Molecular Probes) as recommended by the manufacturer and examined by flow cytometry analysis. For expression of cell surface markers, cells were stained with antibodies for CD4, CD8, CD25, and CD69, and then analyzed by flow cytometry. For mitochondrial membrane potential analysis, cells were staining with TMRE (Molecular Probes) as previously described (Hatle et al., 2013) and examined by flow cytometry analysis. For β-galactosidase activity analysis, cells were incubated in β-galactosidase substrate (FACS Fluorescent Blue lacZ β-Galactosidase Detection Kit, Marker Gene Technologies) as recommended by the manufacturer and analyzed by flow cytometry. WT cells were used as negative controls. For intracellular cytokine staining analysis, cells were fixed after activation in paraformaldehyde, permeabilized with saponin and stained with the corresponding antibodies for IFN-γ or IL-2 (BD Bioscience) as previously described (Yang et al., 1998). No preincubation with brefeldin A or monensin was used prior to intracellular staining. For CD107a mobilization assay, cells were cultured in the presence of monensin (1 µM, Sigma) and anti-CD107a (4 µL/ml, Biolegend) for 4 hours using a modified version of a previously described method (McElroy et al.,
2007). Additionally, cells were cultured with or without ionomycin (250 ng/ml). Cells cultured without anti-CD107a were used as negative controls. All flow cytometry analyses were performed using an LSRII Flow Cytometer (BD Biosciences) and FlowJo software.

7.3.8. Cytokine Analyses

For cytokine gene expression analysis, total RNA was isolated from activated cells using an RNeasy Mini Kit (Qiagen) as recommended by the manufacturer, cDNA was synthesized as previously described (Yang et al., 2015), and IL-2 and IFN-γ mRNA were determined by qRT-PCR using Assays-on-Demand TaqMan Gene Expression Assays (Applied Biosystems). Values were normalized to β2-microglobulin and analyzed by the comparative delta CT method. For cytokine production analysis, IFN-γ and IL-2 in culture supernatants were determined by ELISA as previously described (Yang et al., 1998). For IFN-γ ELISpot assays, cells were washed and plated in an ELISpot assay plate using capture and biotinylated anti-IFN-γ antibodies (MabTech) as previously described (Dienz et al., 2009). Total and larger spots (high IFN-γ producing cells) were quantified using an Axio Imager and software system (Zeiss).

7.3.9. Chromatin immunoprecipitation.

Ten to fifteen million purified CD8+ T cells were activated for 2 days or left unstimulated. ChIP assays were performed as previously described (Navasa, Martin-Ruiz, et al., 2015) using the SimpleChip Enzymatic Chromatin IP kit-
Magnetic beads (Cell Signaling) following the manufacturer’s instructions using anti-Ikaros antibody or normal rabbit IgG as a negative control. Immunoprecipitated DNA was subjected to qPCR using primers encompassing the Ikaros binding site (5’ - TCA TTT GCT GTG AGC GCA AG - 3’ and 5’ - GCC TCC TTA GGT CTA CCT TGA - 3’). Results are presented as fold induction over rabbit IgG immunoprecipitates relative to input (percent input method).

7.3.10. Influenza infection and analyses

WT and MCJ-deficient mice were infected intranasally with a sublethal dose (3×10^3 EIU) of Puerto Rico A/PR/8/34 H1N1 influenza A (PR8) virus as previously described (Dienz et al., 2012). Mice were monitored for weight loss every other day. CD8^+ T cells were isolated by positive selection from the draining mediastinal lymph node (MLN) and spleen 5 weeks later. Equal numbers of NP-tetramer^+ CD8^+ T cells (2 - 2.5 x 10^6) were then transferred to WT mice, and a day later recipient mice were infected with a lethal dose of PR8 virus (10^4 EIU). Mice were monitored for weight loss every other day. Animals that lost >30% of their body weight at the day of infection or became grossly moribund were euthanized. BALF and serum were collected 6 days after the lethal dose. Cytokines and chemokines in BALF and serum were examined using Luminex Mouse Cytokine and Chemokine Panel I (Millipore) according to the manufacturer's protocol. Ex vivo production of IFN-γ by CD8^+ T cells from the MLN and lung were determined by ELISA and/or ELISpot assay as described above. CD8^+ T cells were also stained
with NP366-374/Db tetramer containing peptide from the PR8 influenza virus (Powell et al., 2007) and antibodies for CD44, CD62L, KLRG1, CD127, and CD27 (Biolegend) and analyzed by flow cytometry. For virus copy determination, total RNA extracted from whole lung tissue by RNeasy Kit (Qiagen) was used for cDNA synthesis (2 µg RNA) as previously described (Dienz et al., 2012). Viral titers were determined by quantitative RT-PCR for PR8 virus acid polymerase (PA) gene, a method validated as equivalent to PFU (Jelley-Gibbs et al., 2007).

7.3.11. Confocal microscopy

Cells were activated for 2 days, transferred to glass bottom plates (MatTek), and incubated at 37°C for 1 hour to sediment. Live cells were examined by confocal microscopy prior to staining (background). ATP probe (Rao et al., 2012) was then added (100 µM), cells were incubated for 5 minutes, washed, and visualized using a 405 nm laser. For costaining with Mitotracker, prior to incubation with the ATP probe cells were incubated with Mitotracker (Thermo Fisher) for 30 minutes. Confocal microscopy was performed using a Zeiss LSM 510 Meta Confocal Laser Scanning microscope (Carl Zeiss Microscopy).

7.3.12. Statistical analyses

Statistical significance was determined by t test or long-rank test as indicated. Bars represent the mean with the standard deviation (SD) or standard error of the mean (SEM). p < 0.05 was considered statistically significant.
7.4. Supplementary References


8. Author Contributions

D.P.C., K.A.F., A.D., T.M.T., R.Y., and J.A. conceived and designed experiments, acquired and interpreted data, and drafted and revised the manuscript. K.M.H., D.T., J.T.-C., and L.H. conceived and designed experiments and acquired and interpreted data. Y.W.J., K.H.A., and K.C.H. conceived and designed experiments. M.R. conceived and designed the study, acquired and interpreted data, and drafted and revised the manuscript.

9. Acknowledgments

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CHAPTER 3. ABC Transporter Mediated Cancer Chemoresistance is Overcome by Targeted Disruption of Mitochondrial ATP

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1. Summary

Chemotherapy remains the standard of care for most cancers worldwide, however development of chemoresistance due to the presence of the drug-effluxing ABC transporters remains a significant problem. The development of safe and effective means to overcome chemoresistance is critical for achieving durable remissions in many cancer patients. We have investigated the energetic demands of ABC transporters in the context of the metabolic adaptations of chemoresistant cancer cells. Here we show that mitochondrial-derived ATP, but not glycolysis-derived ATP, provides the energy needed for ABC transporters to efflux drugs out of cancer cells. We further demonstrate that the loss of MCJ (DNAJC15), an endogenous negative regulator of mitochondrial respiration, in chemoresistant cancer cells enhances their ability to produce ATP from mitochondria and fuel ABC transporters. We have developed novel MCJ mimetics that can attenuate mitochondrial respiration and safely overcome chemoresistance in vitro and in vivo. Administration of MCJ mimetics in combination with standard chemotherapeutic drugs could therefore become an affordable new strategy for treatment of multiple cancers.

2. Introduction

Despite the development of novel therapies, including immunotherapies, chemotherapy prior to or following surgery remains the most commonly used systemic treatment for most cancers. However, it is also evident that
chemotherapy treatment still faces major challenges as chemoresistance usually develops in cancer patients. In some cases, patients fail the initial chemotherapy course due to intrinsic properties of tumor cells. Frequently, following a successful response to the first-round chemotherapy, the cancer recurs following acquisition of adaptive mechanisms of chemoresistance, which can extend to families of drugs distinct from the chemotherapy used (i.e., multidrug resistance). Once chemoresistance is established, one of the approaches used to attempt to overcome this problem is increased drug dosing, but this approach is limited by increased toxicity, including off-target effects (e.g., cardio- or neurotoxicity) (Lyman, 2009; Pai and Nahata, 2000). The alternative use of different chemotherapeutic drugs is often hampered by multidrug resistance. Until now, the lack of a mechanistic understanding of the energetics of chemoresistance has hampered the development of effective strategies to overcome this major hurdle in cancer therapy. While there is growing interest in cancer cell metabolism as a novel approach to interfere with cancer growth, little is known about how metabolic changes contribute to the development of chemoresistance.

Different mechanisms of chemoresistance have been identified (Fodale et al., 2011), but the most common mechanism is the upregulation of ATP binding cassette (ABC) transporters that mediate drug efflux and decrease intracellular accumulation of anti-cancer drugs (Kathawala et al., 2015). ABC transporters are a large family of proteins classified in seven subclasses (ABCA, B, C, D, E, F, and G) that use the energy of ATP hydrolysis to mediate the transport of substrates.
(Ambudkar et al., 2003; Dean et al., 2001; Szakacs et al., 2006). In cancer cells, ABC transporters remove chemotherapeutic agents from cells, thereby reducing intracellular concentrations and thus drug efficacy. Several ABC transporters have been associated with the development of multidrug resistance in cancer (Dlugosz and Janecka, 2016). The most characterized ABC transporters associated with chemoresistance in cancer are ABCB1 (also known as P-gp or MDR1), ABCG2 (BCRP), and ABCC1 (MRP1) (Allikmets et al., 1998; Cole et al., 1992; Doyle et al., 1998; Miyake et al., 1999; Roninson et al., 1986; Ueda et al., 1987). A correlation between the expression of ABCB1 and ABCG2 and multidrug resistance in cancer cell lines in vitro is well established (Bugde et al., 2017; Doyle et al., 1998; Ozvegy et al., 2001; Riordan et al., 1985; Roninson et al., 1984), but the correlation between the expression of these transporters and chemoresistance in cancer patients is less clear, and therefore neither can be used as predictive markers for chemoresistance. The presence of ABC transporters therefore may not be sufficient to cause chemoresistance in cancer cells and other factors could be required for these efflux pumps to be active.

ABC transporters are energy consuming pumps that require significant amounts of ATP as biochemical studies have indicated that up to two ATP molecules are required to efflux one molecule of substrate (Patzlaff et al., 2003; Poolman et al., 2005). Despite this energy demand and the distinct metabolism of cancer cells, little is known about the mechanisms that regulate their activity in the chemoresistant cancer cells other than substrate availability as well as how the
activity of these transporters is regulated by the metabolic state of the cell (Robey et al., 2018). Therefore, determining how the metabolic state of the cell affects ABC transporter activity could provide alternative pathways for the generation of novel inhibitors for these transporters to overcome chemoresistance.

The two main pathways for ATP synthesis are glycolysis in the cytosol and oxidative phosphorylation in mitochondria. Cancer cells predominantly utilize aerobic glycolysis to generate ATP for proliferation and biosynthesis (Liberti and Locasale, 2016; Vander Heiden et al., 2009) (Koppenol et al., 2011; Warburg, 1956). While historically switching to a glycolytic metabolism at the expense of mitochondrial respiration was viewed as an approach to promote cancer progression, it is now clear that mitochondria contribute to a number of functions in cancer cells (DeBerardinis and Chandel, 2016; Wallace, 2012; Zong et al., 2016). Several studies have shown an upregulation of the mitochondrial respiratory machinery in slow-cycling, chemoresistant melanoma cells with a quiescent phenotype generated upon drug treatment (Cierlitza et al., 2015; Roesch et al., 2013). This has also been reported in cancer stem cells, which are also more quiescent and resistant to therapy (Abdullah and Chow, 2013). Inhibition of the electron transport chain (ETC) with different pharmacological inhibitors in these slow-cycling resistant cells has been shown to restore drug sensitivity (Roesch et al., 2013). Interestingly, the increased response to drug treatment is not thought to be mediated by the reduction of mitochondrial metabolism but by increasing the production of reactive oxygen species that together with the drug effect promote
death of these quiescent cancer cells (Cierlitza et al., 2015). The regulation of mitochondrial respiration in normal-cycling chemoresistant cancer cells and the contribution of mitochondrial versus glycolytic ATP to ABC transporter mediated chemoresistance remain unclear.

Here we show that mitochondrial ATP is essential for drug efflux mediated by ABC transporters in chemoresistant cancer cells. In contrast, ATP from glycolysis is dispensable, therefore upregulation of mitochondrial metabolism contributes to this mechanism of chemoresistance. Methylation controlled J protein (MCJ, encoded by DnaJC15) is an endogenous brake on mitochondrial respiration that negatively regulates Complex I activity (Barbier-Torres et al., 2017; Champagne et al., 2016; Hatle et al., 2013; Navasa, Martin, et al., 2015). Retrospective and prospective studies have shown that loss of MCJ expression in tumors correlates with chemotherapy resistance and poor prognosis in breast and ovarian cancer patients (Fernandez-Cabezudo et al., 2016; Strathdee et al., 2005). Here we show increased mitochondrial respiration due to the loss of MCJ in normal-cycling chemoresistant cancer cells. Importantly, we have developed therapeutic MCJ mimetics that attenuate mitochondrial respiration and ABC transporter activity in chemoresistant cancer cells both in vitro and in vivo. Restoring MCJ function is therefore a viable therapeutic strategy to inhibit ABC transporter function and overcome chemoresistance in cancer.
3. Results

3.1. Cancer cells reprogram mitochondrial metabolism when acquiring multidrug resistance

Increased mitochondrial respiration due to upregulation of the ETC machinery has been found in slow-cycling drug resistant cancer cells (e.g., stem cells, quiescent cells). However, it remains unclear whether mitochondrial respiration is also enhanced in normal-cycling cancer cells that are multidrug resistant due to mechanisms other than a slow-cycling rate. To examine differences in mitochondrial respiration between chemosensitive and chemoresistant normal-cycling cancer cells, we used the well-known multidrug resistant NCI/ADR-RES ovarian cancer cell line and its chemosensitive parental OVCAR-8 cell line. The difference in drug response between the two cell lines was verified using doxorubicin, a standard clinical chemotherapeutic agent and the selective agent used to obtain NCI/ADR-RES cells. (Supplementary Figure 3-1A).

Mitochondrial respiration was examined using the Seahorse Mito Stress test for mitochondrial oxygen consumption rates (OCR). NCI/ADR-RES cells had higher basal OCR than OVCAR-8 cells (Figure 3-1A and B), indicating that overall mitochondrial respiration is increased in NCI/ADR-RES cells. In contrast, baseline extracellular acidification (ECAR), a parameter for the rate of glycolysis, was comparable between NCI/ADR-RES cells and OVCAR-8 cells (Supplementary Figure 3-1B). The maximal respiratory capacity, determined by subtracting the OCR obtained after rotenone plus antimycin from OCR after the un-coupler FCCP,
was also higher in NCI/ADR-RES cells relative to OVCAR-8 cells (Figure 3-1B), indicating that the overall ability to employ mitochondrial respiration was higher in chemoresistant cells. We also determined the OCR linked to mitochondrial ATP production by subtracting OCR after oligomycin from baseline OCR. The levels of ATP-linked OCR were significantly higher in NCI/ADR-RES cells than OVCAR-8 cells (Figure 3-1B), suggesting that production of mitochondrial ATP was increased in NCI/ADR-RES cells. To further investigate the fraction of total ATP production that was derived from mitochondrial respiration we used the Seahorse XF Real Time ATP Rate assay. The fraction of ATP production derived from mitochondria within the total ATP production was increased in NCI/ADR-RES cells compared with OVCAR-8 cells (Figure 3-1C). Thus, chemoresistant NCI/ADR-RES have an increased ability to undergo mitochondrial respiration and to produce ATP via OXPHOS.

To show that increased mitochondrial respiration is also characteristic of other chemoresistant cells, we used the chemoresistant MES/Dox uterine cancer cell line and its chemosensitive parental MES-SA (MES) cell line. The difference in chemotherapy responses between the two cell lines was verified using doxorubicin (Supplementary Figure 3-1C). Using the Seahorse Mito Stress assay, MES/Dox cells were found to have higher basal OCR compared with MES cells (Figure 3-1D), while no differences were found in baseline ECAR (Supplementary Figure 3-1D). In addition, ATP-linked OCR and maximal respiratory capacity were also elevated in MES/Dox cells (Figure 3-1E). Furthermore, like NCI/ADR-RES
cells, the relative contribution of mitochondrial ATP production to the total ATP production was also higher in MES/Dox cells than in their chemosensitive parental cell line (Figure 3-1F). Therefore, enhanced mitochondrial respiration is a common phenotype in independently generated chemoresistant cancer cell lines from distinct origins (ovarian and uterine cancer).

Since both chemoresistant NCI/ADR-RES and MES/Dox cancer cells were derived from their parental cell lines using doxorubicin as a selective agent, we examined MCF7/Tx400 breast cancer cells that were derived from the chemosensitive MCF7 cell line using paclitaxel as a selective agent. Increased resistance to doxorubicin in MCF7/Tx400 cells was validated (Supplementary Figure 3-1E). Relative to MCF7 cells, MCF7/Tx400 cells exhibited increased basal OCR (Figure 3-1G), but comparable baseline ECAR (Supplementary Figure 3-1F). ATP-linked OCR and maximal respiratory capacity were also higher in MCF7/Tx400 cells (Figure 3-1H). The relative contribution of mitochondrial ATP production to the total ATP production was more prominent in MCF7/Tx400 cells as well (Figure 3-1I). Thus, chemoresistant breast cancer cells also have greater capacity to produce mitochondrial ATP.

To further demonstrate a prominent selective mitochondria component in the overall cell metabolism of chemoresistant cancer cells, we performed mass spectrometry-based, high-throughput metabolic profiling using chemoresistant NCI/ADR-RES cancer cells and their chemosensitive parental OVCAR-8 cells. The nonbiased metabolome analysis revealed distinct metabolic profiles for the two
cancer cell lines (Figure 3-1J and Supplementary Table 3-1). While the levels of some metabolites were decreased, the levels of other metabolites were increased in NCI/ADR-RES cells compared to OVCAR-8 cells (Figure 3-1J). Consistent with the increased mitochondrial respiration of NCI/ADR-RES described above, these cells exhibited (i) an enrichment for the products of substrates of mitochondrial metabolism (including several amino acids), (ii) decreases in glycolytic metabolites and byproducts (pyruvate, lactate) accompanied by increases in carboxylic acids from the TCA cycle (citrate, succinate), (iii) increases in products of amino acid catabolism (urea cycle intermediates and creatine/creatinine), (iv) increases in NADPH and pentose phosphate pathway intermediates (glucose 6-phosphate and sedoheptulose phosphate) as well as glutathione precursors (γ-glutamyl cysteine) and metabolites (glutathione disulfide, ascorbate), (v) increases in high energy phosphate compounds (e.g. ATP) and nucleotide metabolism, and (vi) increased NAD⁺, the product of Complex I of the ETC (Supplementary Figure 3-2). Taken together, these results show an enhanced mitochondrial metabolism and a predominant contribution of mitochondria to overall ATP synthesis in normal-cycling chemoresistant cancer cells.
Chemoresistant cancer cells exhibit increased mitochondrial respiration and ATP production

(A and B) OVCAR-8 and NCI/ADR-RES cells were analyzed by Seahorse MitoStress Assay for (A) oxygen consumption rates (OCR) at baseline and in response to sequential injections of oligomycin (O), FCCP (F), and rotenone with antimycin (RA) and for (B) basal, ATP linked (ATP), and maximal respiration rates (Max) relative to OVCAR-8 cells (n ≥ 5). (C) Rates of mitochondrial production of ATP relative to the total ATP production rate in OVCAR-8 and NCI/ADR-RES cells as determined by Seahorse ATP Production Rate Assay (n ≥ 7). (D and E) OCR of MES and MES/Dox cells were determined as in (A and B) (n ≥ 7). (F) Mitochondrial ATP production rates of MES and MES/Dox cells were determined as in (C) (n = 11). (G and H) OCR of MCF7 and MCF7/Tx400 cells were determined as in (A and B) (n ≥ 6). (I) Mitochondrial ATP production rates of MCF7 and MCF7/Tx400 cells were determined as in (C) (n = 9). (J) Relative abundances of metabolic intermediates in OVCAR-8 and NCI/ADR-RES cells as determined by mass spectrometry based metabolomics (n = 4). Mean ± SD provided. *denotes p < 0.05 by unpaired t test. Results are representative of 2 - 3 experiments.
3.2. Mitochondrial respiration prevents drug accumulation in chemoresistant cancer cells

NCI/ADR-RES cells are resistant to doxorubicin and other chemotherapeutic drugs because drugs fail to accumulate in the cells, and higher doses are needed to cause cell death. To investigate whether increased mitochondrial respiration could contribute to preventing drug accumulation in NCI/ADR-RES cells, we took advantage of the intrinsic fluorescence of doxorubicin. NCI/ADR-RES cells were incubated in the presence or absence of the mitochondrial respiration inhibitors oligomycin, which blocks mitochondrial ATP synthase (Complex V), or rotenone, which blocks Complex I. After 2 h doxorubicin was added to the cells, and they were incubated for an additional 3 h. Doxorubicin cellular accumulation was then examined by confocal microscopy. Neither oligomycin nor rotenone had an effect on cell survival for this short period of time (data not shown). As expected, in the absence of oligomycin or rotenone, NCI/ADR-RES cells treated with doxorubicin contained little to no intracellular doxorubicin. Strikingly, the addition of either rotenone or oligomycin resulted in a pronounced accumulation of doxorubicin in the cells (Figure 3-2A and B). We also examined the effect of inhibiting mitochondrial respiration on doxorubicin accumulation by flow cytometric analysis. NCI/ADR-RES cells were treated with increasing doses of oligomycin or rotenone for 2 h followed by incubation with doxorubicin for an additional 3 h. Cells were then fixed and analyzed by flow cytometry for doxorubicin fluorescence. Similar to the analysis by microscopy,
both oligomycin (Figure 3-2C) and rotenone (Figure 3-2D) robustly increased doxorubicin accumulation in the cells in a dose-dependent manner. Thus, mitochondrial-derived ATP is necessary to prevent doxorubicin accumulation in NCI/ADR-RES cells.

Cancer cells are typically highly dependent on glycolytic pathways for ATP generation and for cell proliferation. We therefore examined whether glycolysis as source of ATP was also necessary to prevent drug accumulation in chemoresistant cells. NCI/ADR-RES cells were treated with 2-deoxyglucose (2-DG), an inhibitor of glycolysis, for 2 h. As a control, we also treated cells with oligomycin as described above. Doxorubicin was added and 3 h later cells were fixed and analyzed by confocal microscopy. In contrast to oligomycin, treatment with 2-DG did not increase doxorubicin accumulation (Figure 3-2E and F). As another approach to inhibit glycolysis, we incubated NCI/ADR-RES cells in medium lacking glucose for 24 h prior to addition of doxorubicin, however a lack of glucose also did not restore doxorubicin accumulation (Figure 3-2E and F). We performed similar experiments examining doxorubicin fluorescence by flow cytometry in NCI/ADR-RES cells treated with increasing doses of 2-DG. The results further showed that inhibition of glycolysis with a high dose of 2-DG does not restore drug accumulation in NCI/ADR-RES cells cancer cells (Figure 3-2G). To verify the activity of the 2-DG used for these studies we measured total ATP levels in the cells. NCI/ADR-RES cells were treated with the highest concentration of oligomycin or 2-DG used in the above assays for 5 h (same period of time used in
the efflux experiments) and then total ATP levels were determined. Relative to untreated cells, ATP levels were strongly reduced in NCI/ADR-RES cells treated with either oligomycin or 2-DG (Figure 3-2H), indicating that 2-DG was active. Thus, inhibition of mitochondrial respiration, but not glycolysis, promotes doxorubicin accumulation. Neither oligomycin, rotenone, nor 2-DG had an effect in the accumulation of doxorubicin in the parental chemosensitive OVCAR-8 cells (Supplementary Figure 3-3A), further demonstrating that regulation of drug accumulation is restricted to chemoresistant cancer cells.

To investigate whether the differential contribution of mitochondrial respiration versus glycolysis to the failure of drug accumulation occurs in other chemoresistant cells, MES/Dox cells were treated with oligomycin, rotenone, and 2-DG, followed by incubation with doxorubicin for an additional 3 h and flow cytometry analysis. Similar to NCI/ADR/RES cells, treatment of MES/Dox cells with either oligomycin or rotenone caused a marked doxorubicin accumulation (Figure 3-2I). In contrast, treatment with 2-DG did not restore doxorubicin accumulation (Figure 3-2I). No effect of oligomycin, rotenone, or 2-DG was detected on doxorubicin accumulation in the parental MES cells (Supplementary Figure 3-3B). Together, these results demonstrate the selective contribution of mitochondrial respiration, but not glycolysis, in preventing chemotherapeutic agent accumulation in chemoresistant cancer cells.
Figure 3-2. Mitochondrial respiration, but not glycolysis, is responsible for reduced drug accumulation in chemoresistant cells

(A and B) NCI/ADR-RES cells were incubated with or without (Veh) oligomycin (Oligo, 5 μM) or rotenone (Rote, 50 μM) for 2 h followed by incubation with doxorubicin (Dox, 3 μM) for 3 h. Cells were then fixed, stained with DAPI (nuclear dye, blue), and analyzed by confocal microscopy for doxorubicin fluorescence (red). (A) Representative images. Scale bars represent 20 μm. (B) Quantification of doxorubicin intensity relative to nuclear area (n ≥ 12 cells). (C and D) NCI/ADR-RES cells were incubated with increasing concentrations of (C) oligomycin or (D) rotenone as indicated for 2 h followed by incubation with doxorubicin as in (A). Cells were then fixed and analyzed for doxorubicin fluorescence by flow cytometry. Median fluorescence intensity (MFI) is shown (n = 3). (E and F) NCI/ADR-RES cells were incubated with oligomycin (5 μM) or 2-deoxyglucose (2-DG, 50 mM) for 2 h or without glucose (No Gluc) for 24 h followed by incubation with doxorubicin as in (A). Cells were then fixed, stained, and analyzed as in (A and B). (E) Representative images. Scale bars represent 20 μm. (F) Quantification of doxorubicin intensity relative to nuclear area (n ≥ 46 cells). (G) NCI/ADR-RES cells were incubated with increasing concentrations of 2-deoxyglucose.
2-deoxyglucose as indicated for 2 h followed by incubation with doxorubicin as in (A). Cells were then fixed and analyzed as in (C and D) (n = 3). (H) NCI/ADR-RES cells were incubated with oligomycin (5 μM) or 2-deoxyglucose (50 mM) for 5 h and then total ATP levels were determined by Luciferase assay (n = 4). (I) MES/Dox cells were incubated with oligomycin (5 μM), rotenone (50 μM), or 2-DG (50 mM) for 2 h followed by incubation with doxorubicin as in (A). Cells were then fixed and analyzed as in (C and D) (n = 3). Mean ± SD provided. *denotes p < 0.05 by one-way ANOVA and Tukey’s multiple comparisons test. Results are representative of 2 - 3 experiments.

3.3. ABC transporter activity in chemoresistant cancer cells is selectively dependent on mitochondrial ATP

A major mechanism that chemoresistant cancer cells use to avoid accumulation of chemotherapeutic drugs is the acquisition of ABC transporters that actively promote drug efflux. ABC transporters utilize the energy of ATP hydrolysis to actively transport substrates against concentration gradients. While the expression of specific ABC transporters has been largely studied in chemoresistant cancer cells, including NCI/ADR-RES cells (Fairchild et al., 1987; Robey et al., 2018), little is known about the regulation of their activity and capacity to promote drug efflux. Since the results above show that drug accumulation in chemoresistant cancer cells is highly dependent on mitochondrial-derived ATP but not glycolytic ATP, we examined the contribution of these two pathways of generating energy on the activity of ABC transporters.

ABCB1 is overexpressed in NCI/ADR-RES cells (Fairchild et al., 1987). We therefore measured ABCB1 activity using a calcein retention assay. NCI/ADR-RES cells were treated with oligomycin or 2-DG for 2 h followed by incubation with calcein for 15 min. Cells were then dissolved in DMSO, and calcein
fluorescence was determined. Inhibition of mitochondrial respiration by oligomycin caused a marked reduction of ABCB1 activity as indicated by increased retention of calcein (Figure 3-3A). In contrast, 2-DG treatment had no effect on ABCB1 activity (Figure 3-3A). To further demonstrate the contribution of mitochondrial respiration to ABCB1 activity, we used HEK 293T cells which do not express ABCB1 (HEK 293T), and HEK 293T-ABCB1 cells generated by stable transfection of ABCB1. As expected, relative to control HEK 293T cells, HEK 293T-ABCB1 cells failed to accumulate calcein due to the presence of ABCB1 (Figure 3-3B). However, treatment of HEK 293T-ABCB1 cells with oligomycin markedly increased calcein levels (Figure 3-3B), while 2-DG treatment had no effect (Figure 3-3B). Since doxorubicin is also a substrate of ABCB1, we examined doxorubicin accumulation by flow cytometry in HEK 293T-ABCB1 cells in the presence or absence of different metabolic inhibitors. As expected, almost no doxorubicin could be detected in HEK 293T-ABCB1 cells relative to control HEK 293T cells (Figure 3-3C). However, treatment with oligomycin or rotenone prevented doxorubicin efflux by ABCB1 transporter, while 2-DG had no effect (Figure 3-3C). Thus, ABCB1 activity requires mitochondrial-derived ATP.

To investigate the need of mitochondrial respiration for the activity of other ABC transporters, we examined the accumulation of Hoechst 33258, a known dye substrate for ABCG2, which is also abundantly expressed by NCI/ADR-RES cells. Hoechst accumulation was examined in NCI/ADR-RES cells after pre-treatment with oligomycin, rotenone, or 2-DG. As expected, no Hoechst could be detected in
NCI/ADR-RES cells, but treatment with either oligomycin or rotenone blocked ABCG2-mediated efflux and restored Hoechst accumulation (Figure 3-3D). In contrast, treatment with 2-DG had no effect (Figure 3-3D). To further verify the effect of mitochondrial respiration on ABCG2 activity, we analyzed HEK 293T cells that stably overexpress ABCG2 (HEK 293T-ABCG2). Relative to control HEK-293T cells that do not express ABCG2, the presence of ABCG2 in HEK 293T-ABCG2 cells prevented these cells from accumulating Hoechst (Figure 3-3E). However, treatment with either oligomycin or rotenone restored the ability of HEK 293T-ABCG2 cells to accumulate Hoechst, while 2-DG had no effect (Figure 3-3E), further demonstrating that, as for ABCB1, efflux activity of ABCG2 is also dependent on mitochondrial respiration. Thus, while ABC transporters can mediate drug efflux and confer resistance to chemotherapy, their activity requires ATP specifically generated by mitochondrial respiration.

To determine the actual ATP cost of ABC transporters relative to the total cellular ATP pool, we examined the impact of the high use of ABC transporters on total ATP levels. NCI/ADR-RES cells were incubated for 5 h with increasing amounts of rhodamine B, a non-chemotherapeutic substrate of ABCB1, with the goal of inducing excess ABCB1 activity mediating rhodamine efflux. Total ATP levels were then determined by Luciferase assay. Interestingly, relative to cells without substrate, rhodamine caused a substantial reduction of total ATP levels in a dose-dependent manner (Figure 3-3F). Although rhodamine does not seem to have harmful effects, to rule out potential toxic effects of high concentrations of
rhodamine that may cause a reduction in the total ATP levels, we performed similar experiments using HEK 293T-ABCB1 cells and control HEK 293T cells. HEK 293T-ABCB1 cells treated with a high concentration of rhodamine also experienced a significant reduction in total ATP levels (Figure 3-3G). In contrast, ATP levels in the control HEK 293T cells were not affected by the high concentration of rhodamine (Figure 3-3G). Taken together, these results indicate that a high concentration of ATP is needed to sustain ABC transporter activity.

Similar to high Ca²⁺ microdomains in the cytoplasm, intracellular ATP-rich microdomains have been identified using an intracellular probe for ATP (Champagne et al., 2016). We have previously shown that these ATP-rich microdomains are dependent on high mitochondrial respiration in CD8⁺ T cells triggered by the lack of the negative regulator of mitochondrial respiration MCJ. Since it is thought that ABC transporters have a low affinity for ATP in the absence of substrate (Linton and Higgins, 2007) and that their activity is highly dependent on mitochondrial ATP based on results presented above, we examined the presence ATP-rich microdomains in NCI/ADR-RES cells using a fluorescent probe for ATP and live cell confocal microscopy analysis. The presence of ATP-rich microdomains was clear in NCI/ADR-RES cells as determined by distinct puncta throughout the cytosol (Figure 3-3H). These areas of high ATP concentration were the result of mitochondrial ATP production as they were significantly reduced by pre-treatment of NCI/ADR-RES cells with oligomycin or rotenone for 2 h prior to analysis (Figure 3-3H). However, treatment with 2-DG
had no effect on the presence of these ATP-rich microdomains (Figure 3-3H). Thus, increased mitochondrial respiration in chemoresistant cells results in the presence of ATP-rich microdomains that serve to increase local ATP levels and can fuel energy-demanding ABC transporters.

**Figure 3-3.** Mitochondrial respiration provides the energy required for ABC transporter activity.

(A) NCI/ADR-RES cells were treated with or without (Veh) oligomycin (Oligo, 5 µM) or 2-deoxyglucose (2-DG, 50 mM) for 2 h followed by incubation with calcein (0.25 µM) for 15 min. Cells were then washed, dissolved in DMSO, and calcein fluorescence relative to untreated cells was determined (n = 3). (B) Calcein fluorescence in HEK 293T cells (293T) and ABCB1-expressing HEK 293T cells
(293T-ABCB1) was determined as in (A) (n ≥ 3). (C) 293T and 293T-ABCB1 cells were treated with oligomycin (5 µM), rotenone (50 µM), or 2-DG (50 mM) for 2 h followed by incubation with doxorubicin (Dox, 3 µM) for 3 h. Cells were then fixed and analyzed for doxorubicin fluorescence by flow cytometry. Median fluorescence intensity (MFI) is shown (n = 3). (D) NCI/ADR-RES cells were treated with oligomycin (5 µM), rotenone (50 µM), or 2-DG (50 mM) for 2 h followed by incubation with Hoechst (100 ng/mL) for 3 h. Cells were then fixed and analyzed for Hoechst fluorescence by flow cytometry. Median fluorescence intensity (MFI) is shown (n = 3). (E) 293T and 293T-ABCB1 cells were treated and analyzed as in (D) (n = 3). (F) NCI/ADR-RES cells were incubated with increasing concentrations of rhodamine (Rho) B as indicated for 5 h and then total ATP levels were determined by Luciferase assay. The percent reduction in ATP relative to untreated cells is shown (n = 3). (G) 293T and 293T-ABCB1 cells were incubated with or without rhodamine B (100 µM) for 5 h and then analyzed as in (F) (n ≥ 3). (H) NCI/ADR-RES cells were incubated with oligomycin (5 µM), rotenone (50 µM), or 2-DG (50 µM) for 2 h, stained with a fluorescent ATP probe (100 µM) for 5 min, and then analyzed by live cell confocal microscopy. ATP probe fluorescence (green) and bright light differential interference contrast (DIC) are shown. Scale bars represent 10 µm. Mean ± SD provided. *denotes p < 0.05 by one-way ANOVA and Tukey’s multiple comparisons test. Results are representative of 2 - 3 experiments.

3.4. Increased mitochondrial respiration due to the loss of MCJ fuels drug efflux

The above results indicate that increased mitochondrial respiration contributes to chemoresistance in cancer cells by promoting drug efflux, suggesting that drug-mediated selection for genetic or epigenetic changes within cancer cells that affect mitochondrial respiration could lead to chemoresistance. The mitochondrial protein MCJ (encoded by the DNAJC15 gene) is an endogenous negative regulator of Complex I that restricts the activity of the ETC (Hatle et al., 2013). In CD8+ T cells and hepatocytes, MCJ deficiency results in increased Complex I activity, increased mitochondrial respiration, and the presence of ATP-rich microdomains (Barbier-Torres et al., 2017; Champagne et al., 2016; Hatle et al., 2013). Interestingly, we and others have shown that MCJ deficiency in cancer cells is linked to increased chemoresistance in breast and ovarian cancer patients and
in mouse models of breast cancer (Fernandez-Cabezudo et al., 2016; Strathdee et al., 2005). However, previous studies have not addressed the effect of MCJ on mitochondrial respiration in cancer cells nor its association with ABC transporter activity.

We first examined whether MCJ acts as a negative regulator of mitochondrial respiration in drug sensitive cancer cells. MCF7 cells express high levels of MCJ (Hatle et al., 2007). We transfected MCF7 cells with an siRNA specific for MCJ (siMCJ) or a control siRNA (C-siRNA). After 2 d, decreased levels of MCJ were verified by Western blot analysis (Figure 3-4A). We then examined mitochondrial respiration by measuring OCR using the Seahorse MitoStress assay. MCF7 cells transfected with siMCJ had higher basal OCR than those transfected with control siRNA (Figure 3-4B and C). ATP-linked OCR and maximal respiratory capacity were also higher in siMCJ transfected cells (Figure 3-4C). Furthermore, the relative production of mitochondrial ATP within the total ATP pool was also elevated after MCJ knock down (Figure 3-4D). To determine whether increased mitochondrial respiration caused by the loss of MCJ could also lead to the presence of ATP-rich microdomains identified in chemoresistant cells, siMCJ-transfected MCF7 cells were stained with the ATP probe and analyzed by live cell confocal microscopy. Almost no ATP puncta could be found in C-siRNA transfected cells (Figure 3-4E). In contrast, MCF7 cells transfected with siMCJ had a large number of well-defined ATP-rich domains (Figure 3-4E). Pre-treatment of siMCJ transfected MCF7 cells with either oligomycin or rotenone for 2 h prior to
analysis reduced the presence of ATP-rich domains, while treatment with 2-DG did not (Figure 3-4E). Thus, increased mitochondrial respiration due to the loss of MCJ in human drug-sensitive breast cancer cells promotes the formation of mitochondrial-derived ATP-rich domains.

To demonstrate the effect of MCJ on the mitochondrial respiration of primary cancers in addition to cancer cell lines, we used mammary tumor cells from MMTV-PyMT mice (WT MMTV) and MMTV-PyMT mice crossed with MCJ-deficient mice (MCJ KO MMTV) previously generated (Fernandez-Cabezudo et al., 2016). We previously showed that mammary tumor development and tumor growth in MCJ KO MMTV mice was comparable to their development in MMTV mice, although tumors from MCJ KO MMTV mice are more resistant to doxorubicin treatment (Fernandez-Cabezudo et al., 2016). We first examined OCR in cells isolated from MMTV and MCJ KO MMTV tumors. MCJ KO tumor cells had higher basal OCR compared with wildtype MMTV tumor cells (Figure 3-4F and G). Moreover, ATP-linked OCR and maximal respiratory capacities were also higher in MCJ KO cancer cells compare to wildtype cancer cells (Figure 3-4G). Thus, loss of MCJ in primary cancer cells results in increased mitochondrial respiration.

Since MCJ deficiency leads to resistance to doxorubicin and other chemotherapeutic drugs, we investigated whether the lack of MCJ in MCJ KO MMTV tumor cells prevented accumulation of doxorubicin due to increased mitochondrial-derived ATP. Primary tumor cells isolated from WT MMTV and
MCJ KO MMTV mice were expanded in vitro and then pretreated with oligomycin for 2 h followed by the addition of doxorubicin for 3 h. Intracellular doxorubicin accumulation was then examined by confocal microscopy. While doxorubicin was clearly present in WT MMTV tumor cells, it was almost undetectable in MCJ KO MMTV cells (Figure 3-4H). However, inhibiting mitochondrial ATP production with oligomycin restored doxorubicin accumulation in MCJ KO MMTV cells (Figure 3-4H). Oligomycin had little to no effect on doxorubicin accumulation in WT MMTV cells (Figure 3-4H). Together these results reveal the mechanism whereby loss of MCJ as a mitochondrial regulator can cause chemoresistance in cancer cells. Loss of MCJ results in enhanced mitochondrial respiration that is necessary to fuel ABC transporter-mediated drug efflux.
Figure 3-4. Loss of MCJ in cancer cells enhances mitochondrial respiration

(A) MCJ expression in MCF7 cells transfected with a control siRNA (C-siRNA) or an siRNA specific for MCJ (siMCJ) as determined by Western blot analysis. GAPDH is shown as a loading control. (B and C) MCF7 cells transfected with C-siRNA or siMCJ were analyzed by Seahorse MitoStress Assay for (B) oxygen consumption rates (OCR) at baseline and in response to sequential injections of oligomycin (O), FCCP (F), and rotenone with antimycin (RA) and for (C) basal, ATP linked (ATP), and maximal respiration rates (Max) relative to C-siRNA transfected cells (n ≥ 5). (D) Rates of mitochondrial production of ATP relative to the total ATP production rate in MCF7 cells transfected with a c-siRNA or siMCJ as determined by Seahorse ATP Production Rate Assay (n = 10). (E) MCF7 cells transfected with C-siRNA or siMCJ were incubated with or without (Veh) oligomycin (Oligo, 5 µM), rotenone (Rote, 50 µM), or 2-deoxyglucose (2-DG, 50µM) for 2 h, stained with a fluorescent ATP probe (100 µM) for 5 min, and then analyzed by live cell confocal microscopy. ATP probe fluorescence (green) and bright light differential interference contrast (DIC) are shown. Scale bars represent 10 µm. (F and G) Cancer cells were isolated from mammary tumors of WT MMTV-PyMT mice (WT MMTV) and MCJ deficient MMTV-PyMT mice (MCJ KO MMTV),
expanded in vitro, and then analyzed for OCR as in (B and C). (B, n = 3; C, n ≥ 8).

(H) WT MMTV and MCJ KO MMTV cells were incubated with oligomycin (5 µM) for 2 h followed by incubation with doxorubicin (Dox, 3 µM) for 3 h. Cells were then fixed, stained with DAPI (nuclear dye, blue), and analyzed by confocal microscopy for doxorubicin fluorescence (red). Scale bars represent 20 µm. Mean ± SD provided. * denotes p < 0.05 by unpaired t test. Results are representative of 2 - 3 experiments.

3.5. MCJ mimetics attenuate mitochondrial respiration in chemoresistant cells

The results above indicate that inhibiting mitochondrial respiration could be a novel approach to overcome cancer chemoresistance. However, rotenone and other inhibitors of the ETC are highly toxic as they potently and indiscriminately block mitochondrial respiration. Ideally, an approach that safely attenuates ETC without a full blockage could be more appropriate as a potential therapeutic. MCJ is the first identified endogenous negative regulator of Complex I and mitochondrial respiration, and it is abundantly expressed in some of the highly metabolically active tissues (e.g., liver, heart) (Hatle et al., 2013). Since the absence of MCJ causes chemoresistance, we investigated whether MCJ mimetics could restore MCJ function as a brake on the ETC in chemoresistant cancer cells. The N-terminal region (35 aa) of MCJ has no significant homology to any other currently known eukaryotic protein, and it has been predicated to interact with the NDUFv1 subunit of Complex I (Hatle et al., 2013). We therefore designed peptide mimetics of MCJ containing the first 20 aa of the N-terminus of human MCJ (N-MCJ). Two different MCJ mimetics were designed with different sequences added to mediate delivery into mitochondria (Supplementary Figure 3-4). The MITOx20 mimetic contains the N-MCJ sequence in addition to the HIV TAT tag that is routinely used
to confer cell permeability to peptides (Nagahara et al., 1998; Schwarze et al., 1999) and a mitochondrial targeting sequence (mts) (Addya et al., 1997) (Supplementary Figure 3-4). For the MITOx30 mimetic, the same N-MCJ sequence was added to a previously described mitochondria-penetrating peptide (MPP) (Horton et al., 2008). The MPP contains hydrophobic, noncanonical amino acids and provides for efficient delivery both into cells and into mitochondria (Supplementary Figure 3-4).

We tested whether these N-MCJ mimetics could restore MCJ function in inhibiting mitochondrial respiration in cancer cells lacking MCJ using the Seahorse Cell MitoStress assay. NCI/ADR-RES cells were incubated with MITOx20 for 12 h prior to analysis. As a control we used a peptide similar to MITOx20 that contains the HIV TAT tag but lacks the mitochondrial targeting sequence (Control-20). Therefore, it can penetrate the cell membrane, but cannot accumulate in mitochondria (Supplementary Figure 3-4). MITOx20-treated cells exhibited lower basal OCR relative to Control-20-treated cells (Figure 3-5A and B). MITOx20 treatment also reduced ATP-linked OCR and the maximal respiratory capacity of NCI/ADR-RES cells (Figure 3-5B). To confirm that the effects of MITOx20 were due to the MCJ N-terminal component, similar experiments were performed using MITOx30. As control for MITOx30, we used a peptide comparable to MITOx30 except that the MCJ sequence was reversed (Control-30). NCI/ADR-RES cells treated with MITOx30 showed lower basal OCR relative to cells treated with Control-30 (Figure 3-5C and D). In addition, ATP-linked OCR and the maximal
respiratory capacity of NCI/ADR-RES cells treated with MITOx30 were also lower compared with cells treated with Control-30 (Figure 3-5D). We also examined whether the treatment with N-MCJ mimetics had an impact on the overall ATP levels in NCI/ADR-RES cells. Both treatment with MITOx20 and MITOx30 significantly lowered total ATP levels after 12 h of treatment compared to untreated cells (Figure 3-5E). In addition, to show that the effect of the N-MCJ mimetics was directly on ETC activity, similar to oligomycin or rotenone, we used a modified Seahorse MitoStress assay where MITOx30 was injected instead of oligomycin (Figure 3-5F). While slower and less pronounced than the effect induced by oligomycin, MITOx30 injection caused a major and prolonged reduction in baseline OCR as well as in the maximal respiratory capacity of NCI/ADR-RES cells (Figure 3-5F). Thus, together these results show that these N-MCJ mimetics can restore MCJ function by attenuating (but not abrogating) mitochondrial respiration in chemoresistant cells.

Our results above (Figure 3-1) show an enhanced mitochondrial metabolism in NCI/ADR-RES cells. To determine whether the treatment with N-MCJ mimetics could also affect overall mitochondrial metabolism, NCI/ADR-RES cells were treated with MITOx30 for 12 h and then analyzed by mass spectrometry-based metabolomics. Results showed that this short period of treatment with MITOx30 was sufficient to alter cell metabolism, primarily by decreasing mitochondrial metabolism, as demonstrated by (i) decreases in TCA cycle intermediates (citrate, fumarate, malate), (ii) decreases in NAD+, the product of
Complex I, and (iii) increases in the levels of low energy phosphate compounds (nucleoside di- and monophosphates) and decreases in nucleoside triphosphates (ATP), creatine, and phosphocreatine – markers of energy reservoirs in mitochondria (Figure 3-5G, Supplementary Figure 3-5, and Supplementary Table 3-2). Overall these results indicate that N-MCJ mimetics are sufficient to attenuate mitochondrial function in MCJ-deficient cells.

**Figure 3-5.** MCJ mimetics attenuate mitochondrial respiration in chemoresistant cancer cells
(A and B) NCI/ADR-RES cells were treated with MITOx20 (25 µM) or Control-20 (25 µM) for 12 h and the analyzed by Seahorse MitoStress Assay for (A) oxygen consumption rates (OCR) at baseline and in response to sequential injections of oligomycin (O), FCCP (F), and rotenone with antimycin (RA) and for (B) basal, ATP linked (ATP), and maximal respiration rates (Max) relative to Control-20 treated cells (n ≥ 4). (C and D) NCI/ADR-RES cells were treated with MITOx30 (25 µM) or Control-30 (25 µM) for 12 h and then analyzed as in (A and B) (n = 4). (E) NCI/ADR-RES cells were incubated with or without (Vehicle) MITOx20 (25 µM) or MITOx30 (25 µM) for 5 h and then total ATP levels were determined by Luciferase assay (n = 4). (F) NCI/ADR-RES cells were analyzed for OCR at baseline and in response to sequential injections of MITOx30 (25 µM), FCCP, and rotenone with antimycin (n ≥ 4). (G) NCI/ADR-RES cells were treated with MITOx30 (25 µM) for 12 h and then the relative abundances of metabolic intermediates compared to vehicle treated cells were determined by mass spectrometry based metabolomics (n = 4). Mean ± SD shown. *denotes p < 0.05 by unpaired t test or one-way ANOVA and Tukey’s multiple comparisons test. Results are representative of 2 - 3 experiments.

3.6. MCJ mimetics decrease chemoresistance in vitro by attenuating mitochondrial respiration

Since ABC transporter-mediated chemoresistance is dependent upon mitochondrial respiration, and N-MCJ mimetics can attenuate mitochondrial respiration and ATP production in chemoresistant cells, we investigated whether N-MCJ mimetics could restore doxorubicin accumulation in NCI/ADR-RES cells. We choose a low concentration of MITOx20 that had no effect on the viability of NCI/ADR-RES cells by itself even after 2 d of treatment (Figure 3-6A). To examine the effect of MITOx20 on drug efflux, NCI/ADR-RES cells were treated with MITOx20 for 2 h followed by incubation with doxorubicin for 3 h. Doxorubicin fluorescence was then examined by confocal microscopy. MITOx20-treated cells accumulated higher levels of doxorubicin compared with cells treated with vehicle (Figure 3-6B). We also tested the effect of MITOx30. Similar to MITOx20, treatment with MITOx30 also restored doxorubicin accumulation in NCI/ADR-RES cells.
To confirm that N-MCJ mimetics could reduce ABC transporter activity, we examined doxorubicin accumulation by confocal microscopy in HEK 293T-ABCB1 cells. Cells were treated with MITOx30 for 2 h followed by incubation with doxorubicin for 3 h and confocal microscopy analysis. MITOx30 treatment enhanced doxorubicin retention in HEK 293T-ABCB1 cells (Figure 3-6D). Thus, by attenuating mitochondrial respiration, N-MCJ mimetics lower ABC transporter activity and drug efflux in chemoresistant cancer cells.

To determine whether the treatment with N-MCJ mimetics could overcome the resistance to doxorubicin in multidrug resistant cancer cells, NCI/ADR-RES cells were incubated with doxorubicin alone or in combination with MITOx30 or Control-30 for 3 d and then the number of viable cells was determined by Trypan blue exclusion. Neither doxorubicin, MITOx30 or Control-30 alone affected cell survival relative to untreated cells (Figure 3-6E). Similarly, the combination of doxorubicin and Control-30 had no effect on cell survival (Figure 3-6E). In contrast, the combination of doxorubicin with MITOx30 markedly reduced NCI/ADR-RES cell viability (Figure 3-6E). Treatment with MITOx20 also enhanced the response of NCI/ADR-RES cells to doxorubicin after 2 d (Figure 3-6F). We also examined the effect of N-MCJ mimetics on doxorubicin responses in the chemoresistant MES/Dox cancer cells. Consistent with results for NCI/ADR-RES cells, while doxorubicin alone had no effect, the combination of doxorubicin and MITOx30 decreased cell survival after 3 d (Figure 3-6G).
To further demonstrate the effect of N-MCJ mimetics in overcoming chemoresistance in cancer cells *in vitro*, we performed clonogenic assays. NCI/ADR-RES cells were treated with doxorubicin alone or in combination with MITOx20 or MITOx30 for 2 d. Cells were then replated at a low density and grown in normal culture medium. After 1 wk, cell colonies were counted. Both MITOx20 and MITOx30 in combination with doxorubicin reduced the number of colonies relative to doxorubicin alone (Figure 3-6H). No effect was observed with either MITOx20 or MITOx30 alone in the absence of doxorubicin (Figure 3-6I). We also examined the effect of MITOx30 in combination with doxorubicin on the clonogenicity of MES/Dox cells. Similar to NCI/ADR-RES cells, MITOx30 together with doxorubicin reduced the number of MES/Dox cell colonies relative to doxorubicin alone (Figure 3-6J). Thus, together these results show the efficacy of combination therapies of N-MCJ mimetics and chemotherapy in overcoming chemoresistance of cancer cells *in vitro*. 
Figure 3-6. MCJ mimetics sensitize chemoresistant cells to chemotherapy treatment

(A) NCI/ADR-RES cells were treated with or without (Vehicle) MITOx20 (5 µM) for 3 d and then surviving cell counts were determined by Trypan blue exclusion. Viability relative to untreated cells is shown (n ≥ 4). (B) NCI/ADR-RES cells were incubated with MITOx20 (5 µM) for 2 h followed by incubation with doxorubicin (Dox, 3 µM) for 3 h. Cells were then fixed, stained with DAPI (nuclear dye, blue), and analyzed by confocal microscopy for doxorubicin fluorescence (red). Representative images and quantification of doxorubicin intensity relative to nuclear area (n ≥ 32 cells) are shown. Scale bars represent 20 µm. (C) NCI/ADR-RES and (D) ABCB1-expressing HEK 293T cells were incubated with MITOx30 (5 µM) for 2 h followed by incubation with doxorubicin and analysis as in (B). Representative images and quantification of doxorubicin intensity relative to nuclear area (C, n ≥ 74 cells; D, n ≥ 106 cells) are shown. Scale bars represent 20 µm. (E) NCI/ADR-RES cells were treated with doxorubicin (3 µM), Control-30 (5 µM), and/or MITOx30 (5 µM) for 3 d and then cell viability was determined as in (A) (n = 4). (F) NCI/ADR-RES cells were treated with doxorubicin (3 µM) and/or MITOx20 (5 µM) for 2 d and then cell viability was determined as in (A) (n = 3). (G) MES/Dox cells were treated with doxorubicin (3 µM) alone or in combination with MITOx30 (5 µM) for 3 d and then cell viability was determined as in (A) (n = 3). (H) NCI/ADR-RES cells were treated with doxorubicin (3 µM) in combination with MITOx20 (5 µM) or MITOx30 (5 µM) for 2 d, replated at a low density (500 cells), grown in normal culture medium for 1 wk, and then the
number of colonies formed was determined (n = 4). (I) NCI/ADR-RES cells were treated with MITOx20 (5 µM) or MITOx30 (5 µM) for 2 d and then analyzed for clonogenicity as in (H) (n = 3). (J) MES/Dox cells were treated with doxorubicin (3 µM) alone or in combination with MITOx30 (5 µM) for 2 d, replated at a low density (400 cells), and then analyzed for clonogenicity as in (H) (n = 4). Mean ± SD provided. * denotes p < 0.05 by unpaired t test or one-way ANOVA and Tukey’s multiple comparisons test. Results are representative of 2 - 3 experiments.

3.7. MCJ mimetics reverse cancer chemoresistance in vivo

To determine whether MCJ mimetics show efficacy in reversing cancer chemoresistance in vivo, we used MCJ KO MMTV mice since the mammary tumors are resistant to doxorubicin and continue to grow with the treatment (Fernandez-Cabezudo et al., 2016). Once tumors reached a measurable size, mice were treated with doxorubicin alone or in combination with MITOx20, MITOx30 or Control-30. We first tested the stability of MITOx20 and MITOx30 to serum proteases by incubating them in the presence of serum in vitro for 3 and 6 h. Spot blot analysis using a specific anti-MCJ antibody that recognizes the N-terminus showed minimal reduction even after 6 h of incubation (Figure 3-7A), indicating that these peptides are relatively stable. Following the doxorubicin treatment schedule used in our previous studies (Fernandez-Cabezudo et al., 2016), treatments with doxorubicin (i.p.) and/or N-MCJ or control mimetics (s.c.) were given every other day for a total of 12 d after which mice treated with doxorubicin alone had to be euthanized as tumors reached the maximum approved size. As determined by the increase in tumor volume at 12 d relative to the initial size, the tumors of mice treated with doxorubicin alone or doxorubicin with Control-20 were markedly increased (Figure 3-7B). In contrast, tumors of mice treated with doxorubicin in
combination with either MITOx20 or MITOx30 showed a prominent reduction in size (Figure 3-7B). We found no obvious harmful effects of MITOx20 or MITOx30 in combination with doxorubicin in these mice even after the 12 d of treatment, and mice remained highly active with no signs of stress. Following H&E staining, histological analysis of the liver and heart showed no evidence of toxicity (Supplementary Figure 3-6). In addition to the final tumor size, we also followed tumor volume over time. Tumors of mice that received either doxorubicin alone or in combination with Control-30 did not respond to treatment and continued growing (Figure 3-7C). In contrast, there was a rapid delay (evident after 4 d) in the growth of tumors followed by a progressive reduction in tumor size in mice treated with doxorubicin in combination with either MITOx20 or MITOx30 (Figure 3-7C). Thus, N-MCJ mimetics show efficacy in vivo in overcoming chemoresistance of mouse tumors that lack MCJ.

To further demonstrate the potential of N-MCJ mimetics to overcome chemoresistance in human cancer, we used a xenograft model with the chemoresistant NCI/ADR-RES cells grafted into immunocompromised NSG mice commonly used for human tumor studies. Once tumors reach sufficient size, mice were treated with doxorubicin alone or in combination with MITOx20 or MITOx30. As determined by the change in tumor volume relative to the initial size, the combination of doxorubicin with either MITOx20 or MITOx30 successfully reduced the final tumor volume (Figure 3-7D). Similar to the mouse tumor model, the response to doxorubicin in combination with N-MCJ mimetics was evident
shortly after initiation of therapy (Figure 3-7E). Thus, restoring MCJ function with N-MCJ mimetics increased responses to standard chemotherapy even in highly resistant human cancer cells that lack MCJ.

![Figure 3-7. Reversal of chemotherapy resistance in mouse models of chemoresistant cancer](image)

(A) Spot blot analysis of MITOx20, MITOx30, and Control-30 prior to (0 h) or after incubation with serum (3 or 6 h) using an antibody specific for the N-terminus of MCJ. (B and C) MCJ deficient MMTV-PyMT mice were treated with doxorubicin alone (Dox, n = 5) or in combination with Control-30 (n = 4), MITOx20 (n = 6), or MITOx30 (n = 5) every other day for 12 d. (B) Tumor volumes at the end of treatment relative to the initial size. (C) Change in tumor volume over time during treatment relative to the initial tumor size. (D and E) NSG mice with NCI/ADR-RES cell xenografts were treated with doxorubicin alone or in combination with MITOx20 or MITOx30 every other day for 8 d (n = 6). (D) Tumor volumes at the end of treatment relative to the initial size. (E) Change in tumor volume over time during treatment relative to the initial tumor size. Dotted lines represent initial tumor volumes prior to treatment (100 %). Mean ± SD provided. * denotes p < 0.05 by one-way ANOVA and Tukey’s multiple comparisons test.
4. Discussion

Chemotherapy (e.g., anthracyclines, taxanes, cyclophosphamides) remains part of the standard of care for most types of cancers worldwide. In addition to being used as frontline therapy, chemotherapy is also used as neoadjuvant therapy to reduce tumor size prior to surgery or in combination with several of the newer biological therapies (e.g., antibodies against CTLA4, PD1, Her2) (Apetoh et al., 2015; Wargo et al., 2015). Thus, while the development of new immunotherapy strategies (e.g., immune checkpoints and adoptive T cell therapies) is currently the main focus in cancer treatment, chemotherapy remains the frontline therapy for many cancers and also represents an affordable option worldwide. However, the development of chemoresistance and the resulting need for high doses of chemotherapeutic drugs with associated toxicity still represent the major limitation of this cancer treatment (Lyman, 2009; Pai and Nahata, 2000). Unraveling the mechanisms of chemoresistance and developing novel strategies to overcome them while minimizing drug toxicity is therefore still a priority (Gottesman et al., 2002; Robey et al., 2018). In this study, we uncover a novel approach to overcome chemoresistance informed by a new understanding of a common mechanism that protects cancer from chemotherapy. We show that mitochondrial metabolism and mitochondrial-derived ATP are essential for the activity of the ABC transporters to promote drug efflux and chemoresistance in cancer cells. In addition, we demonstrate a novel target that acts as negative regulator of mitochondrial metabolism and new compounds that restore activity
of that target and can be further developed as therapeutic agents for combination with standard chemotherapy.

ABC transporters have the ability to transport multiple substrates across the cytoplasmic membrane against the gradient by using ATP as the source of energy (Pan et al., 2016; Ween et al., 2015). Some of these transporters can mediate the efflux of a number of chemotherapeutic drugs. While the correlation between the presence of these transporters and chemoresistance in cancer cell lines is clear, the correlation between the presence of specific drug efflux transporters and poor chemotherapy response in primary tumors in cancer patients is not well established despite a number of clinical studies (Chen et al., 2016; Robey et al., 2018; Ween et al., 2015). As a result, the presence of ABC transporters has not yet been used as biomarker for poor response to chemotherapy, and interest in these transporters in cancer has waned. Other than gene expression, little is known about the regulation of ABC transporter activity. ABC transporters are thought to have a low affinity for ATP in the absence of substrate, and up to two ATP molecules are needed for the transport of one molecule of substrate (Linton and Higgins, 2007; Patzlaff et al., 2003; Poolman et al., 2005). Here we reveal for the first time the unique need of ATP derived from mitochondria for the activity of ABC transporters. Interestingly, we also show that ATP derived from glycolysis is unnecessary for the activity of ABC transporters in cancer cells. Our findings could explain the paradox of why the sole presence of the ABC transporters in tumors may not correlate with poor response to chemotherapy. In line with the Warburg
effect, it is well known that cancer cell metabolism is biased towards glycolysis instead of mitochondria respiration (Koppenol et al., 2011; Warburg, 1956). Thus, expression of ABC transporters in highly glycolytic cancer cells with minimal mitochondrial respiration would not be sufficient to confer chemoresistance. Here we also show that, relative to parental drug-sensitive cancer cells, derived chemoresistant cancer cells have elevated mitochondrial respiratory capacity. Thus, acquisition of ABC transporters as well as highly effective mitochondrial respiration are needed to establish chemoresistance in cancer cells.

In this regard, it is well known that stem cancer cells respond poorly to chemotherapy and are most likely the cause of acquired chemoresistance after the initial successful response of the primary tumor (Abdullah and Chow, 2013). In contrast to glycolytic cancer cells, stem cancer cells display elevated mitochondrial respiration (Peiris-Pages et al., 2016; Viale and Draetta, 2015). Interestingly, ABCB1 and ABCG2 have been used as markers for identification of stem cancer cells (Ding et al., 2010; Haraguchi et al., 2006; Moitra, 2015), although their potential roles in mediating chemoresistance in these cells has not been established. Thus, this is another case where chemoresistance is associated with increased levels of mitochondrial-derived ATP in addition to the overexpression of ABC transporters. An association between mitochondrial respiration and chemoresistance has also been reported in slow-cycling melanoma cancer cell lines, but the potential involvement of ABC transporters was not investigated (Cierlitza et al., 2015; Roesch et al., 2013).
A question that remains following our findings: why is only mitochondrial ATP but not glycolytic ATP required for ABC transporter activity? Because of the potential low affinity of ABC transporters for ATP (Linton and Higgins, 2007), a high concentration of ATP would be needed for their activity. This requirement could be difficult to achieve only with cytoplasmic levels of ATP generated by glycolysis. However, since mitochondria are dynamic organelles that can move throughout the cytosol, they can traffic to the cytoplasmic membrane where most ABC transporters localize and contribute to the generation of ATP-rich microdomains. We have previously shown the presence of ATP-rich microdomains that are dependent on mitochondrial ATP synthesis in CD8+ T cells with high mitochondrial activity (Champagne et al., 2016). Here we also show the presence of these ATP-rich domains in chemoresistant cancer cell lines with high mitochondrial activity. These microdomains with high concentrations of ATP could be required to sustain the activity of ABC transporters.

MCJ has been shown to be an endogenous negative regulator of mitochondrial respiration in a number of primary tissues including the heart, liver, CD8+ T cells, and macrophages (Barbier-Torres et al., 2017; Champagne et al., 2016; Hatle et al., 2013; Navasa, Martin, et al., 2015). MCJ restricts OXPHOS and mitochondrial ATP production by limiting Complex I activity (Barbier-Torres et al., 2017; Hatle et al., 2013). Here we show that MCJ also acts as a negative regulator of mitochondrial respiration in cancer cells and that loss of MCJ leads to increased mitochondrial ATP production. Importantly, we show for the first time that high
levels of mitochondrial derived ATP achieved in the absence of MCJ are sufficient to fuel the activity of ABC transporters and promote drug efflux. Loss of MCJ expression correlates with poor responses to chemotherapy and poor prognoses in ovarian cancer patients (Shridhar et al., 2001; Strathdee et al., 2005). We have shown that loss of MCJ expression causes chemoresistance in vitro in cancer cell lines and in vivo in mouse models of mammary cancer (Fernandez-Cabezudo et al., 2016; Hatle et al., 2007). In addition, retrospective and prospective studies in breast cancer patients revealed that loss of MCJ in primary tumors correlates with poor responses to chemotherapy, but not with responses to hormone therapy (which is not affected by ABC transporters) (Fernandez-Cabezudo et al., 2016). Thus, it is possible that a combination of the expression of MCJ together with ABCB1 or other ABC transporters will serve as a biomarker that effectively predicts chemoresistance with high confidence. Thus, cancer cells that express drug-efflux ABC transporters, but have lost MCJ expression, will have the highest probability of chemoresistance.

A number of ABC inhibitors have been developed and clinically tested, most of which interfere with substrate binding to the transporter (Kathawala et al., 2015; Li et al., 2016; Saraswathy and Gong, 2013). Despite promising preclinical studies, these inhibitors failed to show efficacy in clinical trials. Toxicity due to off target effects was the main problem for first generation inhibitors of ABCB1. A lack of sufficient efficacy in combination with chemotherapy relative to chemotherapy alone was the challenge for second generation inhibitors despite
significant inhibition of the three most commonly expressed ABC transporters (ABCB1, ABCG2, ABCC1) (Coley, 2010; Kohler and Stein, 2003). The third generation of ABCB1 inhibitors showed some efficacy in clinical trials, but their high toxicity has compromised their use for cancer treatment. Thus, no ABC transporters inhibitors have been highly successful (Robey et al., 2018; Ween et al., 2015). Here we show the efficacy of deliverable N-MCJ mimetics in attenuating mitochondrial respiration, reducing ABC transporter drug efflux, and increasing responses to standard chemotherapy in cancer cells in vitro. Moreover, N-MCJ mimetics showed efficacy in reducing tumor size in vivo when administered in combination with doxorubicin without increasing drug toxicity. Together, these results suggest that attenuating mitochondrial respiration by restoring MCJ expression in combination with standard chemotherapy can be a novel therapeutic approach in cancer patients by increasing sensitivity to lower chemotherapy doses in those cancer cells that have lost MCJ. We therefore propose that N-MCJ mimetics could be developed as a novel “adjuvant” chemotherapy to be administered with lower doses of standard chemotherapy.

5. Experimental Procedures

5.1. Cell lines and culture conditions

NCI/ADR-RES (formerly MCF7/Adr), MES, MES/Dox, and MCF7/Tx400 cells were previously described (Batist et al., 1986; Harker et al., 1983; Hatle et al., 2007; Huff et al., 2006; Robey et al., 2003; Robey et al., 2008). MCF7 cells were
purchased from American Type Culture Collection (ATCC). OVCAR-8 cells were kindly provided by Dr. Ernst Lengyel at the University of Chicago. All cancer cell lines were maintained in RPMI 1640 supplemented with 10 % FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. HEK 293T cell lines were previously described (Robey et al., 2003; Robey et al., 2008) and maintained in RPMI as above additionally supplemented with 2 mg/mL Geneticin. Chemotherapeutic agents added to maintain chemoresistant phenotypes were removed at least 1 wk prior to experiments. MCF7 cell transfections were performed using siPORT NeoFX Transfection Agent (ThermoFisher Scientific) following the recommended protocol for an oligonucleotide siRNA based on the previously described MCJ targeting sequence (Hatle et al., 2007). FBS was obtained from Serum Source International, while all other cell culture reagents were from ThermoFisher Scientific. Doxorubicin was obtained from the University of Vermont Medical Center Pharmacy. Oligomycin, rotenone, 2-deoxyglucose, paclitaxel, and Hoechst 33258 were purchased from Sigma Aldrich.

5.2. Mouse models

All mice were maintained in the animal facility at the University of Vermont under Institutional Animal Care and Use Committee (IACUC) approved conditions. WT and MCJ deficient MMTV-PyMT were previously described (Fernandez-Cabezudo et al., 2016). NSG mice (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ,
Jackson Laboratories) were injected with $5 - 7 \times 10^6$ NCI/ADR-RES cells (i.p.) in 150 µL PBS. For both models, mice were treated with vehicle, doxorubicin (i.p., 2 mg/kg for MMTV mice, 1 mg/kg for NSG mice), and/or and MCJ mimetics (s.c., 10 mg/kg) every other day after tumors reached 0.15 - 0.25 cm$^3$. Tumor measurements were performed using a caliper to determine the tumor volumes using the formula $\text{Vol} = \text{length} \times \text{width} \times \text{height}$ (above skin level). Animal studies were performed under the oversight of the University of Vermont IACUC.

5.3. Extracellular flux analysis

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were analyzed under basal conditions and in response to sequential injections of oligomycin (2 µM), FCCP (2 µM), and rotenone with antimycin A (1 µM each) using the Seahorse MitoStress Test Kit. OCR analysis was also performed after incubation with N-MCJ mimetics for 12 h. ATP production rates were determined using the XF Real-Time ATP Rate Assay Kit following the recommended protocol. All extracellular flux analyses were performed using an XF24 or XF96 Extracellular Flux Analyzer as recommended by the manufacturer (all Agilent Technologies).

5.4. Mass spectrometry based metabolomics

Metabolic profile comparisons of OVCAR-8 and NCI/ADR-RES cells were performed on equal numbers of cells. Cells were cultured under normal conditions, detached using trypsin-EDTA (0.05 %), counted, normalized to 0.5 x
in each sample, and then cell pellets were snap frozen in liquid nitrogen prior to analysis. To determine the effect of N-MCJ mimetic treatment, equal numbers of NCI/ADR-RES cells were plated and allowed to adhere for 2 d followed by the addition of vehicle or MITOX30 (25 µM) for 12 h. Cells were then collected and processed as above. Metabolomics and flux analyses were performed as previously reported (D'Alessandro et al., 2015). Briefly, 2 x 10^6 cells and 20 µl of cell media were extracted in 1 mL and 980 µL of cold lysis and extraction buffer (methanol : acetonitrile : water, 5:3:2), respectively. After discarding protein pellets, 10 µL of water and methanol soluble fractions were run through a Kinetex C18 1.7 µm, 100 x 2.1 mm (Phenomenex) reversed phase column (Positive ion mode - phase A: water, 0.1 % formic acid; B: acetonitrile, 0.1 % formic acid; Negative ion mode - phase A: 1 mM NH₄Ac 95:5 water : acetonitrile; phase B: 1 mM NH₄Ac 95:5 acetonitrile : water) via an ultra-high performance chromatographic system (UHPLC - Vanquish, Thermo Fisher). UHPLC was coupled in line with a high-resolution quadrupole Orbitrap instrument run in both polarity modes (QExactive, Thermo Fisher) at 70,000 resolution (at 200 m/z). Gradients and other technical parameters and the variants employed herein have been extensively described (D'Alessandro et al., 2017; Nemkov et al., 2017). Metabolite assignment and peak integration for relative quantitation were performed through the software Maven (Princeton), against the KEGG pathway database and an in-house validated standard library (> 1,000 compounds, Sigma Aldrich, IROATech). Integrated peak areas were exported to Excel (Microsoft) and
elaborated for statistical analysis (unpaired t test) and hierarchical clustering analysis (HCA) through Prism (GraphPad Software) and GENE-E (Broad Institute), respectively.

5.5. Flow cytometry analysis

For detection of ABC transporter substrate accumulation, cells were pretreated with metabolic inhibitors as indicated for 2 h followed by the addition of doxorubicin (3 μM) or Hoechst 33258 (100 ng/mL) for 3 h. Cells were then washed, fixed in PBS supplemented with 1 % paraformaldehyde, and then immediately analyzed using a BD Biosciences LSRII Flow Cytometer at the University of Vermont Flow Cytometry and Cell Sorting Facility. Median fluorescence intensities relative to intreated cells were determined using FlowJo Software.

5.6. Confocal microscopy analysis

All confocal microscopy analyses were performed using a Zeiss LSM 510 Meta Confocal Laser Scanning microscope (Carl Zeiss Microscopy) at the University of Vermont Microscopy Imaging Center as previously described (Champagne et al., 2016). Analysis of doxorubicin pixel fluorescence intensity per nuclear area was performed using ImageJ software (version 1.51h, NIH). For analysis of subcellular ATP distribution, cells were plated in glass bottom plates (MatTek) and allowed to adhere for 3 d. Cells were then pretreated with metabolic inhibitors as indicated for 2 h followed by the addition of a fluorescent ATP/ADP
probe (Rao et al., 2012) (100 μM) for 5 min. Cells were then washed with and immediately imaged in PBS. For analysis of doxorubicin accumulation, cells were plated on glass coverslips and allowed to adhere for 3 d. Cells were then pretreated with metabolic inhibitors as indicated for 2 h followed by the addition of doxorubicin (3 μM) for 3 h. Cells were then washed, fixed in PBS supplemented with 1 % paraformaldehyde, and stained with DAPI. Coverslips were mounted to glass microscope slides using Vectashield Antifade Mounting Medium (Vector Laboratories) and sealed with nail polish prior to imaging.

5.7. Calcein retention

Effects of metabolic inhibitors on calcein retention were determined using the Vybrant Multidrug Resistance Assay Kit (ThermoFisher Scientific). Cells were pretreated with metabolic inhibitors as indicated for 2 h followed by the addition of calcein (0.25 μM) for 15 min. Cells were then washed, dissolved in DMSO, and then calcein absorbance was determined using an ELx800 Absorbance Microplate Reader (BioTek).

5.8. Intracellular ATP concentration

Intracellular ATP concentration in cells (10⁴) was determined using the ATPlite Luminescence Assay System (PerkinElmer) and a TD-20/20 Luminometer (Turner BioSystems) as recommended by the manufacturers.
5.9. MCJ expression

Western blot analyses were performed on whole cell lysates derived using RIPA buffer supplemented with 1 mM PMSF, 1 mM Na$_3$VO$_4$, and 0.5 % Protease Inhibitor Cocktail (Sigma Aldrich). Lysates were separated by electrophoresis, transferred to PVDF membranes, and then analyzed using anti-MCJ and anti-GAPDH antibodies as previously described (Fernandez-Cabezudo et al., 2016; Hatle et al., 2013).

5.10. Cell viability in response to drug treatments

For analysis of cell survival, cells were plated in normal culture medium and allowed to adhere for 2 d. Culture medium was then replaced with medium containing doxorubicin and/or N-MCJ mimetics, and cells were incubated for an additional 2 - 3 d as indicated. Cells were then washed with PBS, detached with 0.05 % trypsin-EDTA, and live cell counts were determined by Trypan blue exclusion. Viability was then calculated relative to untreated cells. For clonogenicity assays, cells were plated and allowed to adhere for 2 d followed by the addition of doxorubicin (1 µM for MES/Dox cells, 3 µM for NCI/ADR-RES cells) and/or N-MCJ mimetics (5 µM) in fresh culture medium for 2 d. Cells were then replated in normal culture medium, incubated for 1 wk, washed with PBS, fixed with PBS supplemented with 1 % paraformaldehyde, stained with crystal violet (0.01 % in water), and then colony counts were determined. MTT cell
viability assays were performed as previously described (Hatle et al., 2007). MTT (thiazolyl blue tetrazolium bromide) was purchased from Acros Organics.

5.11. Peptide stability

N-MCJ mimetic stability was determined by diluting stocks (1 mM in water) 1:1 in human serum and incubating at ambient temperature for the indicated times. For detection, 1 μL of diluent was spotted onto nitrocellulose and allowed to air dry. Blots were then subjected to UV crosslinking for 30 s (UVP Ultraviolet Crosslinker CL-1000) and analyzed by Western blot as described above.

5.12. Statistical analyses

Statistical significance was determined by unpaired t test for two groups or by one-way ANOVA with Tukey’s multiple comparisons test for three or more groups using Graphpad Prism software. P < 0.05 was considered statistically significant. Figures depict mean ± standard deviation (SD) in all cases.
6. Supplementary Information

6.1. Supplementary Figures

Supplementary Figure 3-1. Derived chemoresistant cancer cells do not have increased rates of glycolysis compared to their chemosensitive parental cell lines

(A) Viability of OVCAR-8 and NCI/ADR-RES cells in the presence of increasing concentrations of doxorubicin (Dox) as determined by MTT assay. (B) Baseline extracellular acidification rates (ECAR) of OVCAR-8 and NCI/ADR-RES cells as determined by extracellular flux analysis. (C) Viability of MES and MES/Dox cells in the presence of increasing concentrations of doxorubicin as determined by MTT assay. (D) Baseline ECAR of MES and MES/Dox cells as determined by extracellular flux analysis. (E) Viability of MCF7 and MCF7/Tx400 cells in the presence of increasing concentrations of doxorubicin as determined by MTT assay. (F) Baseline ECAR of MCF7 and MCF7/Tx400 cells as determined by extracellular flux analysis. Mean ± SD provided (n ≥ 4).
Supplementary Figure 3-2. NCI/ADR-RES cells have increased rates of amino acid and nucleotide metabolism compared to OVCAR-8 cells

Altered metabolic pathways in NCI/ADR-RES cells relative to OVCAR-8 cells as determined by mass spectrometry based metabolomics and metabolite sets enrichment analysis (MSEA). Pathway impact, number of metabolites detected that support the assignment of a pathway; -log(p), negative log of the p value determined by unpaired t test (n = 4).
Supplementary Figure 3-3. Metabolic inhibitors do not significantly increase the accumulation of doxorubicin in chemosensitive cancer cells

(A) OVCAR-8 and (B) MES cells were treated with or without (Veh) oligomycin (Oligo, 5 µM), rotenone (Rote, 50 µM), or 2-deoxyglucose (2-DG, 50 mM) for 2 h followed by incubation with doxorubicin (Dox, 3 µM) for 3 h. Cells were then fixed and doxorubicin fluorescence was determined by flow cytometry analysis. Median fluorescence intensity (MFI) is shown. Mean ± SD (n = 3) provided.

Supplementary Figure 3-4. Peptide mimetics of MCJ

(Upper) N-MCJ mimetics compared to the domains of the full length MCJ (not to scale). (Lower) Amino acid sequences of N-MCJ mimetics. Abbreviations used: N-term, N-terminus; TM, transmembrane domain; C-term, C-terminus; TAT, HIV TAT sequences; N-MCJ, first 20 aa of the MCJ N-terminus; mts, mitochondrial targeting sequence; mpp, mitochondrial penetrating peptide; rev, reversed aa sequence.
Supplementary Figure 3-5. MCJ mimetics alter amino acid and nucleotide metabolism in MCJ-deficient cancer cells

Altered metabolic pathways in NCI/ADR-RES cells treated with MITOx30 for 12 h relative to vehicle treated cells as determined by mass spectrometry based metabolomics and metabolite sets enrichment analysis (MSEA). Pathway impact, number of metabolites detected that support the assignment of a pathway; -log(p), negative log of the p value determined by unpaired t test (n = 4).
Supplementary Figure 3-6. MCJ mimetics do not increase doxorubicin toxicity

Representative images from H&E staining of (A) liver and (B) heart sections of control (untreated) mice or mice treated with doxorubicin (Dox) alone or in combination with MITOx20 or MITOx30. Treatments were performed every other day. Mice were sacrificed after 11 d.
6.2. Supplementary Tables

**Supplementary Table 3-1. Metabolome Analysis of OVCAR-8 and NCI/ADR-RES Cells**

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*Median value. †Fold increase of peak area calculated as ADR-RES/OVCAR-8. ‡Determined by unpaired t test (n = 4).
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**Supplementary Table 3-2. Metabolome Analysis of NCI/ADR-RES Cells Treated with MITOx30**

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<tr>
<th>Pathway</th>
<th>Compound</th>
<th>*Peak Area Vehicle</th>
<th>MITOx30</th>
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<th>‡p-value</th>
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*Median value. †Fold increase of peak area calculated as MITOx30/Vehicle. ‡Determined by unpaired t test (n = 4).
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<td>Creatine</td>
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## Supplementary Table 3-2 (continued)

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7. Acknowledgements

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CHAPTER 4. Concluding Remarks

MCJ is a negative regulator of mitochondrial metabolism that functions through direct interactions with Complex I. MCJ is predicted to bind the NDUFv1 subunit of Complex I (Hatle et al., 2013), however the binding interactions that facilitate the function of MCJ are currently unknown. Considering that the NDUFv1 contains the NADH binding site of Complex I (Deng et al., 1990), MCJ may disrupt the binding of NADH and NDUFv1 in order to regulate Complex I activity. It is also possible that NADH (or NAD\(^+\)) may act directly on MCJ, thereby transiently affecting the regulatory activity of MCJ.

While the crystal structure of MCJ has not been solved, the N-terminus is likely the domain governing its regulatory function as it has a unique amino acid sequence compared to all currently known eukaryotic proteins (Hatle et al., 2007; Shridhar et al., 2001). Additionally, as shown in this dissertation (CHAPTER 3) a peptide mimetic of the N-terminus is sufficient to regulate mitochondrial metabolism in MCJ deficient cancer cells. Due to the unique character of the N-terminus, MCJ may be a druggable target, and there may not be significant off-target effects on other proteins. Therefore, the development of small molecule inhibitors or agonists of MCJ function may provide therapeutic benefits.

Inhibitors of MCJ may improve the efficacy of vaccines to viral infections, such as those for influenza. Currently approved vaccines for influenza elicit a strong antibody response to surface viral proteins (notably hemagglutinin), however
genetic drift/shift and strong immune selection of circulating viruses often renders responses ineffective (Krammer, 2015). In addition, antibody titers wane over time, which results in a loss of protection. As such, yearly immunizations are needed to maintain protection. The ultimate goal of influenza vaccine research is to produce a “universal” vaccine that could eliminate the need for annual vaccinations (Pica and Palese, 2013).

CD8+ T cell responses are often targeted to internal viral proteins, which typically undergo less change over time. Therefore, vaccines that elicit stronger CD8+ T cell memory may provide lasting protection. The polio vaccine is a notable example that stimulates strong T cell memory and effectively provides protection for life. As shown previously (CHAPTER 2), loss of MCJ in mouse models is highly protective to secondary influenza virus infection through the promotion of CD8+ T cell effector functions and memory development. Inhibiting MCJ during immunization could therefore lead to improved CD8+ T cell memory development and to enhanced vaccine responses.

In addition, there is ongoing research into the production of vaccines for cancer (Banchereau and Palucka, 2018; Finn, 2018). As for viral infections, the cytotoxic effector function of CD8+ T cells is important for the elimination of transformed cells. While the role of MCJ in cancer immunity has not yet been addressed, it is likely that an MCJ inhibitor could also promote the efficacy of cancer vaccines.

MCJ deficiency in the liver also has several positive outcomes, therefore inhibitors may provide therapeutic benefits in liver diseases. Loss of MCJ in the
liver is highly protective to the development of steatosis (fatty liver) in mouse models of fasting (Hatle et al., 2013). Steatosis is the first clinical manifestation of non-alcoholic fatty liver disease (NAFLD) in humans, and it is thought that excess buildup of fatty acids eventually leads to inflammation, hepatocyte death, and fibrosis in the liver (although the exact mechanisms are currently unclear). NAFLD progression can lead to cirrhosis and liver cancer, however there are currently no specific treatments in the clinic for this disease. Since loss of MCJ function can effectively prevent steatosis, it is possible that inhibitors of MCJ may improve the outcomes of NAFLD patients.

MCJ may also represent a target for the treatment of metabolic syndrome. The most common comorbidity of a number of metabolic diseases is obesity, notably abdominal fat deposition. A large-scale genome wide association study (GWAS) identified MCJ as the closest gene to a single nucleotide polymorphism that strongly correlated with increased waist circumference in humans (Heard-Costa et al., 2009). While this finding is only a correlation that has not been fully examined, it implicates MCJ in the maintenance of fat metabolism and storage. Thus, inhibition of MCJ may provide a therapeutic benefit to metabolic syndrome patients.

In addition, loss of MCJ has been shown to substantially decrease liver toxicity after acetaminophen overdose. The only treatment currently available is administration of N-acetylcysteine (NAC), however the efficacy is substantially diminished in a relative short amount of time post overdose (< 10 hours). It has
been shown that downregulation of MCJ expression up to 24 hours after the overdose substantially protects the liver in mouse models (Barbier-Torres et al., 2017). In addition, high MCJ expression in the liver of patients experiencing drug induced liver injury is predictive of poor outcomes (Barbier-Torres et al., 2017), therefore inhibition of MCJ function may provide a strong benefit to these patients.

Substantial benefits in these systems that occur in the absence of MCJ lead to an unanswered question: what advantageous trait does MCJ provide when its loss is apparently positive? For CD8+ T cells, it is possible that while a deficiency in MCJ is favorable for viral immunity, susceptibility to autoimmune disease is enhanced. CD8+ T cells are implicated in the pathology of several autoimmune diseases due – at least in part – to aberrant cytotoxic activity (Blanco et al., 2005; Gravano and Hoyer, 2013). Since MCJ deficiency enhances the cytotoxic capacity of CD8+ T cells (CHAPTER 2), the risk of developing autoimmunity may be increased in the absence of MCJ. While CD4+ T cells are also implicated in autoimmunity, only a limited number of these cells have been shown to exhibit cytotoxic function (Takeuchi and Saito, 2017). Therefore within the immune system, the beneficial function of MCJ may be to limit cytotoxicity by CD8+ T cells to prevent excessive normal tissue damage and autoimmunity.

While loss of MCJ in the liver is not detrimental under controlled laboratory conditions (Hatle et al., 2013), survival during prolonged periods of metabolic stress may require MCJ function. A notable role of the liver is in the regulation of lipid metabolism (Berg et al., 2002; Voet and Voet, 2011). While mouse models of
fasting (<2 days) indicate that loss of MCJ is beneficial as hepatocytes have an increased capacity to metabolize lipids, the effects of starvation conditions are unknown (Hatle et al., 2013). There may be a decreased length of time that MCJ deficient mice can survive without food, thereby leading to an earlier death due to starvation. Therefore from an evolutionary standpoint, the presence of MCJ in the liver is an advantageous trait when resources are limited. In line with this, NAFLD may be a modern negative consequence of this ancestral need in times of food scarcity as MCJ tips the balance toward lipid storage rather than breakdown.

Similar to starvation conditions, MCJ loss may be detrimental during cold stress. Metabolic reactions produce significant amounts of heat, which maintains body temperature in endotherms (Berg et al., 2002; Voet and Voet, 2011). It was recently shown that mitochondria generate a large amount of heat relative to other cellular components (Chretien et al., 2018), therefore regulation of mitochondrial respiration by MCJ may play a role in thermal regulation. Thus, a deficiency in MCJ may lead to dysregulated body temperatures in times of stress.

MCJ is highly expressed in the heart, however its role in heart function and cardiovascular health has not been addressed. It is known that production of ROS by mitochondria promotes tissue damage during ischemia-reperfusion (IR) injury, which occurs during a myocardial infarction (MI; also known as a heart attack). The current standard of care for MI patients is to immediately remove the clot, which rapidly restores blood flow and increases oxygen concentrations in the ischemic tissue. This results in a burst of mitochondrial ROS production and
causes IR injury. Complex I produces a significant amount of ROS (Chouchani et al., 2014; Scialo et al., 2017), but whether MCJ exacerbates or ameliorates IR injury is unknown. However, considering that MCJ deficiency has been correlated with decreased ROS production in CD8+ T cells (Champagne et al., 2016), it is possible that loss of MCJ in cardiac cells may be protective of IR injury.

While many studies have shown benefits due to the loss of MCJ, deficiency can be detrimental in the context of cancer. As shown previously (CHAPTER 3), loss of MCJ in cancer cells supports chemoresistance through the promotion of drug efflux by ABC transporters. Restoration of MCJ function using peptide mimetics substantially reverses this mechanism of resistance, therefore activating the function of MCJ can provide benefits for the treatment of chemoresistant cancer.

The role of MCJ in metastasis is currently unknown. Mitochondria can promote tumor progression and are likely involved in metastasis (Wallace, 2012; Zong et al., 2016). In line with this, mitochondria can traffic to the leading edge of the cell and provide the ATP required to facilitate migration (Cunniff et al., 2016). While a potential role for MCJ in this process is unknown, it is feasible that loss of MCJ (or other negative regulators of mitochondrial metabolism) improves migratory capacity. Thus, promoting MCJ function during cancer treatment may also have the added benefit of limiting the metastatic potential of tumor cells.

While there is likely a strong benefit in cancer treatment due to limiting ABC transporter activity, there may also be negative consequences. Active transport of substrates generally requires ATP, therefore restricting mitochondria respiration
may have detrimental off-target effects. This is especially true for patients with disorders associated with ABC transporters and similar proteins as disease symptoms may be worsened (e.g., cystic fibrosis, adrenoleukodystrophy, Stargardt disease). Additionally, restriction of TAP (transporter associated with antigen processing), the ABC transporter required to deliver peptide antigen to MHC class I molecules in the ER, may have negative consequences for antigen presentation to T cells and associated immune responses. Therefore, a balance will need to be maintained between inhibiting ABC transporters that mediate chemoresistance and those required for critical cell functions.

Overall this dissertation describes how the regulation of mitochondrial metabolism by MCJ has profound effects on cellular metabolism and how this can be exploited for therapeutic purposes. Future studies will likely elucidate new avenues of manipulating MCJ function for the treatment of human disease.

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