The Contribution Of Metabolism To The Regulation Of Caspase Activity And Cell Death In T Lymphocytes

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THE CONTRIBUTION OF METABOLISM TO THE REGULATION OF CASPASE ACTIVITY AND CELL DEATH IN T LYMPHOCYTES

A Dissertation Presented

by

Michael Anthony Secinaro

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular, and Biomedical Sciences

January, 2019

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Dissertation Examination Committee

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ABSTRACT

During an immune response, T cell activation is mirrored by a dramatic metabolic shift from oxidative phosphorylation to glycolysis. The upregulation of glycolysis allows the cell to generate the molecules needed to rapidly proliferate and to synthesize effector molecules. The resolution of the T cell response is characterized by equally fast death of most effector T cells. The remaining T cells shift back to oxidative phosphorylation, allowing the cell to survive as a memory T cell. The upregulation of glycolysis and proliferation during the effector phase is paralleled by an increased sensitivity to T cell receptor restimulation-induced cell death (RICD). Whereas cellular metabolism and cell death are important in the proper function and response of T cells, it is not clear how metabolism regulates susceptibility to cell death, nor whether T cell proliferation and contraction are directly connected. The work presented in this dissertation provides a mechanistic link between T cell proliferation and contraction by demonstrating the regulation of caspase-3 activity by the metabolic state of T cells.

In effector T cells, the cytokine interleukin (IL)-2 mediates the upregulation of glycolysis, while IL-15 induces oxidative phosphorylation and a memory-like state. IL-2 is known to sensitize T cells to RICD, while IL-15 reduces RICD and increases survival. This results from the ability of IL-2 and glycolysis to increase caspase-3 activity, whereas IL-15 induces the opposite phenotype. Activation of caspase-3 during glycolysis is mediated through clustering in lipid rafts in the plasma membrane. IL-15 is shown to inactivate caspase-3 through the posttranslational modification of protein glutathionylation, which is mediated by ROS generation in the mitochondria as a by-product of oxidative phosphorylation.

We further observe that glycolysis parallels the reduced activity of the electron transport chain and oxidative phosphorylation, further increasing caspase-3 activity. This is mediated by the decreased expression of electron transport chain complexes and an increase in expression of the negative regulator of complex I, methylation-controlled J protein (MCJ). IL-15 promotes reduced expression of MCJ by its methylation. Similar to IL-15-cultured T cells, MCJ-deficient T cells manifest reduced glycolysis, caspase-3 activity, and RICD. Collectively, these findings demonstrate an adaptation that links metabolism to both cell proliferation and cell death to safeguard that proliferating cells do not escape regulation that could result in autoimmune disease or lymphomas.
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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW
1.1 The Immune System

The immune system is a network of proteins, cells, and tissues that protects the host from infections by bacteria, fungi, viruses, and parasites. Defense is achieved through the ability to recognize non-self from self. To do this, the immune system utilizes many different cells that are specialized in recognizing and responding to self and non-self molecules. To maintain both speed and breadth of response to infections, the immune system contains two different branches that evolved at different times, the innate immune system that is found even in insects, and the adaptive immune system found only in vertebrates (Yatim and Lakkis, 2015).

1.1.1 Innate immune system

The innate immune system acts to generate a quick protection from pathogens. It takes the strategy of having a limited number of non-polymorphic receptors that recognize a limited number of motifs from microorganisms. The co-evolution of these receptors and their ligands provides high affinity and hence a rapid response (Martin, 2014).

The complement system represents a group of proteins that cleave in a very precise order to induce inflammation and assist in the attack of microbial pathogens (Tomar and De, 2014). The proteins of the complement system work in three different pathways: the classical pathway, the lectin pathway, and the alternative pathway. These pathways all converge at the activation of the C3 convertase but are initiated by different means (Walport, 2001). The classical pathway is typically activated by antibodies bound to the surface of the pathogen. This leads to the binding of the C1 complex, which activates the C3 convertase (Walport, 2001). The lectin pathway is activated by either the
collectins mannose-binding lectin (MBL) or CL-K1, or the ficollins, which bind to carbohydrates on the surface of the pathogen and activate the MBL-associate serine proteases (MASPs). The MASPs then activate the C3 convertase (Kjaer et al., 2013). The alternative pathway is spontaneously activated in the serum through constant hydrolysis of the complement protein C3 (Roumenina et al., 2011). Upon binding to self surfaces, the pathway is inactivated by the prevention of C3 convertase formation. When hydrolyzed C3 binds to a pathogen, more complement proteins are deposited on the surface of the pathogen, and the C3 convertase becomes activated (Roumenina et al., 2011). Once the C3 convertase is activated, complement proteins are deposited onto the pathogen that result in opsonization and phagocytosis, or the formation of a membrane-attack complex which forms a pore and kills the pathogen (Walport, 2001).

The cells of the innate immune system are varied and act to attack pathogens as well as to activate the adaptive immune system. Innate immune cells recognize components of pathogens called pathogen-associated molecular patterns (PAMPs) via intracellular and surface receptors known as pattern recognition receptors (PRRs) (Martin, 2014). Upon recognition, signaling through PRRs lead to the activation of the innate immune cells and the production of inflammatory cytokines (Martin, 2014). PAMPs can be a number of different antigens from pathogens, ranging from DNA to fragments of bacterial flagella. PRRs include Toll-like receptors, NLRP proteins of the inflammasome, the RNA-sensing RIG-I pathway, and DNA-sensing cGAS/STING pathway (Cai et al., 2014; Janeway and Medzhitov, 2002; Kell and Gale, 2015; Martin, 2014). The cells that express PRRs include granulocytes (basophils, eosinophils, and neutrophils), natural killer (NK) cells, dendritic cells (DCs), macrophages, and mast cells.
Macrophages and DCs are able to phagocytose pathogens, degrade their proteins, and present peptide fragments of them on MHC molecules to T cells in the adaptive immune system (Janeway and Medzhitov, 2002; Yatim and Lakkis, 2015).

1.1.2 Adaptive Immune System

The adaptive immune system generates a wide array of T cell receptors (TCR) and B cell receptors (immunoglobulin) through random gene rearrangements (Tomar and De, 2014; Yatim and Lakkis, 2015). As a result, receptors of the adaptive immune system are highly polymorphic, manifest low affinity for their ligands, but provide great breadth of repertoire (Tomar and De, 2014). Consequently, the adaptive immune response is slower, but provide breadth and memory. B cells express a B cell receptor immunoglobulin that recognizes the three-dimensional structure of antigens. As B cells develop, the immunoglobulin gene undergoes a series of recombination events of its Variable (V), Diversity (D), and Joining (J) regions (termed V(D)J recombination) that allows for each B cell receptor to recognize a unique antigen (Roth, 2014). V(D)J recombination allows for a limited number of V, D, and J regions of DNA to be recombined into a large number of different immunoglobulins (Roth, 2014; Tomar and De, 2014; Yatim and Lakkis, 2015). This process of immunoglobulin gene recombination allows for B cells to respond to a much broader array of antigens compared to PRRs of the innate immune system. Once the B cell receptor interacts with its cognate antigen, the B cell becomes activated and begins proliferating and producing antibodies that recognize their antigenic epitope. During proliferation, B cell antibodies will undergo somatic hypermutation, a process through which the variable region of the antibody gene
is randomly mutated resulting a higher affinity antibody (Di Noia and Neuberger, 2007; Tomar and De, 2014).

T cells express a T cell receptor (TCR) that also undergoes V(D)J recombination, though not somatic hypermutation, allowing T cells to recognize a similarly large array of antigens (Roth, 2014). The difference is that the TCR recognizes antigen complexed with self-major histocompatibility complex (MHC), or self-MHC-like, molecules on APCs (Malissen et al., 2014). Activated T cells perform a variety of functions, depending on the subset of T cell that is activated. The major T cell subsets are discussed further in the next section.

Following expansion in response to antigenic activation during an immune response, most T cells and B cells die, but a few survive to become memory cells. (Quintin et al., 2014; Sun et al., 2014). Immune memory allows for the immune system to respond quickly and with more intensity to a secondary infection.

1.2 T cells

T cells, which express a T cell receptor (TCR), include αβ T cells, γδ T cells, natural killer T (NKT) cells, and mucosa-associated invariant T (MAIT) cells. Conventional αβ T cells are restricted to recognizing peptide antigens presented in complex with MHC I or MHC II. NKT cells, MAIT cells, and γδ T cells, vary in the types of antigens recognized by their limited repertoire of TCRs and are considered to function between the innate and adaptive immune system (Sun et al., 2014). NKT cells, which express an invariant TCR (Vα14-Jα18 α-chain in mice and Vα24-Jα18 α-chain in humans; known as Type I NKT cells) or a non-invariant TCR (known as Type II NKT cells) are restricted to interacting with lipid antigens presented on the MHC I-like
molecule CD1d (Kumar et al., 2017; Macho-Fernandez and Brigl, 2015; Trottein and Paget, 2018). MAIT cells express an invariant TCR (Vα19-Jα33 α-chain in mice and Vα7.2-Jα33 α-chain in humans) and are restricted to non-peptide small-molecule antigens presented on the MHC class I-like molecule MR1 (Trottein and Paget, 2018; Xiao and Cai, 2017). Putative antigens that γδ T cells have been found to recognize include antigens associated with MHC I-like molecules, as well as antigens not associated with MHC molecules. These putative antigens vary from peptides to lipids to proteins (Born et al., 2013; Champagne, 2011).

αβ T cells, henceforth called T cells, can be split into two distinct subsets based on the expression of the coreceptors CD4 or CD8. The TCR recognizes peptide antigen bound to the MHC in mice, or human leukocyte antigen (HLA) in humans on the surface of APCs (Wieczorek et al., 2017). There are two classes of MHC, class I and class II (Rock et al., 2016). MHC class I (HLA-A, -B, and -C in humans) binds small intrinsic peptides (about 8-10 amino acids in length) is expressed ubiquitously among cell types (Rock et al., 2016; Wieczorek et al., 2017). MHC class II (HLA-DR, -DP, -DQ in humans) binds to peptides (about 13-25 amino acids in length) from exogenous antigens that have been phagocytosed and processed within an APC (Rock et al., 2016; Wieczorek et al., 2017). The coreceptor (CD4 or CD8) expressed on the surface of the T cell determines which MHC the T cell will recognize; CD4 recognizes determinants on MHC class II and CD8 recognizes determinant on MHC class I (Rock et al., 2016).

1.2.1 T cell development

T cells begin as hematopoietic stem cells generated in the bone marrow. These stem cells enter the thymus as thymic seeding progenitor cells. In the thymus, the
progenitor cells will undergo successive developmental steps during the CD4\(^{-}\)CD8\(^{-}\) double-negative (DN) stage, which can be divided into four phases based on the expression of CD25 and CD44: DN1, CD25\(^{-}\)CD44\(^{+}\); DN2, CD25\(^{+}\)CD44\(^{+}\); DN3, CD25\(^{-}\)CD44\(^{-}\); DN4, CD25\(^{-}\)CD44\(^{-}\) (Lucas et al., 2016). During the transition from DN2 to DN3, T cell receptor rearrangement begins and developing thymocytes commit to either \(\gamma\delta\) T cells or \(\alpha\beta\) T cells. Between DN2 and DN3, the \(\gamma\), \(\delta\), and \(\beta\) chain genes undergo rearrangement (Carpenter and Bosselut, 2010). If the \(\gamma\) and \(\delta\) chains of the TCR successfully rearrange before the \(\beta\) chain, the cell becomes a \(\gamma\delta\) T cell (Carpenter and Bosselut, 2010). If the \(\beta\) chain successfully rearranges first, then the cell becomes an \(\alpha\beta\) T cell and enters the DN3 stage (Rothenberg, 2011). A pre-\(\alpha\) chain pairs with the \(\beta\) chain to create a surface pre-TCR, and the cell enters the DN4 stage (Rothenberg, 2011). In DN4, CD4 and CD8 are expressed to create a CD4\(^{+}\)CD8\(^{+}\) double-positive (DP) cell. During this DP stage, the \(\alpha\)-chain of the TCR rearranges and is expressed, creating a mature TCR. The TCR on DP cells then interacts with self-peptide:MHC complexes (Carpenter and Bosselut, 2010). DP cells that recognize self-peptide:MHC with low affinity are positively selected, whereas those manifesting high affinity undergo negative selection and cell death (Carpenter and Bosselut, 2010). During this time, either the CD4 or CD8 coreceptor will be downregulated, creating a CD4 or CD8 single-positive (SP) T cell. Mature CD4\(^{+}\) or CD8\(^{+}\) T cells will then leave the thymus and enter the periphery (Koch and Radtke, 2011; Shah and Zuniga-Pflucker, 2014; Zuniga-Pflucker, 2004).

**1.2.2 CD4\(^{+}\) T cells**

CD4\(^{+}\) T cells are known as helper T (Th) cells because they secrete cytokines that promote the effector function of other immune cells. CD4\(^{+}\) T cells can be divided into
different subsets depending on the transcription factor that the cell expresses. Each subset secretes a different combination of effector cytokines. Subsets are identified by those secreted cytokines and their specific transcription factor (Geginat et al., 2013). Th1 cells express the transcription factor T-bet, which induces the expression of interferon-γ (INF-γ), the signature cytokine produced by Th1 cells. INF-γ promotes the production of immunoglobulin IgG2a, induces antiviral responses, and activates macrophages to help protect against microbial pathogens and tumors through upregulation of phagocytosis and antigen presentation (Finkelman et al., 1988; Männel and Falk, 1983; Schoenborn and Wilson, 2007; Szabo et al., 2000; U. Boehm et al., 1997). Th1 cells also produce IL-2, which promotes T cell activation and effector function (Geginat et al., 2013; Liao et al., 2013).

Th2 cells express the transcription factor GATA-3, which induces the expression of IL-4 and IL-5 (Zheng and Flavell, 1997). IL-4 induces antibody class switching in B cells to IgE, while IL-5 induces the proliferation of B cells and eosinophils (Caza and Landas, 2015; Takatsu, 2011).

Th17 cells express the transcription factors RORγT and RORα (Yang et al., 2008). These transcription factors upregulate the expression of IL-17 and IL-17F, which induce the expression of antimicrobial peptides and inflammatory cytokines and chemokines (Kolls and Lindén, 2004; Liang et al., 2006). Th17 cells have been implicated in the pathogenesis of autoimmune diseases such as the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (Komiyama et al., 2006).

Regulatory T (Treg) cells are CD4+ T cells that express the transcription factor FoxP3 and suppress the immune response in part through production of the
immunosuppressive cytokine IL-10. As such, Tregs help to reduce the effector function of immune cells and maintain tolerance for the prevention of autoimmune disease (Caza and Landas, 2015; Geginat et al., 2013).

Since CD4\(^+\) T cells depend on cytokines to promote and maintain subset differentiation, it is also possible for CD4\(^+\) T cells to switch between subsets. This ability to switch subsets is called plasticity, and some subsets are more sensitive to it than others. Th2 cells can easily become Th1 cells if IL-4 is removed from the culture media, though this switch may not be as easy the further these cells are from their initial activation (Murphy et al., 1996). Tregs and Th17 cells show a similar degree of plasticity (Caza and Landas, 2015; Xu et al., 2007).

1.2.3 CD8\(^+\) T cells

CD8\(^+\) T cells are an important defense against intracellular pathogens such as viruses and intracellular bacteria, as well as tumors. Like CD4\(^+\) T cells, there is evidence for the existence of CD8\(^+\) T cell subsets that produce a variety of cytokines when activated, and therefore perform many different functions, though these subsets are not as well understood as the CD4\(^+\) subsets (Mittrucker et al., 2014). The major CD8\(^+\) T cell subset, and the one that is best understood, is called a cytotoxic T lymphocyte (CTL). Once activated, CTLs will secrete proinflammatory cytokines INF-\(\gamma\) and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), which increase both the innate and adaptive immune response. Activated CTLs will also induce death in cells that express their cognate antigen on MHC class I. After recognizing the antigen, CTLs release granules containing granzymes and perforin. Granzymes are serine proteases that enter the target cell through pores created by perforin. Once in the cell, the granzymes will cleave proteins and induce apoptosis.
CTLs also kill by surface expression of Fas-ligand. CTLs are able to combat intracellular pathogens by inducing death in the infected cell, preventing the replication and spread of the pathogen (Mittrucker et al., 2014; Tscharke et al., 2015).

1.2.4 T cell activation

Naïve T cells need to be activated to perform their effector function. Activation occurs when the naïve T cell receives two signals; one from the TCR and another from the costimulatory receptor CD28. The TCR exists in a complex with other proteins, of which the CD3 molecules (CD3 γ, δ, ε, and ζ) are important for signaling (Borst et al., 1984; Samelson et al., 1986). When the TCR and CD4 or CD8 bind to the peptide:MHC complex, the lymphocyte-specific protein tyrosine kinase (Lck) bound to CD4 or CD8 will phosphorylate CD3. The protein ζ-associated protein of 70 kDa (Zap70) is recruited to the phosphorylated CD3ζ, which leads to the phosphorylation of linker for activation of T cells (LAT) (Chan et al., 1992; Zhang et al., 1998a). Once activated, LAT will transduce a number of signals that ultimately lead to the activation of transcription factors activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) (Smith-Garvin et al., 2009). As the TCR and coreceptors are engaged with the peptide:MHC complex, CD28 will bind to CD80 or CD86 on the APC, which results in a phosphorylation cascade leading to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). These transcription factors will upregulate the expression of genes needed for the cell to proliferate and perform its effector function (Malissen and Bongrand, 2015; Malissen et al., 2014). IL-2 is one of the genes expressed as a result of NFAT and AP-1 activation (Liao et al., 2013). IL-2 promotes T cell proliferation, effector function, and induces glycolysis (Friedmann et al., 1996; Liao et al., 2013; Lin et al.,
Not only is glycolysis induced late in T cell activation through IL-2 signaling, it is also induced within hours after TCR stimulation as a result of pyruvate dehydrogenase kinase 1 activation and reduction of pyruvate import into mitochondria (Menk et al., 2018).

1.2.4.1 Role of lipid rafts in T cell signaling and activation

Lipid rafts, also called membrane rafts, are cholesterol-rich microdomains found in cellular membranes. These rafts are typically considered to be small regions of membrane, ranging from 10 nm to 200 nm, which includes caveolae and caveolin-rich regions of the membrane (Anderson and Jacobson, 2002; Pike, 2006). Though the composition of rafts is heterogenous and difficult to define, they are typically enriched in sphingolipids and sterols. Being enriched with these components allows rafts to be conducive for protein-protein interactions, as well as protein-lipid interactions (Pike, 2006). This allows the clustering of proteins, which can stabilize multi-protein interactions and promote the compartmentalization of cellular processes. Stabilization of the rafts can also occur through interactions between raft-associated proteins and the cytoskeleton (Pike, 2006). While rafts are typically small, they can be recruited and stabilized into larger membrane domains (Anderson and Jacobson, 2002; Pike, 2006).

Among the signaling proteins that have been described as localizing to rafts in T cells are Lck, LAT, TCR, CD28, and CD3 complex (Varshney et al., 2016; Zumerle et al., 2017). Relevant to this dissertation is caspase-8 complexed to its activator, c-FLIP, which has been described in lipid rafts in activated T cells (Misra et al., 2007).

A specific lipid raft in T cells is the immunological synapse. The immune synapse is formed where the TCR interacts with the MHC-peptide complex on an antigen-
presenting cell, inducing activation of the T cell (Huppa and Davis, 2003). Many different proteins are involved in this interaction and nearly all the T cell-relevant proteins are found within lipid rafts at the immune synapse. These include the TCR, CD3 complex, Lck, LAT, and CD28 (Varshney et al., 2016; Zumerle et al., 2017). While some of these proteins (e.g. TCR and CD28) have transmembrane domains, others, such as Lck and LAT, are recruited to the cell membrane and lipid rafts by posttranslational addition of fatty acids; LAT can be palmitoylated, whereas Lck can be both acylated and myristoylated (Zhang et al., 1998b). While membrane rafts are important for T cell activation, their function in other aspects of T cell biology is poorly understood.

1.3 Cytokines

Cytokines are small secreted immunomodulatory proteins that act in a paracrine or autocrine manner similar to hormones. Cytokines can be produced by many different cell types including immune cells, fibroblasts, and endothelial cells.

Various groups of cytokines have been identified based on similarities in function or binding to common receptors. The common cytokine-receptor gamma-chain (γc) family is very important in regulating the proliferation and survival of T cells. These cytokine receptors are all heterodimeric or heterotrimeric and contain the γc (also known as CD132) as one of the subunits. The γc cytokines include interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Some of the γc cytokines are important in the differentiation and proliferation of effector T cells (IL-2, IL-4, IL-9, and IL-21), while others are important in the homeostasis and survival of memory and naïve T cells (IL-7 and IL-15) (Rochman et al., 2009). Two of these cytokines, IL-2 and IL-15, are unique because they are the γc cytokines with trimeric receptors. Those receptors contain the γc, the IL-2 receptor β (IL-
2Rβ or CD122), and the IL-2 receptor α (IL-2Rα or CD25) or IL-15Rα, for respectively, IL-2 or IL-15 (Waldmann, 2002). Though they share a similar receptor, IL-2 and IL-15 differ in their contributions to T cell survival.

1.3.1 IL-2 signaling

IL-2 was originally identified as a growth factor for T cells, which remains one of the best understood roles of IL-2 (Morgan et al., 1976). Both CD4+ and CD8+ T cells produce IL-2, though CD4+ T cells produce IL-2 to a greater extent (Paliard et al., 1988). IL-2 binds to the trimeric IL-2R, whose expression increases following T cell activation (Cantrell and Smith, 1983). Binding to the IL-2R induces a signaling cascade through the JAK/STAT pathway (primarily through JAK1 and JAK3, and subsequently STAT5 and STAT3), the PI3K pathway, and the ERK pathway (Fig. 1.1) (Johnston et al., 1995; Ring et al., 2012; Waldmann, 2015). PI3K signaling leads to mTOR activation, and the upregulation of glycolysis (Liao et al., 2013; Ray et al., 2015; Spolski et al., 2018; van der Windt et al., 2012). Through the upregulation of glycolysis, IL-2 increases the synthetic capacity of the cell to allow rapid proliferation during an immune response (Ray et al., 2015). After induction of glycolysis, IL-2 is important for maintaining proliferation and function of effector CD8+ T cells, as well as many of the CD4+ T cell subsets.

In addition to its role in T cell proliferation, IL-2 has a critical role in cell death. In effector T cells, IL-2 increases sensitivity to apoptosis by activation-induced cell death and Fas (CD95)-mediated death (Lenardo, 1991; Refaeli et al., 1998). In this regard, a striking and unanticipated finding was that mice deficient in CD25 accumulate T cells and develop autoimmune symptoms, further supporting the fact that IL-2 is important in
T cell death (Willerford et al., 1995). While it is clear that IL-2 has a role in both proliferation and cell death, the mechanism by which IL-2 increases sensitivity to death is not well understood.

1.3.2 IL-15 signaling

Similar to IL-2, IL-15 binds to a trimeric receptor composed of both the γc and CD122 subunits, as well as IL-15Rα. IL-15 can bind to the IL-15R through cis-presentation (when the IL-15Rα is on the cell receiving the signal) or through trans-presentation (when IL-15 is bound to the IL-15Rα and presented by a neighboring cell) and can be produced by monocytes, dendritic cells, and fibroblasts (McInnes and Liew, Figure 1.1: Signaling through the IL-2 and IL-15 receptors

![Figure 1.1: Signaling through the IL-2 and IL-15 receptors](image)
IL-15 binding initiates a signaling cascade similar to IL-2, activating JAK1 and JAK3, followed primarily by STAT5 (Ring et al., 2012; Rochman et al., 2009). Signaling through the IL-15 receptor also leads to PI3K and ERK signaling (Fig. 1.1) (Ring et al., 2012; Waldmann, 2015). Originally described as a proliferative cytokine for T cells, like IL-2, it was later shown that IL-15 also promotes survival in T cells by reducing restimulation-induced cell death, which is promoted by IL-2 (Burton et al., 1994; Grabstein et al., 1994; Marks-Konczalik et al., 2000). The increased survival promoted by IL-15 is related to an important function of the cytokine, homeostatic proliferation and maintenance of memory CD8+ T cells (Tan et al., 2002). Due to its ability to maintain T cell populations for extended periods of time, as well as activate NK cells, IL-15 can induce tissue damage when it is over expressed. This is apparent in the synovial tissue of patients with rheumatoid arthritis, where the synovium contains high levels of IL-15 and low levels of IL-2, which can lead to prolonged T cell expansion and survival, contributing to the tissue damage in rheumatoid joints (McInnes and Liew, 1998b).

Due to the similarities in signaling with IL-2, there has been much interest in trying to understand the cause for the differences in phenotypes induced by IL-2 and IL-15 signaling. It is thought that differences in receptor binding strength, tissue context, and activation state (in the context of T cells) all play a role in determining these differences (Castro et al., 2011; Ring et al., 2012; Waldmann, 2015). One difference of interest is in the susceptibility to cell death induced by IL-2 versus IL-15. Previous studies from our group have shown the decreased death in IL-15-cultured T cells to be due to inactivation of caspase-3 by the posttranslational modification S-nitrosylation of the critical cysteine
in the enzymatic pocket of caspase-3. (Saligrama et al., 2014). Additional studies have revealed a parallel between the increased T cell survival with IL-15 and its promotion of oxidative phosphorylation and fatty acid utilization (O'Sullivan et al., 2014; van der Windt et al., 2012). However, it is not known how, or if, the metabolic differences between IL-2-cultured and IL-15-cultured T cells directly influence sensitivity to cell death.

1.4 Immunometabolism

Metabolism is the combination of anabolic and catabolic processes within the cell. The anabolic processes are those used to generate sugars, fats, amino acids, nucleotides, among other molecules that are necessary for maintenance of cell integrity and proliferation. Those necessary substrates that cannot be made through anabolic processes are transported into the cell from the environment. The catabolic processes are those that break down large molecules into smaller components, either to feed the anabolic processes or to generate energy.

In most cells, glycolysis and oxidative phosphorylation are the major pathways used to generate ATP. Glucose is catabolized to pyruvate and then acetyl-CoA to fuel the tricarboxylic acid (TCA) cycle in the mitochondria. The energy-carrying products of glycolysis and the TCA cycle are NADH and FADH$_2$, whose electrons fuel the electron transport chain (ETC). The ETC pumps protons across the inner membrane of the mitochondria to produce an energy potential. This energy potential is used by ATP synthase, or complex V of the ETC, to create ATP. Fatty acid oxidation also contributes to ATP generation through catabolism of fatty acids such as palmitate, which results in the production of NADH, FADH$_2$, and acetyl-CoA to fuel the ETC.
In addition to its metabolism in generating ATP, glucose utilization can also be diverted into the pentose phosphate pathway to create intermediates needed for lipid, protein, and nucleotide synthesis. Glucose can also be stored to make glycogen in muscle cells, astrocytes, hepatocytes, and dendritic cells, which is then used to fuel the TCA cycle after being catabolized back to glucose (Roach et al., 2012; Thwe et al., 2017). When glucose is not fueling the TCA cycle, immune cells tend to use glutamine and fatty acids to maintain OXPHOS.

Metabolism plays an important role in the function immune cells, and the metabolites used can dictate which function the cells have. Many techniques have been developed to determine the metabolic pathways used by cells. One of the most powerful tools is the use of heavy isotope-labeled metabolites in cell culture media, combined with mass spectrometry. Heavy isotope-labeled metabolites contain stable, heavy isotopes of carbon, nitrogen, and/or hydrogen that can be differentiated from their normal counterparts by mass spectrometry. When these labeled metabolites are added to the culture medium, the cells will incorporate those heavy atoms as they normally would, but now the atoms can be tracked. This allows for the identification of the metabolic pathways to which a cell will divert a given metabolite (Jang et al., 2018). Techniques such as this help to identify how cells are able to use different metabolites under different growth conditions. The following sections describe the major metabolic pathways of immunometabolism, and how those pathways dictate immune cell function, with an emphasis on T cells.
1.4.1 Glycolysis

Glycolysis is a catabolic process that converts one 6-carbon glucose molecule into two 3-carbon pyruvate molecules using 2 ATP molecules (Fig. 1.2). The process begins with the transport of glucose into the cell through one of fourteen glucose transporters. After entering the cell, glucose is rapidly phosphorylated by hexokinase, resulting in glucose-6-phosphate (G6P). This phosphorylation event uses the first ATP molecule. G6P is converted to fructose-6-phosphate by phosphogluco isomerase. The second ATP is used by phosphofructokinase to convert F6P to fructose-1,6-bisphosphate (F1,6P). At this point, the six-carbon sugar F1,6P is converted into two three-carbon sugars by aldolase. This reaction creates one dihydroxyacetone phosphate (DHAP) and one glyceraldehyde-3-phosphate (GAP). DHAP is rapidly converted to GAP by triose phosphate isomerase. The three-carbon sugar GAP is further converted by glyceraldehyde-3-phosphate dehydrogenase into 1,3-bisphosphoglycerate (1,3-BPG), which is then converted to 3-phosphoglycerate (3-PG) by phosphoglycerate kinase, generating a molecule of ATP in the process. 3-PG is converted into 2-phosphoglycerate (2-PG) by phosphoglycerate mutase. Enolase converts 2-PG into phosphoenolpyruvate (PEP), which is finally used by pyruvate kinase to make pyruvate. Thus, one molecule of glucose results ultimately in two pyruvate molecules.
1.4.1.1 Importance of glycolysis in immune cells

Glycolysis plays an important role in the activation and effector function of many immune cells. Dendritic cells undergo a glycolytic burst after activation by TLR antigens (Everts et al., 2014). T cells, NK cells, B cells, and macrophages all undergo a similar switch to glycolysis after activation (Caro-Maldonado et al., 2014; Hume et al., 1978; Rodriguez-Prados et al., 2010; Roos and Loos, 1973a). For T cells, glycolysis is maintained by effector CD8+ T cells, as well as Th1, Th2, and Th17 CD4+ T cells (Michalek et al., 2011; van der Windt et al., 2012). Whereas glycolysis does generate some ATP (a net yield of 2 ATP per glucose molecule), the reason for the increase in glucose catabolism with immune cell activation is thought to be primarily for the
generation of metabolic intermediates needed to fuel the anabolic processes needed for rapid cell growth and effector function.

Many of the glycolytic intermediates are used in other metabolic pathways. G6P can be used in both glycogen synthesis and to fuel the pentose phosphate pathway. The primary non-glycolytic function for G6P is in the synthesis of nucleotide precursors and the reducing agent NADPH through the pentose phosphate pathway. G6P can also be converted to glycogen, a branched polymer of glucose. Glucose storage as glycogen is most well known in muscle and liver cells, though recent studies have shown glycogen metabolism to play an important role in dendritic cell and T cell biology (Ma et al., 2017; Thwe et al., 2017). Dendritic cells have been shown to generate and store glycogen, which is used to support effector functions such as cytokine production and antigen processing and presentation. Memory T cells have also been found to store glucose as glycogen, which is catabolized to G6P to fuel the pentose phosphate pathway.

Amino acid metabolism is another major use of glucose, fed by 3-PG (serine biosynthesis), PEP (tryptophan biosynthesis), and pyruvate (alanine biosynthesis). Pyruvate, along with contributing to alanine metabolism, is converted into two important metabolites, lactate and acetyl-CoA. Pyruvate is converted into lactate by lactate dehydrogenase, which uses NADH as a cofactor. This replenishes NAD⁺, which can then be used as an electron acceptor in glycolysis and the TCA cycle. Generally, lactate is considered a byproduct of highly glycolytic cells. Recently, however, lactate has been shown to modulate CD8⁺ T cells function, causing the loss of cytolytic activity and migratory function (Haas et al., 2015). Macrophages are polarized toward an M2 phenotype in high lactate environments (Pucino et al., 2017). Pyruvate is converted to
acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA is the base substrate for fatty acid synthesis and can be used to generate lipids and fatty acids for the cell. Acetyl-CoA is also one of the primary carbon donors for the TCA cycle.

1.4.2 Tricarboxylic acid cycle

Also known as the Krebs cycle or the citric acid cycle, the tricarboxylic acid (TCA) cycle is the primary method used by cells to shuttle electron donors, NADH and FADH$_2$, into the electron transport chain at, respectively, complexes I and II. This results in a net yield of 32 ATP, making the TCA cycle plus OXPHOS the major source of ATP in the cell.

The TCA cycle occurs in the matrix of the mitochondria (Fig. 1.3). Pyruvate is transported into mitochondria and converted into acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA can then enter the TCA cycle by reacting with oxaloacetate in a process catalyzed by citrate synthase. Acetyl-CoA is attached to oxaloacetate before the coenzyme A group is removed. The final product is citrate, which is then isomerized to isocitrate by aconitase. Isocitrate dehydrogenase decarboxylates isocitrate to create $\alpha$-ketoglutarate and a molecule of NADH. Another NADH molecule is generated as $\alpha$-ketoglutarate is converted to succinyl-CoA by $\alpha$-ketoglutarate dehydrogenase. Succinyl-CoA is converted to succinate by succinyl-CoA synthetase, creating succinate. Succinate is converted to fumarate by succinate dehydrogenase, an enzyme that is part of complex II of the electron transport chain. This reaction generates one molecule of FADH$_2$, which is used by complex II to transfer electrons to the electron transport chain. Fumarase creates catalyzes the reaction of fumarate to malate, which is followed by the conversion of malate to oxaloacetate by malate dehydrogenase. This final step of the TCA cycle
generates a final molecule of NADH, and completes the cycle, allowing oxaloacetate to be regenerated to react with acetyl-CoA once more. Since two acetyl-CoA molecules are generated from one glucose molecule, the full catabolism of glucose results in 6 NADH molecules and 2 FADH$_2$ molecules to be generated from the TCA cycle.

Figure 1.3: Steps of the tricarboxylic acid cycle

1.4.2.1 Importance of the TCA cycle in immune cells

Carbons can enter and exit the TCA cycle at nearly every intermediate. In fact, immune cells that undergo a high amount of glucose utilization for proliferation need to fuel the TCA cycle with other metabolites, so the glycolytic intermediates can be used for biosynthetic purposes. Citrate is at the first major junction of the TCA cycle. After being generated by citrate synthase, citrate can exit the mitochondria and be converted back to acetyl-CoA, which can then be used for fatty acid synthesis to generate lipids. While it
may seem wasteful for the cell to generate citrate from acetyl-CoA only to turn that citrate back into acetyl-CoA, cells that catabolize a lot of glucose can send those carbons to feed the TCA cycle and generate the lipids needed to support growth and proliferation. Fatty acids generated through this pathway can also be used to support the TCA cycle. Fatty acid synthesis supported by glycolysis is important in the differentiation of CD4+ T cells into either Th17 cells or Treg cells. Th17 cells are shifted toward a Treg phenotype when acetyl-CoA carboxylase 1 (ACC1), an enzyme involved in fatty acid synthesis, is inhibited. Tregs rely on the oxidation of externally derived fatty acids, further indicating the importance of fatty acid synthesis in Th17 development and function (Berod et al., 2014a).

Citrate and succinate also play important roles in inflammatory macrophages. When citrate accumulates in bone marrow-derived macrophages (BMDM) activated by LPS, it can be converted to itaconate, which was found to cause an accumulation of succinate by inhibiting succinate dehydrogenase (Lampropoulou et al., 2016). Reducing the production of itaconate led to an increase in succinate dehydrogenase activity, a decrease in succinate levels, and an increase HIF-1α and the production of inflammatory cytokines such as IL-1β and IL-18. An earlier study of succinate metabolism in macrophages had shown that after LPS stimulation, BMDM use γ-aminobutyric acid (GABA) to make succinate (Tannahill et al., 2013). Reducing succinate generated from GABA led to a reduction in HIF-1α and IL-1β production. Although a reduction in succinate led to two opposing results, the flow of carbons into the TCA cycle was different between studies and this may be more important than the levels of succinate in determining macrophage effector function. Together, these studies demonstrate that
citrate, succinate, and carbon flow through the TCA cycle are important factors in macrophage and T cell function.

An additional notable metabolite is α-ketoglutarate, as it is the second major entry point into the TCA cycle for carbons in immune cells. Glutamine is known to be critically important for supporting immune cell function, and a large reason for that is because glutamine is converted to glutamate, which is then used to generate α-ketoglutarate as a fuel source for the TCA cycle.

### 1.4.3 Glutaminolysis

Glutamine is an important amino acid, as it can be used to generate many different molecules. Glutamine is transported into the cell by a variety of transporters (ASCT2 is an important glutamine transporter on T cells) and can then be used to generate purines, pyrimidines, amino acids, or glutamate (Nakaya et al., 2014; Pochini et al., 2014). Carbons from glutamate can be incorporated into the TCA cycle through α-ketoglutarate, or through succinate. The primary route of glutamine into the TCA cycle is through the conversion to glutamate by glutaminase. Glutamate dehydrogenase then converts glutamate to α-ketoglutarate, which then continues into the TCA cycle to be converted into glutamate.

The other path for glutamine to enter the TCA cycle is through conversion of glutamate into GABA, a neuroinhibitory neurotransmitter. Glutamate is converted into GABA by glutamate decarboxylase, and then converted into succinate semialdehyde. Finally, succinate semialdehyde is converted into succinate and can enter the TCA cycle or perform other functions. This non-canonical pathway for succinate synthesis in immune cells has been shown in BMDM. GABA was detected by mass spectrometry in
BMDM and $^{13}$C-labeled succinate was detected after culturing BMDM with $^{13}$C-labeled glutamine, indicating the use of this pathway in immune cells (Tannahill et al., 2013).

GABA has also been implicated in modulating T cell function, having been reported to reduce T cell proliferation and inhibit Th1 cell responses (Bhat et al., 2010; Bjurstom et al., 2008; Tian et al., 2004).

### 1.4.4 Fatty acid oxidation

Fatty acid oxidation, also known as β oxidation, is the process through which fatty acids are broken down into individual acetyl-CoA molecules that can enter the TCA cycle. Fatty acid oxidation begins in the cytosol with the addition of coenzyme A (CoA) to a fatty acid by acyl-CoA synthetase. This process creates an acyl-CoA and requires one ATP molecule to complete. Fatty acid oxidation occurs in the mitochondria, but acyl-CoA molecules cannot be transported into the mitochondria and must first be converted to acyl-carnitine. These acyl-carnitine molecules can be transported into the mitochondria. Carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2) are the enzymes responsible for translocating fatty acids into the mitochondria. CPT1 is located on the external side of the inner mitochondrial membrane and is responsible for converting the acyl-CoA into acyl-carnitine. The acyl-carnitine can then be shuttled across the inner mitochondrial membrane by a carnitine carrier protein. CPT2, located in the mitochondrial matrix, converts acyl-carnitine back into acyl-CoA. The free carnitine is then transported out of the matrix by the carnitine carrier protein.

Once in the mitochondrial matrix, the acyl-CoA undergoes fatty acid oxidation. This is a four-step process that results in the release of one acetyl-CoA molecule from the fatty acid chain. Fatty acid oxidation generates energy not only by adding acetyl-CoA to
the carbon pool for the TCA cycle, but also through the release of that acetyl-CoA. Two steps in the process, reactions catalyzed by acyl-CoA dehydrogenase and 3-L-hydroxyacyl-CoA dehydrogenase, generate FADH$_2$ and NADH, respectively. These cofactors can then donate electrons to the ETC. Fatty acid oxidation ends with the addition of a CoA molecule onto the acyl chain, regenerating acyl-CoA. The acyl-CoA, now two carbons shorter due to the release of acetyl-CoA, can undergo another round of fatty acid oxidation. The whole process repeats until the fatty acid is fully oxidized.

1.4.4.1 The role of fatty acid oxidation in immune cells

Fatty acid oxidation is generally considered a contributor to the anti-inflammatory state of immune cells. In macrophages, CPT1A (an isoform of CPT1) expression has been shown to be correlated with increased fatty acid oxidation. Overexpression of the protein in macrophages resulted in the increase of fatty acid oxidation and the decrease of pro-inflammatory molecules, such as IL-1β (Malandrino et al., 2015).

In T cells, fatty acid oxidation has been shown to be important for both memory T cell and Treg cell generation. CD8$^+$ T cells that have been shifted to a memory phenotype through supplementation of the culture medium with IL-15 display increased fatty acid oxidation and OXPHOS, which is important for maintaining that memory state. Memory CD8$^+$ T cells use glucose to generate fatty acids, which are then used to support OXPHOS via fatty acid oxidation (O'Sullivan et al., 2014). IL-15-generated memory-like CD8$^+$ T cells also express a greater amount of CPT1A compared to effector cells and blocking CPT1A with the drug etomoxir results in a decrease in OXPHOS (O'Sullivan et al., 2014; van der Windt et al., 2012). Treg cells generated in vitro were shown to have increased OXPHOS and fatty acid oxidation, as measured by oxidation of radiolabeled
palmitate, which was decreased with etomoxir treatment. Th17 cells, compared to Treg cells, undergo less fatty acid oxidation and more glycolysis (Michalek et al., 2011).

1.4.5 Oxidative phosphorylation

Unlike the previously mentioned metabolic pathways, oxidative phosphorylation (OXPHOS) is not a series of enzymatic reactions that are responsible for the catabolism or anabolism of metabolites. OXPHOS encompasses glycolysis, the TCA cycle, and electron transport. The name oxidative phosphorylation refers to the oxidation of glucose and TCA intermediates which results in the phosphorylation of ADP to create ATP. The key to the whole process is the transfer of electrons between protein complexes and ultimately to oxygen. This process results in the generation of a proton gradient across the inner mitochondrial membrane which is needed for complex V, or ATP synthase, to generate ATP from ADP. Having covered the “oxidative” half of OXPHOS in the previous sections, the next section will focus on the second half, that is electron transport and ATP synthesis.

1.4.5.1 The electron transport chain

The electron transport chain (ETC) is composed of five different protein complexes in the inner mitochondrial membrane: complex I (NADH-coenzyme Q oxidoreductase), complex II (succinate-coenzyme Q oxidoreductase), complex III (coenzyme Q-cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase). Electrons removed at various points during glycolysis, the TCA cycle, and fatty acid oxidation, are transported to the ETC in the form of the cofactors NADH and FADH₂ (Fig 1.4). NADH donates electrons to complex I, where the electrons traverse the complex through a series of coenzymes and iron-sulfur redox
centers before being transferred to coenzyme Q (CoQ). The process of moving electrons from NADH to CoQ results in the translocation of protons from the matrix of the mitochondria to intermembrane space. Complex II, which also contains succinate dehydrogenase, transfers electrons from succinate to CoQ, using FAD as the initial cofactor. Electrons are transferred to FAD to generate FADH$_2$, which then transfers the electrons to redox centers in the complex before finally reducing oxidized CoQ. Reduced CoQ, generated either at complex I or complex II, transports electrons to complex III through the lipid bilayer. Complex III then transfers the electrons from CoQ to cytochrome C, translocating protons to the intermembrane space in the process. Cytochrome c then transfers the electrons to complex IV, where the electrons are used to reduce oxygen to water. Complex IV also translocates protons to the intermembrane space in the process of reducing oxygen.

The purpose of the electron transport chain is to translocate protons from the mitochondrial matrix to the intermembrane space. This creates an electrochemical gradient of protons and charge, with the intermembrane space being much more positively charged than the matrix. This gradient, called the proton motive force or mitochondrial membrane potential, is what allows complex V of the ETC to generate ATP. Complex V allows protons to flow back down this gradient while using the energy of that proton flow to phosphorylate ADP. Protons enter the matrix, where they are again translocated through the complexes of the ETC, replenishing the electrochemical gradient.
1.4.5.2 The role of oxidative phosphorylation in immune cells

OXPHOS is often referred to as an opposing metabolic state from glycolysis in immunometabolism literature. This can be misleading, as OXPHOS can encompass glycolysis through the oxidation of carbons derived from glucose. Therefore, the term OXPHOS has taken on a related meaning of mitochondrial respiration. Since oxygen is the final electron acceptor in OXPHOS, measurements of oxygen consumption by cells can be used as a proxy measurement of mitochondrial respiration and electron transport. Electron transport is directly tied to the TCA cycle through the oxidation of FADH$_2$ and NADH, so an increase in oxygen consumption is considered to be linked to an increase in carbon flow through the TCA cycle, and electron transport.

Figure 1.4: Electron transport chain
T cells will switch between OXPHOS and glycolysis during an immune response. Both naïve and memory T cells are known to favor OXPHOS as the primary metabolic pathway of homeostasis (van der Windt et al., 2012). These metabolic differences are not only seen between effector and non-effector T cells, but also between subsets, particularly in the CD4⁺ T cells. Th1 and Th2 cells favor glycolysis, whereas Treg cells favor OXPHOS. Th17 cells appear to utilize both glycolysis and OXPHOS. Th17 and Treg cells even utilize different sources of fatty acids to fuel mitochondrial metabolism, with Treg cells importing fatty acids and Th17 cells making fatty acids to use in fatty acid oxidation (Berod et al., 2014a; Michalek et al., 2011). This difference in fatty acid generation could be one reason for the different glycolytic states between these two subsets.

Similar to T cells, macrophages also favor different metabolic states depending on function. M1 macrophages utilize glycolysis when activated, which is thought to promote the quick response these cells need for antibacterial responses. Conversely, M2 macrophages utilize OXPHOS. This is thought to help the cells maintain survival and effector function for a much longer period, allowing these cells to better fight parasites such as helminths (O'Neill et al., 2016; O'Neill and Pearce, 2016). Considering the differences in metabolism between macrophage and T cell subsets, it can be appreciated that metabolism plays an important role in the function of immune cells.

1.5 Methylation-controlled J-protein

Methylation-controlled J-protein (MCJ) is a 15-17 kDa member of the DNAJ family of proteins encoded by the gene dnak15 (Hatle et al., 2007; Shridhar et al., 2001). DNAJ proteins can perform a variety of functions from protein folding to protein
regulation. Expression of MCJ is regulated through DNA methylation, and expression is decreased when CpG rich regions within the gene are methylated (Shridhar et al., 2001; Strathdee et al., 2004). MCJ is highly expressed in the heart, liver, kidneys, and testis. In the immune system, MCJ is expressed in T cells, though at a much higher level in CD8$^+$ T cells compared to CD4$^+$ T cells (Hatle et al., 2013). Low expression of MCJ has been correlated with increased chemoresistance in some cancers due to excretion of the chemotherapeutic from the cell. When MCJ is re-expressed in these cancer cells, chemotherapeutics are retained within the cell, making the cancers chemosensitive (Hatle et al., 2007; Shridhar et al., 2001).

1.5.1 Structure, location, and function

MCJ is a unique member of the DNAJ family of proteins. Similar to other DNAJ family members, MCJ contains a DNAJ domain, though it is located at the C-terminus as opposed to the N-terminus. The N-terminus of MCJ is unique among the DNAJ family members. MCJ also contains a transmembrane domain, unlike many DNAJ family members (Hatle et al., 2013; Hatle et al., 2007).

MCJ is located in mitochondria, and the transmembrane domain allows the protein to associate with the inner mitochondrial membrane (Hatle et al., 2013) where MCJ performs its function as a regulator of mitochondrial metabolism. MCJ associates with complex I of the electron transport chain (Hatle et al., 2013) and reduces electron flow through the electron transport chain (ETC) by reducing both complex I activity and supercomplex formation (Hatle et al., 2013). Supercomplexes are associations of complexes I, III, and IV of the ETC. These supercomplexes can have a variety of combinations (e.g. supercomplex I/III/IV or supercomplex I/III) and can even contain...
multiples of a complex (e.g. supercomplex I/III2/IV, where the subscript denotes the number of a given complex) (Vartak et al., 2013). MCJ was found to reduce supercomplex formation, which is thought to reduce the efficiency and rate of electron flow through the ETC (Hatle et al., 2013).

1.5.2 Role in the immune system

Studies on the role of MCJ in the immune system have been carried out primarily through the use of mice deficient in MCJ (Hatle et al., 2013). As a negative regulator of complex I, it was not surprising that the loss of MCJ in CD8+ T cells resulted in an increase in oxygen consumption in mitochondria, increased ATP production, and an accumulation of amino acids (Champagne et al., 2016). MCJ-deficiency increased cytokine secretion in activated CD8+ T cells. Loss of MCJ. Loss of MCJ in CD8+ T cells had no effect on cell proliferation or death immediately after activation in vitro, but did allow activated effector T cells to survive after cytokine withdrawal, which is one mediator of apoptotic death (Champagne et al., 2016; Snow et al., 2010). The ability to withstand cytokine withdrawal could potentially explain the fact that MCJ deficient CD8+ T cells have an increased memory response to viral infection (Champagne et al., 2016). While it is apparent that MCJ affects the metabolism and memory function of T cells, it is not clear why the loss of MCJ promotes the survival of CD8+ T cells.

1.6 Cell Death

Cell death is an important process in all animals. It plays a key role in many aspects of biology, from embryo development to tissue homeostasis and even immune responses. For the immune system, cell death is critical during T cell development. In the thymus, autoreactive T cells undergo death to eliminate autoreactive T cells. In addition,
following an immune response, effector T cells that have proliferated need to die to prevent unintentional tissue damage. The timely death of effector T cells is possible due to a dramatic increase in susceptibility to death signals once the cell is activated (Green et al., 2003; Snow et al., 2010). Emerging evidence suggests that the increased sensitivity is linked to the upregulation of glycolysis after T cell activation, though the full mechanism of how glycolysis increases sensitivity to death signals is not well understood (Larsen et al., 2017). Defects in T cell death can lead to lymphomas as well as autoimmune disease, so a better understanding of how susceptibility to cell death is regulated in T cells is critical for finding therapeutic interventions for these diseases.

1.6.1 Forms of cell death

There are many ways for a cell to die. Cell death can be split into two general categories; necrosis versus apoptosis. Necrotic death occurs through rupture of the cytoplasmic membrane and is highly inflammatory. Apoptosis occurs through a mechanism initiated by the cell itself or externally by death ligands such as TNF, Fas-Ligand, or TRAIL. and as such, is often called programmed cell death (Tait et al., 2014). Apoptotic cell death can take many forms, and each one utilizes a unique pathway in the cell. Some of those pathways utilize caspases whereas others do not.

One caspase-independent pathway is necroptosis, which begins with the stimulation of a death receptor ligand such as TNF (Degterev et al., 2005). When caspase-8 is inhibited, either chemically or by interaction with an inhibitory protein, receptor-interacting serine/threonine-protein kinase (RIPK) 1 and 3 will interact at the death receptor and become activated. RIPK3 then phosphorylates mixed-lineage kinase
domain-like (MLKL), which oligomerizes and forms pores in the plasma membrane, leading to necrotic cell death (Murphy et al., 2013; Tait et al., 2014).

Ferroptosis is an iron-dependent form of caspase-independent cell death. Originally identified through the use of the small molecule erastin, ferroptosis results from the inhibition of the glutamate/cystine transporter. This leads to antioxidant starvation through the loss of glutathione. The decrease in antioxidants causes an increase in oxidative stress and lipid peroxidation, which ultimately induces cell death (Dixon et al., 2012; Gao et al., 2018).

Autophagic cell death has been documented in various eukaryotes such as slime molds and fruit flies but has not been fully appreciated in vivo in mammals. When studied in vitro, autophagic cell death is dependent on autophagic proteins such as Beclin-1, ATG5, and ATG7. The autophagy pathway can be initiated when other routes of cell death are inhibited, such as through the inhibition of caspase-8 or with the loss of BAX and BAK (Shimizu et al., 2004; Yu et al., 2004). As autophagy is initiated, ROS and oxidative stress will increase in the cell, partly due to the degradation of catalase, and induce cell death (Yu et al., 2006).

Unlike the previously summarized forms of cell death, pyroptosis is induced by caspases. Caspase-1 and caspase-11 (in mouse) are inflammatory caspases that are responsible for processing and activating the cytokines IL-1β and IL-18. During pyroptosis, DNA is fragmented, and the cell will swell and lyse. It is not completely understood why pyroptosis occurs. One reason could be to increase the release of IL-1β and IL-18. This process is pro-inflammatory and helps control bacterial infections (Bergsbaken et al., 2009; Tait et al., 2014).
1.6.2 Apoptosis and caspases

First defined in 1972 (Kerr et al., 1972), apoptosis is the form of death that cells undergo during tissue development, and post-development tissue homeostasis. The term “apoptosis” is Greek meaning “falling off” and refers to the way in which flower petals or leaves fall off a flower or tree. The “falling off” that happens in cell death is the blebbing of small membrane-bound compartments, known as apoptotic bodies, from a cell undergoing apoptosis. Apoptosis begins with the condensation of nuclear chromatin and fragmentation of DNA, which results in the engulfment and degradation of apoptotic bodies by phagocytic cells (Kerr et al., 1972).

Apoptosis is mediated by proteases called caspases (cysteine-containing aspartate-cleaving proteases). Cleavage of caspases can be done by Caspases involved in apoptosis can be sorted into two major groups, upstream initiator caspases that respond to death stimuli, and downstream effector caspases that carry out the destruction of the cell. Caspases are translated as zymogens, meaning they need to be cleaved to become active. Cleavage of caspases is usually done by upstream caspases. In the case of apoptosis, effector caspases are cleaved by initiator caspases. Those initiator caspases can be activated by other initiator caspases, or they can be activated through a process called proximity induced autoactivation (Fig. 1.5). In this method of activation, the inactive zymogen has an intrinsic activity and will autocleave when in close proximity to other
caspase zymogens. This is the mechanism through which caspase-8 is activated, initiating the extrinsic pathway of apoptosis (Muzio et al., 1998).

Through the extrinsic pathway of apoptosis, an extracellular signal is transduced across the cellular membrane to activate caspases and induce apoptosis (Fig. 1.6). This signal transduction begins with the ligation of a death receptor (e.g. Fas, TNFR, TRAILR) with its ligand, which results in oligomerization of the receptor. The death receptors contain a cytosolic death domain (DD), which will form a homodimer with the DD of the adapter protein Fas-associated death domain protein (FADD) (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD then recruits pro-caspase-8 or pro-caspase-10 through dimerization of a death effector domain (DED) found on both FADD and certain
caspases. Recruitment of caspases-8 or -10 through DED interactions forms the death-inducing signaling complex (DISC) (Kischkel et al., 1995; Muzio et al., 1996). The caspases in the DISC become active and cleave pro-caspase-3 and/or pro-caspase-7 to activate the caspases, which then go on to induce apoptosis. The DISC can also result in the activation of the cell intrinsic pathway of apoptosis through the cleavage of BID to form active pro-apoptotic tBID (Luo et al., 1998).

Figure 1.6: Extrinsic initiation of apoptosis
In the intrinsic pathway of apoptosis, the release of cytochrome c from the mitochondria results in the activation of the caspase cascade. This process begins with the activation or expression of pro-apoptotic molecules, such as PUMA, BID, or BAD, as a result of cell stress or damage (Kuwana et al., 2005; Leibowitz and Yu, 2010). The presence or activation of these molecules triggers the activation of BAX and/or BAK, which form pores in the outer membrane of the mitochondria (Gross et al., 1999). Cytochrome c is then released and binds to Apaf-1, forming a complex that can recruit pro-caspase-9 through a caspase recruitment domain (CARD) found on both Apaf-1 and caspase-9. Once bound to the complex, known as the apoptosome, pro-caspase-9 becomes active, then cleaves and activates pro-caspase-3 and pro-caspase-7 (Li et al., 1997; Srinivasula et al., 1998).

As proteases, the way in which effector caspases execute apoptosis is through the cleavage of critical proteins. Caspases can cleave proteins, at particular aspartate residues, important in maintaining the cytoskeleton, such as actin, myosin, and filamin (Browne et al., 2000; Taylor et al., 2008). While cleavage of the cytoskeleton helps to weaken the structural integrity of the cell, the blebbing of the membrane is due to the cleavage of a protein called ROCK1, a kinase that increases myosin contraction of actin filaments through phosphorylation. Cleavage of ROCK1 by caspases results in a truncated form of ROCK1 that has increased kinase activity, resulting in the blebbing of portions of the cell that have been structurally weakened through the degradation of the cytoskeleton (Coleman et al., 2001).

ROCK1 has also been implicated in another hallmark of apoptosis, the fragmentation of the nuclear envelope. Similar to cell membrane blebbing, ROCK1
facilitates the tearing of the nuclear membrane by increasing the activity of myosin (Croft et al., 2005). This causes the actin filaments attached to the nuclear envelope to contract and pull the envelope apart. This also requires weakening of the nuclear envelope, which happens through the cleavage of nuclear lamins by caspases (Orth et al., 1996; Rao et al., 1996).

Within the nucleus, DNA also undergoes fragmentation due to caspase-3 cleavage of the inhibitor of caspase-activated DNase (ICAD), which is found heterodimerized with caspase-activated DNase (CAD). Once liberated from ICAD, CAD fragments nuclear DNA (Liu et al., 1997; Taylor et al., 2008), which is important to prevent the activation of the innate immune system and autoinflammatory disease (Kawane et al., 2003; Napirei et al., 2000).

In addition to the nuclear degradation, the Golgi apparatus, ER, and mitochondria are fragmented during apoptosis. While the fragmentation of the Golgi and ER are dependent on caspases, mitochondrial degradation is due to the disruption of the mitochondrial membrane by BAX and BAK (Taylor et al., 2008). Ultimately, the fragmentation of these organelles, along with the proteolysis of the cytoskeleton and blebbing of the membrane, allow the apoptotic cell to break into smaller apoptotic bodies that can be engulfed by phagocytes.

Since the intracellular components of the dying cell are contained within the apoptotic bodies, apoptosis is considered a non-inflammatory form of cell death. In fact, phagocytes have been shown to release anti-inflammatory molecules (IL-10, TGF-β), and reduce the production of pro-inflammatory molecules (IL-12, IL-1β, TNF-α) upon phagocytosis of apoptotic bodies (Fadok et al., 1998; Kim et al., 2004; Nagata, 2018).
Efferocytosis, or the phagocytosis of apoptotic bodies, is potentially so important for the regulation of the immune system during tissue homeostasis that dysregulation of this process has been suggested to induce autoimmune diseases such as systemic lupus erythematosus (SLE) (Hanayama et al., 2004; Nagata et al., 2010; Ravichandran, 2010). Recent evidence suggests efferocytosis is a specialized type of phagocytosis called LC3-associated phagocytosis, which recruits autophagy machinery to complete the phagocytosis of apoptotic bodies (Martínez et al., 2016).

### 1.6.3 Anti-apoptotic and pro-apoptotic molecules

Mitochondria play an important part of the intrinsic apoptotic pathway. The release of cytochrome c from mitochondria contributes to the formation of the Apoptosome, which also includes caspase-9 and ATP. This leads to activation of downstream effector caspases and cell death. Due to the implications of mitochondrial integrity in cell death, there are many proteins in the cell involved in preventing or promoting death through mitochondrial disruption. These proteins are members of the B-cell lymphoma-2 (BCL-2) family of proteins.

BCL-2 proteins can be divided into two categories: anti-apoptotic and pro-apoptotic. All members of the BCL-2 family contain at least one BCL-2 homology (BH) domain. The anti-apoptotic proteins contain four BH domains, while the pro-apoptotic proteins contain three BH domains (Leibowitz and Yu, 2010; Taylor et al., 2008).

The BCL-2 family functions by interacting with each other to prevent or promote cell death. The BCL-2 pro-apoptotic proteins, specifically BAX and BAK, form pores in the mitochondrial membrane allowing the release of cytochrome c, as well as Smac/DIABLO, which blocks the function of inhibitors of apoptosis (IAPs). The BCL-2
anti-apoptotic members, such as BCL-2, MCL-1, and BCL-xL, primarily function by preventing the formation of BAX/BAK pores the outer mitochondrial membrane. This can happen through the direct interaction with BAX/BAK, or by binding to BH3-only proteins that can promote BAX/BAK oligomerization (Antonsson and Martinou, 2000; Leibowitz and Yu, 2010; Taylor et al., 2008).

Members of the BCL-2 family are not the only proteins that are involved in apoptosis. Some proteins induce apoptosis by activating caspases, such as apoptotic protease activating factor 1 (Apaf-1), which promotes the activation of caspase-9 (Li et al., 1997; Srinivasula et al., 1998). Others prevent apoptosis by binding to pro-apoptotic proteins, such as ICAD which binds to CAD to prevent DNA fragmentation (Liu et al., 1997; Taylor et al., 2008). Apaf-1 and ICAD, along with many other pro- and anti-apoptotic proteins, either activate or are cleaved by caspases.

1.6.4 Non-death functions of caspases

Caspases are known as inducers of cell death, but some caspases have functions that are not related to cell death. For instance, caspase-3 is important for the differentiation of skeletal muscle as it is required for the cleavage and activation of mammalian sterile twenty-like kinase, which is critical in the differentiation process (Fernando et al., 2002). Caspase-3 also helps to negatively regulate B cell cycling by cleaving p21, which can increase cycling in B cells (Woo et al., 2003). Some caspases were even discovered for their non-death functions. Caspase-1 was initially discovered as a protease that cleaves pro-IL-1 into the proinflammatory cytokine IL-1β and caspase-11 was found to be involved in the processing of another proinflammatory cytokine, IL-18.
(Black et al., 1989; Viganò and Mortellaro, 2013; Wang et al., 1996). While both caspases can induce death via pyroptosis, cytokine processing is their canonical function.

Caspase-8 is needed to transduce death signals through death receptors. However, caspase-8 is also necessary for the proliferation in T cells upon TCR stimulation (Misra et al., 2007). This makes caspase-8 interesting because these two opposing functions are dependent upon the location of caspase-8 in the cell. During activation and T cell proliferation, caspase-8 is located in lipid rafts at the plasma membrane. When Fas is stimulated to induce cell death, caspase-8 is found in the cytosol of the cell (Koenig et al., 2008). The location of the caspase is just as important as its activation state when considering the function of the enzyme.

1.6.5 Posttranslational regulation of caspases

Posttranslational modifications are changes made to proteins after they are synthesized and can range from cleavage to the addition of a small molecule to an amino acid. Some posttranslational modifications, such as phosphorylation, can change the function of the protein, while others will lead to the destruction of the protein (i.e. polyubiquitylation). Caspases normally undergo at least one posttranslational modification, which is the cleavage or the protein into an active state. Other posttranslational modifications have been reported for caspases, specifically caspase-3. Since caspases have a cysteine that functions as the proteolytic residue of the enzyme, any modification of that cysteine can inactivate the caspase. Cysteines are particularly sensitive to redox-dependent posttranslational modifications, such as s-nitrosylation and glutathionylation. Caspase-3 has been shown to be inactivated by both s-nitrosylation and glutathionylation, and s-nitrosylation of caspase-3 was reported to be important for
maintaining T cell survival in the presence of IL-15 (Huang et al., 2008b; Mannick, 2007; Mitchell et al., 2007; Saligrama et al., 2014).

1.7 Glutathionylation

Oxidative stress caused by reactive oxygen species (ROS) can induce a variety of deleterious aberrations in cells, such as irreversible protein damage, DNA damage, lipid peroxidation, and cell death (Perl, 2013). Cells have several antioxidant mechanisms through which they can reduce oxidative stress. One of the most important of these mechanisms is the use of glutathione ($\gamma$-glutamyl-cysteinyl-glycine). This tripeptide exists in both the reduced (GSH) and oxidized (GSSG) state and can be converted between the two states by glutathione reductase or thioredoxin and thioredoxin reductase (Deponte, 2013). Glutathione has two main functions in reducing oxidative stress. It can function as an antioxidant by reducing ROS and forming GSSG, and by protecting proteins from over-oxidation (Popov, 2014).

Thiols (R-SH), often those found on cysteines, are very sensitive to oxidative stress. Due to this sensitivity, the cell will add glutathione to the thiol to protect it from irreversible oxidation. Glutathionylation is mediated by glutathione S-transferases and deglutathionylation is mediated by glutaredoxin, thioredoxin, and sulfiredoxin (Tew and Townsend, 2012). Thiols can be modified when in a reduced state (-SH) or a mildly oxidized sulfenyl state (-SOH) (Giustarini et al., 2004). The result of protein glutathionylation is a protein modified with a glutathione moiety, which can be actively removed by the cell.
1.7.1 Synthesis of glutathione

Glutathione is composed of three amino acids: glutamate, cysteine, and glycine. The first step in the synthesis of glutathione is to ligate glutamate and cysteine. This is done by glutamate-cysteine ligase and requires one ATP molecule. This step is also rate-limiting due to the low concentration of cysteine in the cell (Lu, 2013). The second and last step of glutathione synthesis is the addition of glycine by GSH synthase and results in the formation of GSH (Lu, 2013).

1.7.2 Modification of proteins

Protein S-glutathionylation serves to both protect proteins from irreversible oxidation and to regulate protein function. Glutathione can be added and removed from proteins by enzymes, making the modification reversible. Protein glutathionylation can change the function of proteins and if the modified cysteine is involved in the activity of an enzyme, then the modification may result in inactivation (Giustarini et al., 2004). These modifications typically happen when a cell is under oxidative stress. In T cells, GAPDH is known to be glutathionylated and its activity is reduced when the cell is under oxidative stress (Ghezzi et al., 2002). Previous studies have shown that the oxidative state of T cells can change depending on the environment they are in and this oxidative state can influence T cell survival (Saligrama et al., 2014). However, further studies are needed to fully understand how the environment can affect protein glutathionylation in T cells, which proteins are glutathionylated, and how changing the function of those proteins impacts the cell.
1.8 Dissertation Aims

The metabolic state of immune cells is highly important in determining their function and fate. Varying the balance of oxidative phosphorylation and glycolysis allows a cell to perform different functions, which is exemplified by CD4⁺ T cell subsets (Berod et al., 2014b; Michalek et al., 2011; Pearce and Pearce, 2013). CD8⁺ T cells are known to utilize glycolysis during activation, and oxidative phosphorylation in the memory state (Roos and Loos, 1973a; van der Windt et al., 2012), though it is not known how metabolism influences the contraction that leads to memory. The work presented in the following chapters of this dissertation aims to answer two important questions related to T cell metabolism during proliferation and death:

1. Does metabolism regulate caspase activity in effector T cells?
2. Are T cell proliferation and contraction linked?

The answers to these questions will fill the gaps in knowledge of how deeply metabolism is integrated into the resolution of the immune response and how the immune system is able to mitigate the autoimmune risk associated with the rapid proliferation of effector immune cells.
1.9 References


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CHAPTER 2: GLYCOLYSIS PROMOTES CASPASE-3 ACTIVATION IN LIPOPRAFTS IN T CELLS
Glycolysis promotes caspase-3 activation in lipid rafts in T cells

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Running Title: Glycolysis promotes caspase-3 activity
2.1 Abstract

Resting T cells undergo a rapid metabolic shift to glycolysis upon activation in the presence of IL-2, in contrast to oxidative mitochondrial respiration with IL-15. Paralleling these different metabolic states are striking differences in susceptibility to restimulation induced cell death (RICD); glycolytic effector T cells are highly sensitive to RICD whereas non-glycolytic T cells are resistant. It is unclear whether the metabolic state of a T cell is linked to its susceptibility to RICD. Our findings reveal that IL-2-driven glycolysis promotes caspase-3 activity and increases sensitivity to RICD. Neither caspase-7, caspase-8, nor caspase-9 activity is affected by these metabolic differences. Inhibition of glycolysis with 2-deoxyglucose reduces caspase-3 activity as well as sensitivity to RICD. By contrast, IL-15-driven oxidative phosphorylation actively inhibits caspase-3 activity through its glutathionylation. We further observe active caspase-3 in the lipid rafts of glycolytic but not non-glycolytic T cells, suggesting a proximity-induced model of self-activation. Finally, we observe that effector T cells during influenza infection manifest higher levels of active caspase-3 than naïve T cells. Collectively, our findings demonstrate that glycolysis drives caspase-3 activity and susceptibility to cell death in effector T cells independently of upstream caspases. Linking metabolism, caspase-3 activity, and cell death provides an intrinsic mechanism for T cells to limit the duration of effector function.
2.2 Introduction

The balance of cell proliferation and cell death is critical for the maintenance of stable cell numbers and tissue homeostasis. Thus, it is perhaps not surprising that these opposing processes may be mechanistically linked in various cell types, including cancer (Evan et al., 1994; Wang et al., 2011). During an immune response, T lymphocytes undergo a period of very rapid proliferation. During this expansion, T cells also become susceptible to T cell receptor (TCR) restimulation-induced cell death (RICD) (Green et al., 2003; Snow et al., 2010). However, the link between proliferation and susceptibility to death remains poorly understood (Hedrick et al., 2010).

Changes in cellular metabolism are well recognized to play a critical role during an effective immune response. Resting naïve T lymphocytes, upon activation, undergo a dramatic metabolic shift from oxidative phosphorylation to aerobic glycolysis (Hume et al., 1978; Roos and Loos, 1973a; Roos and Loos, 1973b). The switch to a largely glycolytic state allows the cell to generate the synthetic capacity needed for rapid proliferation and effector function, such as cytokine production. In a similar manner, B cells and dendritic cells also utilize glycolysis upon activation to enable their effector functions (Santambrogio et al., 2005; Woo et al., 2003). Recent studies have further indicated that the metabolic state of effector T cells helps determine their subset differentiation (Michalek et al., 2011). Differing metabolic states are also known to be involved in the specification of T cell memory, with central memory T cells exhibiting high oxidative phosphorylation and effector memory T cells being more glycolytic (O'Sullivan et al., 2014; Phan et al., 2016; van der Windt et al., 2012).
It is well appreciated that cell death and metabolism are closely linked. Glycolytic enzymes can be induced by the same transcription factors that upregulate the expression of anti-apoptotic proteins such as BCL-xL (Green et al., 2014). Other proteins with metabolic function, such as cytochrome c, are directly involved in cell death (Green et al., 2014; Mason and Rathmell, 2011). When released from the mitochondria, cytochrome c activates caspase-9, which then cleaves caspase-3 and induces apoptosis. Caspase-3 can be alternatively activated through cleavage by caspase-8, which is activated by death receptors such as Fas (CD95). However, little is known regarding possible links between metabolism and caspase activity.

Though caspases were originally defined for their role in cell death, it is now appreciated that caspases perform many functions in cells in addition to cell death (Lamkanfi et al., 2007; Shalini et al., 2015). This is particularly well established for caspase-8, an initiator caspase that can induce apoptosis upon ligation of a death receptor (Medema et al., 1997), but can also allow cell proliferation by inhibiting formation of the necroptosome and induction of necroptosis (Dillon et al., 2012). Active caspase-3, a critical downstream mediator of apoptosis, has also been observed in non-dying cells and is implicated in skeletal muscle differentiation (Fernando et al., 2002), T cell anergy (Puga et al., 2008), B cell cycling (Woo et al., 2003), and erythrocyte maturation (Lamkanfi et al., 2007). However, these studies did not examine how caspase-3 activity is being regulated in these non-apoptotic situations. Moreover, an explanation has been lacking for the molecular switch between TCR-stimulated proliferation of naïve T cells versus induction of cell death in effector T cells (Green et al., 2003; Snow et al., 2010).
Given the involvement of caspases in both cell death and non-death functions, regulation of caspase activity and its location in cells are of paramount importance in determining cell fate. We have observed that T cells grown in interleukin (IL)-2 versus IL-15 have similar amounts of total pro-caspase-3, but IL-2-cultured T cells have a substantially higher level of active caspase-3, and as a result are much more susceptible to RICD (Saligrama et al., 2014). IL-15-cultured T cells are resistant to this form of cell death, in part due to the high levels of reactive oxygen and nitrogen species that lead to the redox modification of a critical cysteine in the active site of caspase-3, resulting in its inactivation (Huang et al., 2008a; Mitchell and Marletta, 2005a; Saligrama et al., 2014). IL-2 and IL-15 also induce very different metabolic states in T cells; IL-2 promotes glycolysis, whereas IL-15 upregulates oxidative phosphorylation (van der Windt et al., 2012). We now observe that caspase-3 activity in non-dying effector T cells is largely a function of their glycolytic state. Inhibition of glycolysis by a variety of methods reduces caspase-3 activity and protects T cells from RICD. In addition, IL-15-cultured T cells further reduce caspase-3 activity through its inactivation by glutathionylation. These findings underscore the importance of cellular metabolism in the regulation of caspase-3 activity and susceptibility toward cell death.
2.3 Results

2.3.1 IL-15 induces mitochondrial ROS and glutathionylation of caspase-3

To investigate the influence of cell metabolism on caspase activity, we initially modeled two metabolic states in vitro using cytokines that are known to promote very different levels of glycolysis following T cell activation: IL-2, which upregulates glycolysis, and IL-15, known to induce a non-glycolytic state of mitochondrial respiration (van der Windt et al., 2012). To mimic an immune response in vitro, purified naïve T cells were activated with anti-CD3/CD28 in the presence of IL-2 for 2 days, then removed from stimulation and propagated in IL-2 for an additional day. The activated T cells were then washed thoroughly to remove exogenous cytokines and recultured in medium containing either IL-2 or IL-15 for an additional 3 days. In agreement with previous observations (van der Windt et al., 2012), IL-15-cultured T cells manifested high mitochondrial respiration, as reflected by a high oxygen consumption rate (OCR) (Fig. 2.1A). Consistent with these findings, complex I activity of the electron transport chain was higher in IL-15-cultured T cells than in IL-2-cultured T cells (Fig. 2.1B and C). Complex I is known to generate reactive oxygen species (ROS) (Grivennikova and Vinogradov, 2006), and we determined that IL-15 induced a greater amount of mitochondrial ROS compared to IL-2 (Fig. 2.1D) as measured using the mitochondria-targeted probe mitoboronic acid (MitoB) (Cochemé et al., 2011). MitoB is converted to mitophenol (MitoP) upon reaction with mitochondrial hydrogen peroxide, and the levels of MitoB and MitoP are then measured by liquid chromatography tandem mass spectrometry and expressed as a ratio of MitoP to MitoB (Cochemé et al., 2011).
ROS can promote protein glutathionylation, a process that results in the reversible modification of a cysteine by glutathione (Giustarini et al., 2004). Caspase-3 has been shown previously to be inactivated in HL-60 cells by the glutathionylation of the critical cysteine in the enzymatic pocket (Huang et al., 2008a). We therefore investigated the possibility that caspase-3 is glutathionylated in IL-15-cultured T cells. Glutathionylated proteins were immunoprecipitated and immunoblotted for caspase-3 (Fig. 2.2A). Caspase-3 was glutathionylated to a considerably greater extent in the IL-15-cultured T cells compared to IL-2 (Fig. 2.2B). Of interest, the glutathionylation was observed specifically on cleaved caspase-3 (Fig. 2.2A). This implies that full-length pro-caspase-3 was initially cleaved and then inactivated by glutathionylation.

Given that IL-15 induced a greater amount of mitochondrial ROS compared to IL-2 (Fig. 2.1D), we examined the effect of quenching mitochondrial ROS on overall caspase activity in activated T cells. IL-2- and IL-15-cultured T cells were incubated with the mitochondria-targeted antioxidant mitoquinone (MitoQ) to quench mitochondrial ROS (Kelso et al., 2001). MitoQ treatment of IL-15-cultured T cells, but not IL-2-cultured T cells, induced a reduction in ROS (Fig. 2.2C). The lack of effect of MitoQ in IL-2-cultured T cells may reflect a non-mitochondrial source of ROS, such as cytoplasmic NADPH oxidases. Consistent with these findings, MitoQ did not alter caspase activity in IL-2-cultured T cells, but did slightly increase caspase activity in IL-15-cultured T cells, though not to the level of caspase activity in IL-2-cultured T cells (Fig. 2.2D). Consistent with these findings, treatment with MitoQ did not affect the sensitivity of IL-2- or IL-15-cultured T cells to death, either before or after restimulation (Fig. 2.2E). Thus, mitochondrially-derived ROS partially contributed to the low level of
caspase activity in IL-15-cultured T cells, but were not solely responsible for the decreased sensitivity to RICD. We therefore considered that the pronounced metabolic shift to glycolysis during T cell activation might also influence the activity of certain caspases and sensitivity to RICD.

2.3.2 Glycolysis drives caspase-3 activation

Glycolysis was measured by extracellular acidification rate (ECAR) in naïve T cells, effector T cells (day 6 after activation), and late effector T cells (day 10 after activation). Glycolysis increased initially from naïve to day 6 T cells, then decreased from day 6 to day 10 (Fig. 2.3A). These changes were greater in the IL-2-cultured T cells than IL-15. IL-2 signaling is known to drive glycolysis (van der Windt et al., 2012), and the kinetics of glycolysis in T cells grown in IL-2 closely paralleled the levels of surface IL-2 receptor α (CD25) expression, which also peaked and declined over a nearly identical time span (Fig. 2.3B). These results are consistent with previously reported kinetics of CD25 expression (Cantrell and Smith, 1983). Concomitant with the rise of glycolysis and surface CD25 expression following T cell activation, we observed an increase in total caspase activity. T cells propagated in IL-2 continued to increase caspase activity until a peak at day 6, after which it declined through day 10, closely paralleling ECAR and CD25 expression (Fig. 2.3C). As a result of the culturing system and the introduction of IL-15 at day 3, the first measurements of caspase activity and CD25 expression occurred on day 4 for IL-15-cultured T cells. In the presence of IL-15, CD25 expression and caspase activity remained at a consistently low level throughout the same 10-day period (Fig. 2.3C). The largest difference in caspase activity between IL-2- and IL-15-cultured T cells was at day 6. We also measured the level of active caspase-3 by
flow cytometry, and found it to be greater in IL-2-cultured T cells compared with IL-15-cultured T cells at day 6 (Fig. 2.3D). Whereas caspase-3 activity was detected in live IL-2-cultured T cells, activity was much higher in cells in which death was induced through Fas stimulation (Fig. 2.3D). Thus, the intermediate levels of active caspase-3 in proliferating T cells were not due to contaminating dead cells, but rather were present in all IL-2-cultured T cells.

To extend the observed correlation of glycolysis with caspase activity, we examined CD4+ T cell functional subsets, which have been shown to have varying levels of glycolytic activity (Michalek et al., 2011). In vitro-differentiated T helper (Th)1 and Th2 cells were reported to have high levels of glycolysis, being slightly higher in Th2 cells. By contrast, in vitro-differentiated T regulatory (Treg) cells had low levels of glycolysis. Purified naïve CD4+ T cells were differentiated into Th1, Th2, and Treg cells in vitro and their caspase activity was measured. Caspase activity was highest in the Th2 subset and lowest in the Treg subset, matching glycolytic activity (Fig. 2.3E and F).

These collective findings suggested that glycolysis and caspase activity might be causally linked. We thus examined more directly the effect of glycolysis on caspase activity. To inhibit glycolysis, IL-2-cultured T cells were incubated with 2-deoxy-D-glucose (2-DG), a glucose analog that blocks glucose catabolism after phosphorylation by hexokinase (Wick et al., 1957). As an inhibitor of glycolysis, 2-DG could potentially induce cell death in highly glycolytic IL-2-cultured T cells. To reduce contamination by dead cells, the 2-DG cell cultures were centrifuged over Histopaque. The inhibition of glycolysis by 2-DG was confirmed by measuring a decrease in ECAR (Fig. 2.4A). In parallel, caspase activity was also decreased, in a dose-dependent manner, in IL-2-
cultured T cells cultured with 2-DG (Fig. 2.4B). To confirm that the decrease in caspase activity was due to the inhibition of glycolysis and not an off-target effect of 2-DG, we also inhibited glycolysis by two other methods, culturing IL-2-cultured T cells with rapamycin or in a low-glucose medium. These treatments also reduced caspase activity in IL-2-cultured T cells (Fig. 2.4B) but not in IL-15-cultured T cells (Fig. 2.4C).

To determine whether the regulation of caspase activity by glycolysis was selective for certain caspases, we examined the levels of specific caspase activity in IL-2-cultured T cells in the presence or absence of 2-DG, using biotin-VAD (bVAD) to selectively precipitate only active caspases with streptavidin-coated Sepharose beads. Immunoblot analysis of the precipitates revealed that 2-DG caused a profound decrease in caspase-3 activity, but had no effect on the activities of the related effector caspase-7, nor the upstream caspase-8 or caspase-9 (Fig. 2.5A and B). The disparate levels of active caspase-3 in glycolytic versus non-glycolytic T cells suggested they might differ in their susceptibility to cell death following TCR restimulation. Day 6 IL-2-cultured T cells cultured with and without 2-DG were restimulated with anti-CD3. While 2-DG induced more cell death prior to restimulation (Fig. 2.5C), the 2-DG-treated cells were considerably more resistant to restimulation-induced cell death (RICD) (Fig. 2.5D). RICD is primarily mediated by Fas-ligand (FasL) signaling (Alderson et al., 1995). Therefore, we measured FasL by western blot and found expression to be highest in glycolytic IL-2-cultured T cells, and slightly less in non-glycolytic IL-15-cultured and 2-DG-treated T cells (Fig. 2.5E and F). However, the fact that the activities of caspases-8 and -9 were not affected by 2-DG suggests that FasL did not mediate caspase-3 activity in viable IL-2-cultured T cells.
2.3.3 Active caspase-3 is sequestered in membrane lipid rafts in effector T cells

Self-cleavage of caspases can occur when pro-caspases are clustered in close proximity (Muzio et al., 1998), such as in lipid rafts where caspase-8 is known to be active in effector T cells (Koenig et al., 2008; Misra et al., 2007). To determine if caspase-3 was localized to membrane lipid rafts in effector T cells, lipid rafts were purified from both glycolytic, IL-2-cultured T cells, and non-glycolytic, IL-15-cultured T cells and the resulting fractions were analyzed for caspase-3 cleavage and activity. In IL-2-cultured glycolytic T cells, pro-caspase-3 was present primarily in the non-raft fractions, and to a much lesser extent in the raft fractions (Fig. 2.6A, raft fractions: 3-5; non-raft fractions: 10 and 11). However, caspase-3 was extensively cleaved only in the lipid raft fractions. In the IL-15-cultured non-glycolytic T cells, pro-caspase-3 was also found predominantly in the non-raft fractions. Although caspase-3 was also observed to be cleaved primarily in the raft fractions of IL-15-cultured T cells, it was to a lesser extent than in IL-2-cultured T cells (Fig. 2.6B). Hence the ratio of cleaved caspase-3 to pro-caspase-3 was greater in the lipid rafts of IL-2-cultured glycolytic T cells than in non-glycolytic IL-15-cultured T cells (Fig. 2.6C).

To confirm that the cleaved caspase-3 observed in lipid rafts was active, we performed a bVAD precipitation on the lipid raft fractions 3-5 and the non-raft fraction 11. Caspase-3 was found to be active at a much greater extent in the raft fractions of IL-2-cultured glycolytic T cells, compared with IL-15-cultured non-glycolytic T cells (Fig. 2.6D and E).
2.3.4 Caspase-3 activity increases in effector T cells during influenza infection

To determine whether active caspase-3 is observed in activated T cells in vivo, mice were infected intranasally with influenza virus strain A/Puerto Rico/8/1934 H1N1 (Flu) and lymphocytes from the lung-draining mediastinal lymph node were analyzed after 6 days. Caspase-3 activity was measured in naïve (CD44\textsuperscript{low}) and proliferating effector (CD44\textsuperscript{high}) CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (Fig. 2.7A). The percentage of active caspase-3-positive cells, as well as the median fluorescence intensity (MFI) of active caspase-3 staining, were increased in the CD44\textsuperscript{high} population of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells after Flu infection, compared to the CD44\textsuperscript{low} population (Fig. 2.7B).
2.4 Discussion

The present findings reveal that IL-2-mediated glycolysis increases caspase-3 activity and the consequential susceptibility of effector T cells to caspase-3-mediated cell death. IL-15, by contrast, drives low levels of glycolysis that favor low caspase-3 activity. The high amount of oxidative metabolism in IL-15-cultured T cells also promotes a further decrease in caspase-3 activity through its glutathionylation. Our data support a model by which glycolytic metabolism sets the stage for a high proliferative rate of effector T cells that is coupled to enhanced susceptibility to death cues through the activity of downstream effector caspase-3.

Cleavage of caspase-3 can be induced by upstream caspase-8 following Fas ligation, and by caspase-9 following cytochrome c release from the mitochondria. It is also possible for cleavage of caspase-3 to occur by self-activation when caspase-3 molecules are clustered in close proximity (Muzio et al., 1998; Shi, 2004). We observed that the levels of both caspase-8 and caspase-9 activity were similar between glycolytic and non-glycolytic T cells suggesting that they are not likely to be the regulators of caspase-3 activity in these different metabolic states. The findings are also consistent with our previous observations that caspase-3 activity persists in effector T cells in which caspase-8 was deleted (Koenig et al., 2014). It is possible that translocation to the cell membrane, and particularly to lipid raft domains, allows the clustering of full-length inactive caspase-3, and could promote its proximity-induced autocleavage and activation (MacCorkle et al., 1998; Shi, 2004). Furthermore, while confined to lipid rafts, active caspase-3 may also be restricted in its access to substrates that might induce cell death.
Effector T cells and memory T cells have very different metabolic profiles. Similar to memory T cells derived in vivo, memory-like T cells can be generated in vitro through IL-15 signaling that promotes oxidative phosphorylation (O'Sullivan et al., 2014; van der Windt et al., 2012). Consistent with those previous reports, we observed that compared to IL-2, IL-15-cultured T cells have elevated oxidative phosphorylation and mitochondrial ROS. Our findings further reveal that IL-15-cultured T cells manifest glutathionylation of caspase-3, which is known to inactivate caspase-3 at the critical cysteine in the enzymatic pocket (Huang et al., 2008a). Given that glutathionylation of caspase-3 was detected only of cleaved caspase-3 in IL-15-cultured T cells, this suggests that caspase-3 is being inactivated by glutathionylation after its cleavage. Along with our previous report of the inactivation of caspase-3 by S-nitrosylation (Saligrama et al., 2014), these data show that in T cells, caspase-3 can be inactivated by redox-dependent post-translational modifications, which are influenced by the metabolic state of the cell. Metabolically-induced modifications of apoptotic caspases may be an important mechanism for memory T cell formation.

The correlation between glycolysis and CD25 expression for in vitro-generated effector T cells is not surprising given the known role of IL-2 in the induction of glycolysis in T cells. However, in vitro-differentiated T reg cells, despite expressing high levels of CD25, are not glycolytic, likely due to their derivation with TGFβ (Delisle et al., 2013; Michalek et al., 2011). We observed that these cells have low caspase activity, indicating that caspase activity is a function of glycolysis rather than CD25 expression. Proliferating T cells are known to be susceptible to cell death cues such as RICD (Kabelitz and Janssen, 1997; Peter et al., 1997). Our data demonstrate independent
increases in both FasL and caspase-3 activity in glycolytic IL-2-cultured T cells prior to TCR restimulation. Consistent with our observations, a study recently reported that human CD8+ T cells rapidly induced FasL protein following TCR restimulation only when glycolytic, rendering them more susceptible to RICD (Larsen et al., 2017). However, we do not feel that the caspase-3 activity or the capase-8 activity in proliferating glycolytic T cells is due to Fas/FasL interactions for a few reasons. First, we observed no increased levels of active upstream caspase-8 in IL-2-cultured T cells despite more active caspase-3. Second, we have previously shown that Fas-deficient effector T cells express as much active caspase-8 as wild-type T cells (Misra et al., 2007). Finally, reducing caspase-8 activity in effector T cells by deleting its regulator, c-FLIP, had no effect on the levels of active caspase-3 (Koenig et al., 2014). Hence, the collective findings would indicate that the levels of active caspase-8 and caspase-3 in effector T cells are largely independent of one another as well as independent of Fas/FasL interactions.

At present the full spectrum of function of caspase-3 activity in viable effector T cells is unclear. Other reports have noted a role for caspase-3 in T cell anergy (Puga et al., 2008), cleavage of p21 and cell cycling in B cells (Woo et al., 2003), skeletal muscle differentiation (Fernando et al., 2002), and maturation of erythrocytes, macrophages, and dendritic cells (Lamkanfi et al., 2007). The localization of active caspase-3 in membranes could provide access to substrates important to these functions, while simultaneously preventing the enzyme from cleaving pro-death substrates. While it is possible that the glycolytic state of cells may influence these other reported functions of caspase-3, the
current findings nonetheless link metabolism to caspase-3 activity and thus provide a plausible regulatory mechanism of immune cell homeostasis.
2.5 Materials and Methods

*Mice*

C57BL/6NJ male mice (Jackson Laboratory, Bar Harbor, ME) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-approved facility at the University of Vermont Larner College of Medicine. Mice were used at 2-6 months of age and protocols were approved by the Institutional Animal Care and Use Committee.

*Cell Culture*

T cells were purified from mouse lymph nodes (axillary, inguinal, brachial, cervical) and spleens by negative selection as described previously (Saligrama et al., 2014). Briefly, lymph nodes and spleens were homogenized through nylon mesh and red blood cells were lysed with Gey’s solution. Combined lymphocytes and splenocytes were incubated on ice for 30 min with the following antibodies: anti-CD11b (M1/70), anti-MHC class II (M5/114/15/2; a kind gift from M Rincón, Larner College of Medicine, University of Vermont, Burlington, VT, USA), and anti-B220 (RA3-6B2). Cells were then rocked over magnetic goat anti-rat beads (Qiagen, Germantown, MD, USA) at a 10:1 bead:cell ratio for 45 min at 4°C. Beads and bound cells were removed with a magnet. Naïve T cells were cultured in RPMI-1640 (Corning, Manassas, VA, USA), supplemented with 25 mM HEPES, 100 U/mL penicillin-streptomycin (ThermoFisher Scientific, Waltham, MA, USA), 5% bovine calf serum (GE Healthcare HyClone, Logan, UT, USA), 2.5 mg/L glucose, 2 mM glutamine, 10 μg/mL folate, 1 mM pyruvate, and 50 μM 2-mercaptoethanol (RPMI-C) and stimulated on 10 μg/mL plate-bound anti-CD3 clone 145-2C11 (Bio X Cell, West Lebanon, NH, USA) and soluble anti-CD28 ascites
clone 37-51 (1:1000), supplemented with 50 U/mL IL-2 (Cetus, Emeryville, CA, USA) at 37°C, 5% CO₂. After 2 days, activated T cells were removed from stimulation and cultured for an additional day in RPMI-C and 50 U/mL IL-2. Cells were then washed 3 times to remove cytokines and cultured for 2-3 more days in RPMI-C supplemented with either 50 U/mL IL-2 or 20 ng/mL IL-15 (a kind gift from Amgen, Thousand Oaks, CA, USA). For studies of the inhibition of glycolysis, T cells were activated for 2 days in RPMI-C medium supplemented with 50 U/mL IL-2. Cells were then washed 3 times and cultured in RPMI-C medium with 50 U/mL IL-2 and the indicated doses of 2-deoxy-D-glucose (Sigma-Aldrich, St. Louis, MO, USA) or rapamycin (MP Biomedicals, Solon, OH, USA) for 3 days, replacing media, cytokine, and inhibitor each day. IL-2-cultured T cells cultured in 2-deoxy-D-glucose were centrifuged over Histopaque-1077 (Sigma-Aldrich) to minimize the number of dead cells in the cultures prior to use in the assays. Alternatively, cells were washed and cultured with glucose-free RPMI-1640 (Corning) supplemented with 100 U/mL penicillin-streptomycin, 5% bovine calf serum, 2 mM glutamine, 10 μg/mL folate, 1 mM pyruvate, and 50 μM 2-mercaptoethanol (GF RPMI-C). Cells were then cultured in GF RPMI-C supplemented with the indicated decreasing concentrations of glucose for 3 days, replacing the media each day.

**Caspase activity assay**

Caspase activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s specifications. Samples were analyzed using a Synergy HT Plate Reader (BioTek, Winooski, VT, USA).

**Cell lysis and immunoblot analysis**
Cells were lysed for 20 min on ice in Lysis Buffer A (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10% glycerol, 2 mM sodium orthovanadate, and Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN, USA)). Protein concentration was determined by Bradford Assay (Bio-Rad, Hercules, CA, USA). Lysates were boiled for 5 min in Laemmli loading buffer supplemented with 2-mercaptoethanol (2-ME). Proteins within the lysates were separated by SDS-PAGE on a 12% acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in 4% milk in Tris-buffered saline with 0.1% Tween-20 (American Bioanalytic, Natick, MA, USA) at room temperature for 1 hr. The following antibodies were used for protein detection: anti-caspase-3, 585 rabbit polyclonal antibody (a kind gift from Dr. Yuri Lazebnik, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA), anti-caspase-8 (a kind gift from Dr. Andreas Strasser, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), anti-caspase-9, clone 5B4 (Stressgen Assay Designs, Ann Arbor, MI, USA), anti-flotillin (BD Biosciences), GM-1 HRP (Sigma-Aldrich), anti-paxillin, clone 165 (BD Biosciences), anti-FasL MAB5262 (R&D Systems, Minneapolis, MN, USA), anti-β-actin (Sigma-Aldrich), anti-mouse IgG HRP, anti-rabbit IgG HRP, and anti-rat IgG HRP (all from Jackson Laboratory). Densitometry was performed using Image Studio Lite v5 (LI-COR Biotechnology, Lincoln, NE, USA).

*bVAD active caspase precipitation*

Cells were washed once with PBS containing 1% bovine serum albumin (PBS/1% BSA) and once with PBS, then incubated on ice for at least 20 min with Lysis Buffer B (0.2% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10% glycerol, 2 mM
sodium orthovanadate, and Complete Protease Inhibitor (Roche Diagnostics)) supplemented with 20 μM biotin-VAD-fmk (bVAD) (MP Biomedicals). Protein was quantified by Bradford assay (Bio-Rad). 400–600 μg of protein in 300 μL of Lysis Buffer B (or 250 μg of protein for lipid raft fractions with no extra buffer added) was pre-cleared by rocking over 40 μL of Sepharose 4B beads (Sigma-Aldrich) for 2 hr at 4°C. Supernatants were then rocked over streptavidin-Sepharose beads (ThermoFisher Scientific) overnight at 4°C. Beads were then washed 3 times with Lysis Buffer B without protease inhibitor and boiled for 5 min in Laemmli loading buffer supplemented with 2-ME. For precipitations with lipid raft and non-raft fractions, an equal amount of protein was used from each fraction for the assay.

*Glutathione Immunoprecipitation*

Cells were washed once with PBS/1% BSA and once with PBS, then incubated in Lysis Buffer B supplemented with 1 mM N-ethylmaleimide (NEM) for at least 20 min on ice. Protein was quantified by Bradford assay (Bio-Rad). 300 μg of lysate was brought to 250 μL with lysis buffer. The reduced control was incubated with 50 mM dithiothreitol (+DTT) and rocked at 4°C for 1 hr. The reduced sample was then centrifuged through a Micro Bio-Spin P-6 Gel Column (Bio-Rad). For the IgG control, 5 μg of mouse IgG (Jackson Laboratory) was added to the sample. For all other samples, 2 μg of anti-glutathione antibody (Virogen, Watertown, MA, USA) was added. All samples were rocked overnight at 4°C. Samples were added to 50 μL of washed Protein G Plus Agarose (ThermoFisher Scientific) and rocked at 4°C for 1 hr. Beads were washed and boiled in Laemmli buffer before being loaded onto a 15% acrylamide gel. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane for immunoblot analysis.
Metabolic analyses

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the Seahorse XFe 96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s specifications. Analysis was performed using the Wave Software v2.2.0 or v2.3.0.2 (Agilent Technologies).

Flow Cytometry

For surface staining, cells were washed in PBS and stained with Live/Dead Fixable Blue Dead Cell Stain (ThermoFisher Scientific) for 25 min on ice. Cells were washed and incubated for 25 min on ice with anti-CD25-BV421 (BioLegend, San Diego, CA, USA), anti-CD44-PE (BioLegend), anti CD4-PE-TexasRed (ThermoFisher Scientific), anti-CD8-PerCP-Cy5.5 (BioLegend), anti-TCRβ-PE-Cy7 (BioLegend), anti-CD45RB-FITC (BioLegend). Cells were washed, fixed in 1% (v/v) methanol-free formaldehyde, and analyzed on an LSRII (BD Biosciences).

For intracellular staining, after staining with Live/Dead Fixable Blue Dead Cell Stain and surface antibodies as described above, cells were washed and fixed with 2% formaldehyde (v/v) for 15 min on ice. Fixed cells were washed and permeabilized with PBS/1% BSA supplemented with 0.03% saponin for 10 min on ice. Cells were washed and incubated with anti-cleaved caspase-3 Alexa 647 (Cell Signaling) for 30 min on ice. Cells were washed, fixed in 1% formaldehyde (v/v), and analyzed on an LSRII (BD Biosciences).

All flow cytometry data was analyzed with FlowJo v10 software (FlowJo, Ashland, OR, USA).

Cellular and mitochondrial ROS measurement
Cellular ROS was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (ThermoFisher Scientific). 5 x 10^5 cells were incubated with 1 μM DCFDA in PBS at 37°C in the dark for 30 min. Cells were washed with cold PBS/1% BSA and analyzed immediately by flow cytometry.

Mitochondrial ROS was quenched using MitoQ (Kelso et al., 2001). Cell cultures were incubated with MitoQ or a DMSO control for 4 h. Cells were then stained with DCFDA, as described above, for ROS detection.

Mitochondrial ROS was measured using the MitoB probe as previously described (Cochemé et al., 2011). MitoB specifically targets the mitochondria and is converted to MitoP in the presence of ROS. The ratio of MitoP/MitoB was measured using liquid chromatography tandem mass spectrometry.

**Complex I activity and mitochondrial extraction**

Cells were washed with PBS/1% BSA and resuspended in STE buffer (250mM sucrose, 5mM Tris, 1mM EGTA, pH 7.4 with HCl). Cells were homogenized and centrifuged at 1 000 x g for 3 min at 4°C. The supernatant was then centrifuged at 10 000 x g for 10 min at 4°C. The supernatant (cytosol) was saved and the pellet (mitochondria) was washed with STE buffer. The mitochondria were then resuspended and assayed for Complex I activity using the Complex I Enzyme Activity Microplate Kit (MitoSciences, Eugene, OR, USA) according to the manufacturer’s specifications. Samples were run in parallel with mouse liver mitochondrial extracts as a positive control. Complex I rates were calculated by dividing Complex I activity by time. Complex I rates were normalized to mouse liver mitochondrial complex I rate.

**Cell Death**
Restimulation-Induced Cell Death was induced in day 6 or 7 effector T cells by incubation on plate-bound anti-CD3 (10 μg/mL) for 16-18 hr at 37°C. Cells were removed and stained with Live/Dead Fixable Blue Dead Cell Stain (ThermoFisher Scientific), fixed in 1% formaldehyde (v/v), and analyzed by flow cytometry. As a positive control for cell death, day 6 effector T cells were incubated with 400 ng/mL FLAG-tagged FasL (Enzo Life Sciences, Farmingdale, NY, USA) and 2 μg/mL anti-FLAG antibody M-2 (Sigma-Aldrich) for 1.5 hr at 37°C. Cells were then stained with Live/Dead Fixable Blue Dead Cell Stain (ThermoFisher Scientific) fixed in 1% formaldehyde (v/v), and analyzed by flow cytometry.

**CD4+ T cell subset differentiation**

Purified CD4+ T cells were activated in complete medium on plate-bound anti-CD3/soluble anti-CD28 for 2 or 3 days in the presence of differentiating cytokines (Th1: 4 ng/mL IL-12 (PeproTech, Rocky Hill, NJ, USA) plus 10 μg/mL anti-IL-4 (BioLegend); Th2: 10 ng/mL IL-4 (PeproTech) plus 10 μg/mL anti-IFN-γ (BioLegend); Treg: 2 ng/mL TGF-β (PeproTech) plus 100 U/mL IL-2). After differentiation, cultures were propagated for 2 or 3 days in complete medium containing 50 U/ml IL-2 for Th1 and Th2 cells, and 100 U/mL IL-2 for Treg.

**Lipid Raft Purification**

Lipid rafts were separated as previously described (Misra et al., 2007). Briefly, cells were lysed on ice for 25 min in TNE buffer (5 mM iodoacetic acid (ThermoFisher Scientific), 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 15 mM EDTA, Complete Protease Inhibitor) supplemented with 100 mM Na2CO3 and 0.5% Triton X-100. Lysates were sonicated and added to 60% Opti-Prep sucrose substitute (Sigma-Aldrich) to make a final
concentration of 40% Opti-Prep. Lysates were placed in an ultracentrifuge tube and layered over with 30% and 5% solutions of Opti-Prep diluted with TNE buffer. Samples were centrifuged for 18 hr at 200 000 x g at 4°C. Eleven 1 mL fractions were taken sequentially from each sample. An equal volume of each fraction was used for immunoblot analysis.

*Influenza infection of mice*

Mouse-adapted influenza virus strain A/Puerto Rico/8/1934 H1N1 (PR8) virus (Charles River Laboratories, Wilmington, MA, USA) was used to infect mice. Mice were briefly anesthetized using 2.3% isoflurane in oxygen and infected intranasally with a sublethal dose of PR8 (0.2 LD50) in 0.05 ml of PBS. Control mice received 0.05 ml PBS without virus. Mice were monitored daily until harvest on day 6, at which time the mediastinal lymph node was harvested from the influenza infected mice, and axillary, brachial, and inguinal lymph nodes were extracted from the PBS control mice. Lymphocytes were collected and stained for analysis by flow cytometry.

*Statistical Analysis*

Statistical analyses were performed using the graphing software Prism v7 (GraphPad Software, La Jolla, CA, USA). The statistical test used for each experiment is indicated in the figure legends. The following statistical tests were used: paired and unpaired t-test when comparing two conditions (e.g. IL-2 compared to IL-15), one-way ANOVA with Tukey test or Dunnett test (when comparing to one control) for correction for multiple comparisons when comparing multiple conditions for a single variable (e.g. dose titration of 2-DG), two-way ANOVA with Sidak test or Tukey test for correction for multiple comparisons when comparing multiple variables across multiple conditions (e.g.
IL-2 with and without anti-CD3 compared to IL-15 with and without anti-CD3). All data met the assumptions of the statistical tests used and variation between the compared groups was similar.

2.6 Acknowledgments

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2.7 Conflict of Interest

The authors declare no conflict of interest.
2.8 Figure Legends

Figure 2.1. IL-15 drives increased oxygen consumption, complex I activity, and mitochondrial ROS. Anti-CD3/CD28-activated T cells were cultured for 3 days in IL-2 or IL-15. (A) Oxygen consumption rate (OCR) was measured by extracellular flux analysis. O = Oligomycin A (inhibitor of ATP synthase), F = FCCP (uncoupler of the electron transport chain), R/A = Rotenone and Antimycin (inhibitors of complexes I and III, respectively) (mean ± SD of 4 replicates within the one experiment shown. The graph is representative of 2 independent experiments). (B) Complex I activity was measured in mitochondrial (Mito) and cytosolic (Cyto) fractions of IL-2- or IL-15-cultured T cells (the graph is representative of 2 independent experiments). (C) Complex I rates of activity in (B) for mitochondrial fractions, normalized to the rate of activity of complex I in mouse liver mitochondria (unpaired t-test; * indicates p<0.05; mean ± SD of data from 2 independent experiments). (D) Mitochondrial ROS were measured by the conversion of mitoboronic acid (MitoB) to mitophenol (MitoP), and the ratio of MitoP/MitoB was measured by liquid chromatography tandem mass spectrometry (paired t-test; * indicates p<0.05; mean ± SEM of means from 3 independent experiments).

Figure 2.2. IL-15 induces glutathionylation of caspase-3. Anti-CD3/CD28-activated T cells were cultured in IL-2 or IL-15 for 3 days before lysis. (A) Glutathione immunoprecipitation (GSH IP) was performed on whole cell lysates (WCL). Immunoprecipitations were conducted alongside a reduced control (+DTT) and an IgG control (IgG). The WCL and GSH IP were immunoblotted for caspase-3 (the immunoblot is representative of 3 independent experiments). (B) Densitometry of cleaved caspase-3 detected in the IL-2 and IL-15 GSH IP lanes in (A), indicative of glutathionylated
cleaved caspase-3 (paired t-test; * indicates p<0.01; mean ± SD; n = 3 independent experiments). (C) ROS were measured by flow cytometry using DCFDA in IL-2- or IL-15-cultured T cells in the presence or absence of 200 nM mitoquinone (MitoQ) for 4 h (two-way ANOVA with Sidak’s correction; data indicate a fold change compared to IL-2 DMSO control; NS = not significant; * indicates p<0.05; mean ± SEM; n = 3 independent experiments). (D) Caspase activity was measured by DEVD rhodamine fluorescence in IL-2- or IL-15-cultured T cells with or without MitoQ (two-way ANOVA with Sidak’s correction; NS = not significant; * indicates p<0.05; mean ± SD of 3 replicates within the one experiment shown. The graph is representative of 2 independent experiments). (E) IL-2- or IL-15-cultured T cells were incubated with MitoQ (200 nM) or DMSO for 24 h. Restimulation-induced cell death was then promoted by 16 h anti-CD3 restimulation followed by Live/Dead staining and flow cytometry (two-way ANOVA with Tukey’s correction; NS = not significant; mean ± SD of 2 replicates).

Figure 2.3. Caspase activity parallels glycolysis and CD25 expression in T cells. (A-C) T cells were activated with anti-CD3/CD28 for 2 days and cultured in IL-2 for a third day. T cells were then washed and cultured in IL-2 or IL-15 for an additional 7 days. (A) Baseline extracellular acidification rate (ECAR) was measured by extracellular flux analysis at days 0, 6, and 10 (two-way ANOVA with Tukey’s correction; NS = not significant; * indicates p<0.01; mean ± SEM of means from 3 independent experiments). (B) CD25 expression measured daily by flow cytometry (the graph is representative of 2 independent experiments). (C) Caspase activity was measured daily by DEVD rhodamine release (mean ± SD of 3 replicates within the one experiment shown. The graph is representative of 2 independent experiments). (D) Anti-CD3/CD28-activated T cells were
cultured in IL-2 or IL-15 for 3 days. For a positive control, IL-2-cultured T cells were incubated with FasL for 1.5 h to induce caspase-3 activity and cell death. Cells were stained for intracellular active caspase-3 and analyzed by flow cytometry (the graph is representative of 3 independent experiments). (E, F) Purified CD4+ T cells were differentiated into Th1, Th2, and Treg subsets by activation with anti-CD3/CD28 for 2 or 3 days along with the appropriate cytokines (see Materials and Methods). T cell subsets were then cultured for an additional 2 or 3 days in IL-2 according to an established protocol (Michalek et al., 2011). (E) Baseline ECAR was measured by extracellular flux analysis (one-way ANOVA with Tukey’s correction; ** indicates p<0.0001; mean ± SD of 5 replicates within the one experiment shown. The graph is representative of 2 independent experiments). (F) Caspase activity was measured by DEVD rhodamine fluorescence (one-way ANOVA; ** indicates p<0.0001; mean ± SD of 3 replicates within one experiment. The graph is representative of 2 independent experiments).

**Figure 2.4. Glycolysis regulates caspase activity in effector T cells.** Anti-CD3/CD28-activated T cells were cultured for 3 days in IL-2 with (A) 2-deoxyglucose (2-DG, 0-5 mM). Baseline ECAR was measured (one-way ANOVA with Dunnett’s correction; ** indicates p<0.001; mean ± SEM of means from 3 independent experiments). (B, C) Activated T cells were cultivated for 3 days in (B) IL-2 or (C) IL-15, in medium, 2-deoxyglucose (2-DG, 5 mM), without glucose (No glucose), Rapamycin (300 nM), or DMSO control. Caspase activity was measured by DEVD rhodamine fluorescence. Data indicate a fold change compared to the control (one-way ANOVA with Dunnett’s correction; * indicates p<0.05; ** indicates p<0.001; mean ± SD; n = 3 independent experiments).
Figure 2.5. Activity of caspase-3, but not caspase-8 or -9, is regulated by the glycolytic state of effector T cells. Activated T cells were cultured for 3 days in IL-2 with or without 5 mM 2-DG. (A) Active caspases were selectively precipitated using biotin-VAD (bVAD) and streptavidin Sepharose beads, and immunoblotted for caspases-8, -9, -7, and -3 (the blot is representative of 2 independent experiments). (B) Densitometry of active caspase-8, -9, -7, and -3 in (A) (For caspase-8, -9, and -3: two-way ANOVA with Sidak’s correction; For caspase-7: unpaired t-test; NS = not significant; * indicates p<0.05; mean ± SD of 2 replicates). (C) Cell death was determined by Live/Dead staining after centrifugation over Histopaque. (unpaired t-test; mean ± SD; ** indicates p<0.01; n = 3 independent experiments). (D) Restimulation-induced cell death was promoted by 16 h anti-CD3 restimulation of IL-2-cultured T cells cultured with or without 2-DG, and cell death determined by Live/Dead staining using flow cytometry. Data indicate fold change compared to the unstimulated medium control (two-way ANOVA with Sidak’s correction; NS = not significant; * indicates p<0.05; mean ± SD of 2 replicates; n = 2 independent experiments). (E) Cell lysates of T cells grown in the indicated conditions were immunoblotted for FasL. Controls included FasL-transfected 3T3 cells or mock-transfected 3T3 cells (the immunoblot is representative of 2 independent experiments). (F) Densitometry of FasL in (E) (one-way ANOVA; * indicates p<0.05; ** indicates p<0.01; mean ± SD of 2 replicates).

Figure 2.6. IL-2-mediated glycolysis promotes the localization and cleavage of caspase-3 in lipid rafts. Activated T cells were cultured for 3 days in IL-2 or IL-15. (A, B) Cells were sonicated before fractionation in an ultracentrifuge over an Optiprep density gradient. Fractions of 1 mL each were collected. A volume of 30 μL of each
fraction was used to perform an immunoblot for capsase-3, GM-1 (lipid rafts), and paxillin (non-raft) (the blot is representative of 2 independent experiments). (C) Densitometry of the ratio of cleaved capsase-3 to pro-caspase-3 in raft and non-raft fractions (n = 2 independent experiments). (D) bVAD precipitation of active caspases was performed on the indicated raft and non-raft fractions and precipitates were immunoblotted for active caspase-3 (the blot is representative of 2 independent experiments). (E) Densitometry of active caspase-3 in raft and non-raft fractions (n = 2 independent experiments).

**Figure 2.7. Caspase-3 is active in vivo in proliferating T cells after influenza infection.** Mice received influenza virus strain A/Puerto Rico/8/1934 H1N1 virus (FLU) or PBS control intranasally and lymph nodes were extracted 6 days after infection. Mediastinal lymph node cells (flu-infected mice) or non mediastinal lymph node cells (PBS control mice) were stained and analyzed by flow cytometry. (A) Representative contour plots of live CD4+ (left) or CD8+ (right) T cells from PBS control mice (top) or flu-infected mice (bottom) for CD44 versus active caspase-3. The two rectangular inserts identify the CD44^{high} (upper) and CD44^{low} (lower) populations. All events to the right of the vertical line are positive for active caspase-3. Numbers represent the percentage of CD44^{high} or CD44^{low} gated cells that are positive for active caspase-3 (%+), and the median fluorescent intensity of active caspase-3 (MFI). (B) Graphs of the percent positive cells for active caspase-3 (top) and MFI (bottom) (two-way ANOVA with Tukey’s correction; mean ± SD; * indicates p<0.001, ** indicates p<0.0001; NS = not significant; n = 5 mice per group).
Figure 2.1: IL-15 drives increased oxygen consumption, complex I activity, and mitochondrial ROS
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2.9 References


CHAPTER 3: GLYCOLYSIS INDUCES MCJ EXPRESSION THAT LINKS T CELL PROLIFERATION WITH CASPASE-3 ACTIVITY AND DEATH
Glycolysis induces MCJ expression that links T cell proliferation with caspase-3 activity and death

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3.1 Abstract

An effective adaptive immune response requires rapid T cell proliferation, followed by equally robust cell death. These two processes are coordinately regulated to allow sufficient magnitude of response followed by its rapid resolution, while also providing the maintenance of T cell memory. Both aspects of this T cell response are characterized by profound changes in metabolism; glycolysis drives proliferation whereas oxidative phosphorylation supports the survival of memory T cells. While much is known about the separate aspects of T cell expansion and contraction, considerably less is understood regarding how these processes might be connected. We report a link between the induction of glycolysis in CD8\(^+\) T cells and upregulation of the inhibitor of complex I and oxidative phosphorylation, methylation-controlled J protein (MCJ). MCJ acts synergistically with glycolysis to promote caspase-3 activity. Effector CD8\(^+\) T cells from MCJ-deficient mice manifest reduced glycolysis and considerably less active caspase-3 compared to wild-type cells. Consistent with these observations, in non-glycolytic CD8\(^+\) T cells cultured in the presence of IL-15, MCJ expression is repressed by methylation, which parallels their reduced active caspase-3 and increased survival compared to glycolytic IL-2-cultured T cells. Elevated levels of MCJ are also observed in vivo in the highly proliferative and glycolytic subset of CD4\(^+\)CD8\(^-\) T cells in Fas-deficient lpr mice. This subset also manifests elevated levels of activated caspase-3 and rapid cell death. Collectively, these data demonstrate tight linkage of glycolysis, MCJ expression, and active caspase-3 that serves to prevent the accumulation and promote the timely death of highly proliferative CD8\(^+\) T cells.
3.2 Introduction

The adaptive immune response is characterized by rapid proliferation of responding T cells followed by equally rapid cell death. These two processes need to be tightly coordinated, lest this result in excessive expansion or loss of T cells. Whereas much is known about the metabolic shifts leading to T cell proliferation and the death pathways resulting in contraction, less is appreciated about the possible close linkage of these two processes. Understanding this connection is critical to the design of T cell immunotherapy where enormous expansion of T cells is performed *ex vivo* using exogenous cytokines followed by the need for the cells to survive *in vivo* when infused in patients.

T cell activation induces IL-2 and CD25 signaling, promoting IL-2-induced glycolysis that is characterized by the activation of mTOR and the upregulation of Glut1 (Finlay et al., 2012; Ray et al., 2015). The increase in glycolysis allows cells to generate the synthetic molecules needed for rapid proliferation and proper effector function. Proliferative effector T cells are highly sensitive to various forms of cell death, including Fas stimulation and cytokine withdrawal (Alderson et al., 1995; Larsen et al., 2017; Snow et al., 2010). The cytokine IL-15 is also important in proliferation. By contrast, IL-15 reduces glycolysis and promotes oxidative phosphorylation and T cell survival to the memory stage, although the mechanism of survival is not clear (Saligrama et al., 2014; van der Windt et al., 2012).

In addition to the critical role of metabolism in T cell activation and proliferation, the metabolic state of T cells may greatly influence their susceptibility to cell death. Given that caspases are frequently the mediators of cell death, we considered that
metabolism might regulate the activity of certain caspases, and as such, set a level of susceptibility to cell death. We have previously observed that IL-2 selectively promotes caspase-3 activity whereas IL-15 inhibits its activation. Knowing that IL-15 promotes activity of complex I of the electron transport chain and oxidative phosphorylation (Secinaro et al., 2018; van der Windt et al., 2012), we considered that other mechanisms of reducing glycolysis and enhancing complex I activity might also reduce caspase-3 activity.

Methylation-controlled J protein (MCJ) was recently identified as a negative regulator of complex I (Hatle et al., 2013). MCJ is a member of the DNAJ family of proteins, encoded by the gene *dnajc15* (Hatle et al., 2013; Hatle et al., 2007; Shridhar et al., 2001). MCJ is located at the inner mitochondrial membrane and interacts with complex I of the electron transport chain (Hatle et al., 2013). This interaction decreases complex I activity and reduces supercomplex formation of members of the electron transport chain, which results in a decrease in mitochondrial respiration (Champagne et al., 2016). MCJ-deficient T cells thus manifest increased complex I activity, mitochondrial respiration, and provide more effective memory than wild-type T cells (Champagne et al., 2016). We therefore considered that regulation of MCJ expression may be a component of the linkage between metabolism and cell death.

Here, we observe that as T cells enter glycolysis via IL-2 *in vitro* to become effector T cells they strongly upregulate MCJ. Paralleling this was an increase of caspase-3 activity. Similar findings were observed *in vivo* with rapidly proliferating glycolytic CD4^+^CD8^-^ T cells from Fas-deficient *lpr* mice. By contrast, in MCJ-deficient IL-2 effector T cells caspase-3 activity was decreased. IL-15-cultured T cells downregulated
MCJ expression through its gene methylation, which also paralleled reduced caspase-3 activity. These findings establish a close relationship between glycolysis, MCJ, and mitochondrial respiration, with a level of caspase-3 activity that is independent of Fas engagement.

3.3 Results

3.3.1 Induction of glycolysis by IL-2 increases expression of MCJ and reduced complex I activity which is reversed by IL-15

We modeled in vitro the metabolic switch that occurs in CD8\(^+\) T cells during the transition from naïve to effector and then to memory T cells by analyzing freshly purified CD8\(^+\) T cells before, and at various times after, activation with anti-CD3/CD28. After two days, cells were removed from the activation stimuli and cultured for an additional day in IL-2, then washed and recultured for an additional three days in cytokines known to induce differing metabolic states; IL-2 to induce glycolysis and effector T cells versus IL-15 to induce oxidative phosphorylation and memory T cells (van der Windt et al., 2012). IL-2-cultured activated CD8\(^+\) T cells manifested a rapid increase in glycolysis, as measured by extracellular acidification rate (ECAR) and a low oxygen consumption rate (OCR) on day 6, whereas IL-15-cultured CD8\(^+\) T cells had the opposite metabolic profile, consistent with oxidative phosphorylation, in agreement with previous reports (van der Windt et al., 2012) (Fig. 3.1A).

As oxygen consumption is primarily due to mitochondrial activity of the electron transport chain, we examined complex I activity in naïve versus IL-2-cultured CD8\(^+\) T cells. Corresponding to the decrease in OCR as naïve T cells became glycolytic effector
T cells with IL-2 stimulation, we observed a decrease in complex I activity in IL-2 CD8\(^+\) T cells (Fig. 3.1B). We further investigated the possibility that the metabolic switch between oxidative phosphorylation and glycolysis reflected changes in methylation-controlled J protein (MCJ), which physically associates with complex I and inhibits its activity (Hatle et al., 2013). MCJ expression in naïve CD8\(^+\)T cells increased following activation with IL-2, peaking at days 3-5. In contrast, MCJ expression decreased rapidly in IL-15-cultured CD8\(^+\) T cells compared to IL-2, resembling resting naïve CD8\(^+\)T cells (Fig. 3.1C). Of further interest was that paralleling the increase in MCJ expression with IL-2, CoxIV (a subunit of complex IV) decreased, reaching low expression levels at days 3-4, and then increasing toward initial levels. This shows that the increase in MCJ was not the result of a global increase in mitochondrial mass, as reflected in the MCJ:CoxIV ratio (Fig. 3.1C). IL-15-cultured-CD8\(^+\) T cells manifested the opposite profile with persistently high CoxIV, compared to their low levels of MCJ (Fig. 3.1C).

### 3.3.2 Inhibition of DNA methyltransferases results in the expression of MCJ in IL-15-cultured CD8\(^+\) T cells

Given the differences in MCJ expression between glycolytic IL-2-cultured cells and non-glycolytic IL-15-cultured cells, we investigated the possibilities that IL-15 reduces MCJ expression or glycolysis drives MCJ expression. The gene encoding MCJ, *dnajc15*, is known to be regulated by methylation-induced suppression (Shridhar et al., 2001; Strathdee et al., 2004). To investigate whether the reduction in MCJ by IL-15 was related to gene methylation we cultured activated CD8\(^+\) T cells in IL-15 with 1 \(\mu\)M 5-azacytidine (5azaC), a DNA methyltransferase inhibitor, or with an equivalent concentration of DMSO as a vehicle control for 2 days starting at day 3. We observed
that 5azaC treatment resulted in a substantially increased expression of MCJ protein, and this was to a much greater extent than for complex I component NDUFA9, or complex IV component CoxIV (Fig. 3.2). This was reflected in the MCJ:NDUFA9 ratio, indicating 5azaC treatment did not globally increase mitochondrial proteins (Fig. 3.2).

3.3.3 Glucose utilization increases MCJ protein expression

We directly examined the influence of glycolysis on MCJ expression using 2-deoxy-D-glucose (2-DG), which inhibits glycolysis after its phosphorylation by hexokinase (Wick et al., 1957). We cultured activated CD8+ T cells in IL-2 for 3 days in the absence or presence of 5 mM 2-DG, starting at day 3. Cells cultured with IL-2 plus 2-DG expressed dramatically less MCJ compared to IL-2 alone (Fig. 3.3). Such decreases were not observed for NDUFA9 or CoxIV, as reflected in the MCJ:NDUFA9 ratio (Fig. 3.3). These data demonstrate a link between MCJ expression and glucose utilization in CD8+ T cells.

3.3.4 Reduced glycolysis and caspase-3 activity of T cells from MCJ-deficient mice.

Given the link between glucose utilization and MCJ expression, we measured glycolysis in IL-2-cultured effector CD8+ T cells derived from either MCJ-deficient (MCJ−/−) mice or wild-type (WT) control mice by extracellular flux analysis at day 4. The MCJ−/− T cells were less glycolytic than the WT T cells (Fig. 3.4A). We have previously reported that glycolysis drives caspase-3 activity (Secinaro et al., 2018). Since MCJ−/− T cells were less glycolytic than WT T cells, we hypothesized the MCJ−/− T cells would have reduced caspase-3 activity. Cell lysates were prepared in the presence of biotin-VAD (bVAD), which labels active caspases, on day 6 (Zhou et al., 2006). Active caspases were then precipitated from lysates with avidin-sepharose, and precipitates
immunoblotted for caspase-3 and caspase-8. We observed that MCJ⁻/⁺ CD8⁺ T cells manifested a pronounced decrease in active caspase-3 and a modest increase in active caspase-8 (Fig. 3.4B). CD8⁺ T cells deficient in MCJ have been shown previously to be resistant to cell death during a simulated immune contraction phase using cytokine withdrawal (Champagne et al., 2016). However, the mechanism for this increased survival is not known. Since cell death by either cytokine withdrawal or T cell receptor restimulation is mediated by caspases (Snow et al., 2010), we examined whether this resistance to cell death extended also to restimulation-induced cell death (RICD). We restimulated day 6 effector WT or MCJ⁻/⁺ T cells with anti-CD3 overnight, and measured cell death by LiveDead stain and flow cytometry. This revealed a trend toward a decrease in cell death and caspase activity after restimulation in the MCJ⁻/⁺ T cells compared to WT, though the difference did not reach statistical significance (Fig. 3.4C and D).

3.3.5 Highly proliferative and glycolytic T cells in vivo manifest high levels of MCJ and increased active caspase-3

To examine whether these observations in vitro extend to proliferative glycolytic T cells in vivo, we examined a highly proliferative T cell subset that exists in Fas-deficient lpr mice. CD4⁺CD8⁻ (double negative, DN) T cells accumulate in lpr mice with age and our previous studies have shown that by in vivo BrdU labeling a remarkable 18% of this subset undergoes cell cycling in a 24 h period compared with only about 5-6% of the CD4⁺ and CD8⁺ (B220⁻) T cells from the same mice (Fortner et al., 2017). Consistent with this, we observed that freshly isolated lpr DN T cells were highly glycolytic, as revealed by ECAR, compared to the B220⁻ T cells (Fig. 3.5A). In parallel with their high glycolytic state, the DN T cells also expressed high levels of MCJ compared to the B220⁻
T cell subset (Fig. 3.5B). Similar increases in NDUFA9 or CoxIV were not observed in the DN population, as reflected in the MCJ:NDUFA9 ratio (Fig. 3.5B).

Given the increased glycolysis and MCJ expression in the DN T cells, we examined whether there was also an increase in caspase-3 activity. We first measured caspase-3 activity by flow cytometry and found a five-fold increase in cells positive for active caspase-3 in the DN T cells compared to the B220− T cells (Fig. 3.6A). This was confirmed by bVAD precipitation (Fig. 3.6B). This paralleled an almost two-fold increase in spontaneous cell death in the DN T cells compared to B220− T cells (Fig. 3.6C).

3.4 Discussion

The current findings highlight the coordinated regulation between induction of glycolysis and the negative regulation of mitochondrial respiration and oxygen consumption in the electron transport chain (ETC) in T cells. We show as resting naïve CD8+ T cells are activated and increase glycolysis in the presence of IL-2, they decrease electron transport and oxygen consumption by reducing expression of the ETC complexes. Congruently, expression of the complex I negative regulator methylation-controlled J protein (MCJ) is increased, further reducing electron transport and oxidative phosphorylation (OXPHOS). The presence of MCJ also acts synergistically with glycolysis to promote caspase-3 activation. This likely acts as a safeguard to prevent the accumulation of highly proliferative CD8+ T cells that might cause damage to self-tissues.

The metabolic switch from OXPHOS in naïve and memory T cells to glycolysis in effector T cells is crucial for supporting their rapid proliferation and synthetic capacity.
Under such proliferative conditions, it is important to shunt glucose metabolites into synthetic pathways, such as the pentose phosphate pathway, rather than the Krebs cycle and ATP production by the ETC. It may thus be beneficial in certain instances for glycolytic T cells to actively suppress the ETC by reducing expression of its components as well as upregulating an inhibitor, MCJ. This notion is consistent with the observation that inhibition of glycolysis with 2-DG decreased the MCJ:NDUFA9 ratio.

The expansion of proliferative effector T cells is paralleled by an increase in caspase-3 activity, which sensitizes them to restimulation-induced cell death. We have previously shown that caspase-3 activity is regulated in part by the glycolytic state of the cell, and caspase-3 activity is also reduced in the presence of 2-DG (Secinaro et al., 2018). Activated CD8+ T cells deficient in MCJ manifest increased oxygen consumption compared to wild-type cells, and an increased memory response to influenza (Champagne et al., 2016). This resembles IL-15-cultured T cells, which also have increased oxygen consumption and reduced active caspase-3 compared to IL-2-cultured T cells (Saligrama et al., 2014; Secinaro et al., 2018; van der Windt et al., 2012). As both IL-15 and MCJ deficiency contribute to increased T cell memory, their common reduction in active caspase-3 is likely a significant contributing factor in enhancing their survival.

How IL-15 and MCJ-deficiency might inhibit caspase-3 activity is uncertain at present, but two possible mechanisms are conceivable. One is that the increased oxygen consumption might result in increased reactive oxygen species that could inactivate caspase-3 by glutathionylation or nitrosylation of caspase-3 at the critical cysteine in the enzymatic pocket (Huang et al., 2008b; Mitchell and Marletta, 2005b). We have observed this for IL-15-cultured T cells compared to IL-2-cultured T cells (Saligrama et al., 2014;
Secinaro et al., 2018). A second process would be preventing caspase-3 acylation and hence migration to lipid rafts and its self-activation. In preliminary studies we have observed that IL-2 promotes acylation of caspase-3.

The lpr CD4⁺CD8⁻ (DN) T cells provided a useful *in vivo* source of rapidly proliferating T cells in which to test our *in vitro* observations. We have previously shown using *in vivo* BrdU labeling of proliferating cells that a remarkable 18% of the DN T cells in lpr mice divide within a given 24 h period (Fortner et al., 2017). This extraordinary rate of proliferation was paralleled by a high rate of glycolysis as well as elevated levels of MCJ. The high levels of caspase-3 activity in lpr DN T cells paralleled their rapid cell death *ex vivo*, which was obviously independent of Fas expression since Fas is absent on lpr T cells. This serves to underscore that caspase-3 activity in highly proliferative T cells is likely independent of death receptor ligation. This is also consistent with our previous observations that whereas caspase-3 activity is higher in IL-2-cultured T cells than IL-15-cultured T cells, caspase-8 activity is quite similar, or even slightly higher, in IL-15-cultured T cells (Saligrama et al., 2014). Similarly, in the current studies MCJ-deficient IL-2-cultured T cells manifested as much or more active caspase-8 than wild-type IL-2-cultured T cells, despite MCJ-deficient T cells having greatly reduced active caspase-3. Thus, caspase-3 activity in proliferating T cells is independent of active upstream caspase-8.

The elevated expression of MCJ in glycolytic T cells may serve a variety of critical functions. By inhibiting complex I and electron transport, it may reduce ROS generation, which could reduce ROS-mediated inactivation of caspase-3 (Saligrama et al., 2014; Secinaro et al., 2018) and promote susceptibility to cell death of rapidly
proliferating T cells. Increased MCJ could thus help divert carbon fuel sources away from mitochondrial respiration and toward anabolic pathways, as is seen in actively proliferating IL-2-cultured T cells.

The current findings establish a close linkage between active T cell proliferation, glucose utilization, and activation of caspase-3. That glycolysis promotes activation of selectively caspase-3, whereas oxidative phosphorylation actively inhibits caspase-3 activity through glutathionylation and S-nitrosylation, underscores the tight regulation of caspase-3 activity by the metabolic state of T cells. Such a linkage may represent an adaptation to ensure that proliferating glycolytic T cells do not escape regulation that might promote tumor progression or an autoimmune diathesis. These observations may also be highly relevant to providing optimal numbers of T cells for immunotherapy, such as with chimeric antigen receptor (CAR)-T cells. This requires the ex vivo expansion of very large numbers of T cells often with IL-2, followed by infusion into patients, where there may be negligible amounts of IL-2, which could result in considerable rapid loss of T cells (Geyer et al., 2018; Hollyman et al., 2009; Tumaini et al., 2013). However, reducing caspase-3 activity through the inhibition of MCJ in the infused T cells might considerably augment their survival.

3.5 Materials and methods

Mice

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA), MRL/MpJ-Fas<sup>bt</sup>/J mice (Jackson Laboratory), MCJ<sup>-/-</sup> mice (Hatle et al., 2013), were housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility at the University of Vermont Larner College of Medicine.
Tissues from MCJ\(^{-}\) and C57BL/6J mice were harvested at 2-6 months of age and tissues from MRL/MpJ-Fas\(^{lp}\)/J mice were harvested at 4 months of age. Protocols were approved by the Institutional Animal Care and Use Committee.

**Cell purification**

CD8\(^{+}\) T cells were purified by negative selection from mouse lymph nodes (axillary, inguinal, brachial, cervical) and spleens as described previously (Saligrama et al., 2014). In brief, tissues were homogenized through nylon mesh and Gey’s solution was used to lyse red blood cells. Combined splenocytes and lymph node cells were incubated for 30 min on ice with the following antibodies: anti-MHC class II (M5/114/15/2), anti-B220 (RA3-6B2), anti-CD11b (M1/70), and anti-CD4 (GK1.5). Cells were then rocked with goat anti-rat coated magnetic beads (Qiagen, Germantown, MD, USA) at a 10:1 bead:cell ratio for 45 min at 4\(^{\circ}\)C. Beads and bound cells were removed with a magnet.

CD4\(^{-}\)CD8\(^{-}\) B220\(^{+}\) double negative (DN) and B220\(^{-}\) (CD4\(^{+}\) + CD8\(^{+}\)) T cells were purified from the lymph nodes of MRL/MpJ-Fas\(^{lp}\)/J in the same manner with the following antibodies: DN T cells; anti-MHC class II (M5/114/15/2), anti-CD11b (M1/70), anti-CD4 (GK1.5), anti-CD8 (Tib105), and anti-kappa (187.1). B220\(^{-}\) T cells; anti-MHC class II (M5/114/15/2), anti-CD11b (M1/70), and anti-B220 (RA3-6B2). Wild-type lymphocytes were obtained from C57BL/6J mouse lymph nodes.

**Cell culture**

Naïve CD8\(^{+}\) T cells were cultured in RPMI-1640 (Corning, Manassas, VA, USA), supplemented with 25 mM HEPES, 100 U/mL penicillin-streptomycin (ThermoFisher Scientific, Waltham, MA, USA), 50 μM 2-mercaptoethanol, 5% bovine calf serum (GE Healthcare, Pittsburgh, PA, USA).
Healthcare HyClone, Logan, UT, USA), 2.5 mg/L glucose, 1 mM pyruvate, 2 mM glutamine, and 10 μg/mL folate (RPMI-C), and stimulated on 10 μg/mL plate-bound anti-CD3 clone 145-2C11 (Bio X Cell, West Lebanon, NH, USA) and soluble anti-CD28 ascites clone 37-51 (1:1000), supplemented with 50 U/mL IL-2 (Cetus, Emeryville, CA, USA) at 37°C and 5% CO₂ for 2 days. The activated CD8+ T cells were then removed from stimulation and cultured in RPMI-C and 50 U/mL IL-2 for an additional day. For studies comparing IL-2- to IL-15-cultured CD8+ T cells, the cells were then washed 3 times to remove cytokines and cultured in RPMI-C supplemented with either 50 U/mL IL-2 or 20 ng/mL IL-15 (a kind gift from Amgen, Thousand Oaks, CA, USA) for 3 days or the number of days indicated. For studies of the inhibition of glycolysis, cells were cultured in RPMI-C medium with 50 U/mL IL-2 with or without 5 mM 2-deoxy-D-glucose (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. For studies inhibiting DNA methylation, cells were washed 3 times to remove cytokines and cultured in RPMI-C supplemented with 20 ng/mL IL-15 and 1 μM 5-azacytidine (Sigma-Aldrich) or an equal concentration of DMSO for 2 days.

**Biotin-VAD precipitation of active caspases**

Cells were washed once with PBS/1% BSA, washed again with PBS, then lysed for 30 min on ice in Lysis Buffer supplemented with 20 μM biotin-VAD-fmk (bVAD) (MP Biomedicals, Solon, OH, USA). Protein was quantified by Bradford assay. 400-600 μg of protein in 300 μL of Lysis Buffer was rocked with 40 μL of Sepharose 4B beads (Sigma-Aldrich) at 4°C for 2 hr. The beads were removed and the supernatants were rocked with 60 μL streptavidin-Sepharose beads (ThermoFisher Scientific) at 4°C overnight. The beads bound with active caspases were washed 3 times with Lysis Buffer.
without protease inhibitor, then boiled for 5 min in Laemmli loading buffer supplemented with 2-ME and analyzed by immunoblot for active caspases as described below.

**Immunoblot assays**

Cells were washed in PBS containing 1% bovine serum albumin (PBS/1% BSA), washed again with PBS, then lysed for 30 min on ice in Lysis Buffer (0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM sodium orthovanadate, 10% glycerol, and Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN, USA)). Protein concentration of each lysate was determined by Bradford Assay (Bio-Rad, Hercules, CA, USA). Lysates were boiled in Laemmli loading buffer supplemented with 2-mercaptoethanol (2-ME) for 5 min. Proteins were separated by SDS-PAGE on a 15% acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in 4% milk in Tris-buffered saline with 0.1% Tween-20 (American Bioanalytic, Natick, MA, USA) at room temperature for 1 hr. The following antibodies were used for protein detection: anti-caspase-3, 585 rabbit polyclonal antibody (a kind gift from Dr. Yuri Lazebnik, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA), anti-caspase-8 (a kind gift from Dr. Andreas Strasser, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), anti-MCJ (Hatle et al., 2007), anti-CoxIV (Cell Signaling, Danvers, MA, USA), anti-NDUFA9 (Abcam, Cambridge, UK), anti-β-actin (Sigma-Aldrich), anti-mouse IgG HRP, anti-rabbit IgG HRP, and anti-rat IgG HRP (all from Jackson Laboratory). Densitometry was performed using ImageQuant v8.1 software (GE Healthcare, Chicago, IL, USA).

**Metabolic analysis**
Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were measured with the Seahorse XFe 96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s specifications. Analysis was performed with the Wave Software v2.4 or v2.6 (Agilent Technologies).

Flow cytometry of active caspase-3

Cells were washed in PBS and stained in PBS with Live/Dead Fixable Blue Dead Cell Stain (ThermoFisher Scientific) on ice for 25 min. Cells were washed with PBS/1% BSA and fixed with 2% formaldehyde (v/v) on ice for 15 min. The fixed cells were then washed with PBS/1% BSA and permeabilized with PBS/1% BSA supplemented with 0.03% saponin (PBS/1% BSA/0.03% saponin) on ice for 10 min. Fixed and permeabilized cells were then washed and incubated with anti-cleaved caspase-3 Alexa 647 (Cell Signaling) in PBS/1% BSA/0.03% saponin on ice for 30 min. Cells were washed, fixed in 1% formaldehyde (v/v), and analyzed on an LSRII (BD Biosciences).

Cell death

Restimulation-induced cell death was induced in day 6 wild type (WT) or MCJ⁻/⁻ CD8⁺ T cells by incubation on plate-bound anti-CD3 (10 μg/mL) for 16-18 hr at 37°C. Cells were removed from the plate and stained with Live/Dead Fixable Blue Dead Cell Stain (ThermoFisher Scientific), fixed in 1% formaldehyde (v/v), and analyzed by flow cytometry.

Complex I activity measurement

Cells were washed twice with PBS then resuspended in PBS and assayed for complex I activity using the Complex I Enzyme Activity Microplate Kit (MitoSciences, Eugene, OR, USA) according to the manufacturer’s specifications. 40 – 100 μg of protein
was used for each sample. Complex I rates were calculated per microgram of protein used in the assay.

**Statistical analysis**

Statistical analyses were performed using the graphing software Prism v7 (GraphPad Software, La Jolla, CA, USA). The following statistical tests were used: paired and unpaired t-test when comparing two conditions (e.g. DMSO compared to treatment), one-way ANOVA with Tukey’s test for correction for multiple comparisons when comparing multiple conditions (e.g. naïve compared to Day 2 compared to Day 6) and two-way ANOVA with Sidak test for correction for multiple comparisons when comparing multiple variables across multiple conditions (e.g. wild-type versus MCJ<sup>−/−</sup> with and without anti-CD3 restimulation). All data met the assumptions of the statistical tests used and variation among the compared groups was similar.

**3.6 Acknowledgements**

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**3.7 Author contributions**

MS, KF, MR, and RB designed the experiments presented in this manuscript, which were performed by MS, KF, and RB. MS and RB analyzed the resulting data. MS and RB wrote the manuscript, which was edited by MS, KF, MR, and RB.
3.8 Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
3.9 Figure Legends

Figure 3.1. MCJ expression is upregulated with the induction of glucose utilization in CD8+ T cells. (A-C) CD8+ T cells were activated for 2 days with adherent anti-CD3/CD28, removed and cultured for a third day in IL-2, then washed and cultured for 3 additional days in either IL-2 or IL-15. (A) Relative baseline extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of CD8+ T cells, measured by extracellular flux analysis at days 0, 2, and 6 (one-way ANOVA with Tukey’s correction; mean ± SD; * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.0001; n = 3 independent experiments). (B) Complex I activity in naïve or day 6 IL-2-cultured CD8+ T cells (t-test; mean ± SD; *** indicates p<0.0001; n = 3 independent experiments). (C) Immunoblot and densitometry (normalized to actin) for methylation-controlled J (MCJ) protein, cytochrome c oxidase subunit IV (CoxIV), and actin in whole cell lysates of IL-2- or IL-15-cultured CD8+ T cells, day 0 through day 6 (blot and graphs are representative of 2 independent experiments). Graph of the MCJ:CoxIV ratio (representative of 2 independent experiments).

Figure 3.2. Inhibition of DNA methylation increases MCJ expression in IL-15-cultured CD8+ T cells. Anti-CD3/CD28-activated CD8+ T cells were cultured in IL-15 supplemented with 1 μM 5-azacytidine (5azaC) or an equivalent volume of DMSO for 48 hours. Whole cell lysates were immunoblotted for MCJ, NDUFA9, CoxIV, and actin. Bar graph represents densitometry quantification of MCJ normalized to actin and the MCJ:NDUFA9 ratio (t-test; mean ± SD; * indicates p<0.05; MCJ:NDUFA9 ratio p = 0.1707; n = 3 independent experiments; blot is representative of 3 independent experiments).
Figure 3.3. Inhibition of glycolysis decreases MCJ expression. Anti-CD3/CD28 activated CD8+ T cells were cultured in IL-2 ± 5 mM 2-deoxyglucose for 3 days. Whole cell lysates were immunoblotted for MCJ, NDUFA9, CoxIV, and actin. Bar graphs represent the densitometry quantification of MCJ normalized to actin, and the MCJ:NDUFA9 ratio (t-test; mean ± SD; * indicates p<0.05; ** indicates p<0.01; n = 3 independent experiments; blot is representative of 3 independent experiments).

Figure 3.4. MCJ deficiency reduces glycolysis and caspase-3 activity CD8+ T cells.
Day 2 anti-CD3/CD28-activated wild-type (WT) or MCJ-deficient (MCJ−/−) CD8+ T cells were cultured in IL-2 for an additional (A) 2 or (B-D) 4 days. (A) ECAR was measured by extracellular flux analysis. Bar graph is a summary of 5 samples in one experiment (t-test; mean ± SD; *** indicates p<0.0001; representative of 2 independent experiments). (B) Active caspases were precipitated from WT or MCJ−/− whole cell lysates using biotin-VAD (bVAD). Whole cell lysates and bVAD precipitates were immunoblotted for caspase-8 and caspase-3. Bar graph indicates fold change in active caspase-3 compared to WT (t-test; mean ± SD; *** indicates p<0.0001; n = 3 independent experiments). (C-D) On day 6 WT or MCJ−/− cells were restimulated overnight ± 10 μg/mL anti-CD3. (C) Cell death was measured by Live/Dead staining and flow cytometry (Two-way ANOVA; p = 0.1263; n = 3 independent experiments). (D) Caspase activity was measured by DEVD-rhodamine fluorescence (Two-way ANOVA; p = 0.0912; n = 3 independent experiments).

Figure 3.5. MCJ expression is increased in vivo in highly glycolytic CD4−CD8+ T cells from lpr mice. Freshly isolated CD4−CD8+ double negative (DN) T cells or CD4+ + CD8+ (B220+) T cells from the same lpr mice were purified from lymph nodes by negative
selection. (A) ECAR was measured by extracellular flux analysis. Bar graph is a summary of 5 samples in one experiment (t-test; mean ± SD; *** indicates p<0.0001; representative of 2 independent experiments). (B) Whole cell lysates were immunoblotted for MCJ, NDUFA9, CoxIV, and actin. Bar graphs represent the densitometry quantification of MCJ normalized to actin, and the MCJ:NDUFA9 ratio (mean ± SD; n = 2 independent experiments).

Figure 3.6. Cell death and active caspase-3 are increased in CD4⁺CD8⁻ T cells from lpr mice. Freshly isolated CD4⁺CD8⁻ double negative (DN) T cells or CD4⁺⁺ CD8⁺ (B220⁻) T cells from the same lpr mice were purified from lymph nodes by negative selection. (A) Representative contour plot of flow cytogram for active caspase-3. The box indicates the gate for active caspase-3 based on isotype control staining. Number inserts indicate the percent active caspase-3-positive cells. Bar graph is a summary of quantification of percent active caspase-3-positive cells from 3 samples in one experiment (t-test; mean ± SD; *** indicates p<0.0001; plots and bar graph are representative of 2 independent experiments). (B) bVAD precipitation of active caspases from DN T cells, B220⁻ T cells, and wild-type lymphocytes (WT), immunoblotted for caspase-3. Graph is of the relative densitometry of active caspase-3. (C) Cell death was measured by Live/Dead staining and flow cytometry in DN or B220⁻ T cells after overnight incubation at 37°C (t-test; mean ± SD; *** indicates p<0.0001; bar graph depicts 3 samples from one experiment and is representative of 2 independent experiments).
Figure 3.1: MCJ expression is upregulated with the induction of glucose utilization in CD8⁺ T cells.
Figure 3.2: Inhibition of DNA methylation increases MCJ expression in IL-15-cultured CD8+ T cells
Figure 3.3: Inhibition of glycolysis decreases MCJ expression
Figure 3.4: MCJ deficiency reduces glycolysis and caspase-3 activity CD8+ T cells
Figure 3.5: MCJ expression is increased in vivo in highly glycolytic CD4⁺CD8⁺ T cells from lpr mice.
Figure 3.6: Cell death and active caspase-3 are increased in CD4⁺CD8⁻ T cells from \textit{lpr} mice
3.10 References


CHAPTER 4: DISCUSSION

The work presented in this dissertation aimed to answer two questions related to the links among T cell metabolism, proliferation, and death:

1. Does metabolism regulate caspase activity in effector T cells?
2. Are T cell proliferation and contraction linked?

The results are summarized in the following section, and the implications of those results are discussed in the context of what is currently known. Lastly, some potential future studies are presented as the future directions of this dissertation.

4.1 Summary of Results

The work of this dissertation is presented in full in Chapter 2 and Chapter 3. The findings describe a novel metabolic regulation of caspase-3 activity by glycolysis in proliferating T cells, and thus a metabolic link between T cell proliferation and contraction.

4.1.1 Metabolism regulates caspase activity in effector T cells

The findings presented in Chapter 2 describe the metabolic regulation of caspase-3 activity in proliferating T cells. Glycolysis, which is promoted in proliferating T cells cultured in IL-2, drives caspase-3 activity, and inhibition of glycolysis reduces caspase-3 activity. Caspase-3 clusters in lipid rafts in glycolytic T cells, but not non-glycolytic cells, indicating a potential mechanism through which glycolysis drives caspase-3 activity.

Non-glycolytic T cells cultured in IL-15, which favors oxidative phosphorylation, produce mitochondrial ROS that is responsible for decreasing caspase-3 activity through glutathionylation of a critical cysteine in the enzymatic pocket. Finally, T cells that
proliferate in vivo in response to a flu infection also have increased caspase-3 activity compared to non-proliferating T cells.

**4.1.2 T cell proliferation and contraction are linked by metabolism**

The findings in Chapter 3 describe a connection between proliferation and contraction in T cells. T cell contraction is marked by cell death and a transition from glycolysis to oxidative phosphorylation in the surviving cells. The results show that as glycolysis increases in proliferating T cells, the expression of certain electron transport chain (ETC) proteins decreases whereas the expression of the complex I negative regulator, methylation-controlled J protein (MCJ), increases. As T cells become memory-like, through culture in IL-15, glycolysis and MCJ expression decrease, while the ETC proteins increase in expression. Inhibition of glucose utilization reduces MCJ expression, indicating expression is driven by glucose utilization. T cells deficient in MCJ were found to have a decrease in caspase-3 activity as well as a decreased sensitivity to cell death. Finally, CD4\(^+\)CD8\(^-\) T cells, which are highly proliferative, display elevated levels of glycolysis and MCJ expression.

**4.2 Synthesis of Findings and Implications**

The findings presented in this dissertation help to fill gaps in the overall understanding of how T cells integrate signals from the tissue environment, such as the cytokine milieu, into changes in metabolism, and how those changes in metabolism contribute to the survival or death of the cell. This work has implications not only in autoimmune disease, but also in the immune response to cancer or an infection. In particular, these findings help explain why stronger T cell activation, such as that provided by superantigens like staphylococcal enterotoxin B (SEB), is correlated with
faster proliferation and faster contraction (Weber et al., 2000). Faster proliferation would need a greater induction of glycolysis and, consequently, result in greater caspase-3 activation. The way in which T cells integrate an external signal into a change in metabolism and how metabolism influences cell fate may be more broadly applicable to other cells in the immune system, and even non-immune cells.

4.2.1 Influence of the tissue cytokine environment on T cell metabolism

The cytokine environment is a critical regulator of T cell function. During activation and the effector response, IL-2 supports T cell growth and proliferation. This support is mediated by the induction of glycolysis, which allows for carbons coming from glucose to be used to generate amino acids, nucleotides, energy intermediates such as NADPH, and lipids, all of which are needed as the cell grows and produces effector molecules. IL-15 plays a role in the maintenance of T cells through homeostatic proliferation (Alves et al., 2003). Dysregulation of IL-15 expression can cause autoimmune disease, such as rheumatoid arthritis, in which the synovial fluid is rich in IL-15 and low in IL-2 (McInnes and Liew, 1998a; Waldmann, 2013). Elevated expression of IL-15 could promote excessive homeostatic proliferation and the generation of cytotoxic CD8+ T cells that have the potential to induce autoimmune disease (Alves et al., 2003; King et al., 2004). Targeting the cytokine environment is one potential method to reduce autoimmune disease. For example, anti-IL-15 therapy has been used to treat both rheumatoid arthritis and celiac disease (Baslund et al., 2005; Richmond et al., 2018; Waldmann, 2013).

It is possible that targeting metabolism could be a useful stand-alone treatment or combination therapy for autoimmune diseases. IL-15 induces mitochondrial metabolism
and oxidative phosphorylation, so reducing those metabolic pathways could reverse the survival effect that IL-15 has on T cells. Mitochondrial ROS generation could be targeted with antioxidants such as mitoQ, which could increase caspase activity enough to induce death in the autoreactive cells.

Understanding the relationship between the cytokine environment and T cell metabolism can also assist in finding novel ways to activate Tregs. Whereas IL-15 induces cytotoxic T cell proliferation and survival, Tregs are often inactive or decreased in a number in autoimmune diseases, and promoting Treg function in affected tissues is a potential treatment (Bluestone et al., 2015; Grant et al., 2015). A recent study identified TGF-β as a cytokine that can induce different metabolic states in two Treg subsets; inducible Tregs (iTregs, generated in vitro and similar to peripheral Tregs generated from naïve CD4+ T cells in vivo) and thymic Tregs (tTregs, generated in the thymus during T cell development) (Priyadharshini et al., 2018). Currently, it is thought that tTregs recognize self-peptide:MHC complexes and are important for restraining immune cell migration to within the lymphatic system. iTregs are thought to promote immune suppression during inflammation (Shevach and Thornton, 2014). The study found that tTregs undergo mTOR-dependent glycolysis, which is reduced in the presence of TGFβ. Conversely, iTregs undergo oxidative phosphorylation due to the presence of TGFβ. These data suggest that manipulation of the cytokine environment with TGFβ could induce a metabolic switch and promote tolerance-inducing iTregs.

4.2.2 Mechanisms of metabolic switching

Proliferating T cells are known to induce glycolysis through IL-2 signaling while naïve and memory T cells induce oxidative phosphorylation, which has been linked to IL-
15 signaling (van der Windt et al., 2012). Less is known about the mechanism through which these cytokines induce the corresponding metabolic state, and how T cells can switch between metabolic states as they proceed from a naïve cell to a memory cell. IL-2 signaling activates mTOR, and since mTOR induces the upregulation of glycolysis, this is likely the mechanism through which IL-2 drives glycolysis (Ray et al., 2015). The mechanism through which IL-15 drives oxidative phosphorylation is less clear. The studies presented here provide one piece of that mechanism. Through the methylation of the MCJ gene, IL-15 can increase mitochondrial metabolism and oxidative phosphorylation. This can lead to increased carbon flow through the TCA cycle to support increased ETC activity, which provides a partial explanation for the observed utilization of fatty acids to support oxygen consumption seen in IL-15-cultured T cells (O'Sullivan et al., 2014). The regulation of MCJ expression by IL-15 signaling provides an example for an understudied method through which metabolism can be regulated in T cells. Mitochondrial metabolism can be controlled by changing the expression level of ETC regulators. DNA methylation, specifically of genes related to metabolic pathways, is another understudied but important avenue of investigation that could be very insightful in the context of T cell metabolism. As such, IL-15 may promote regulation of several genes through epigenetic programming.

4.2.3 Linkage of glycolysis, proliferation, and cell death

One gap in knowledge this dissertation aimed to address was a mechanistic explanation for the high degree of sensitivity to death seen in rapidly proliferating T cells (Green et al., 2003; Snow et al., 2010). Glycolysis has now been tied to an increase in
caspase-3 activity that is seen in proliferating T cells, which enhances their susceptibility to cell death.

Glycolysis is a metabolic state common to many proliferating cells, and the upregulation of caspase-3 activity may also be common in other cell types. In fact, intestinal epithelial cells, which renew every 4-5 days, have been shown to become glycolytic as they migrate from the crypt to the tip of the villus (Yang et al., 2016). These cells then undergo apoptosis and are replaced by newer cells. This apoptosis coincides with an increase in caspase-3 activity (Bullen et al., 2006; Grossmann et al., 1998; Gunther et al., 2013). It is possible that glycolysis is directly linked to the caspase-3 activity seen in these cells.

This linkage of glycolysis to proliferation and death may also be relevant in cancer cells, which are highly glycolytic (Warburg, 1956). Glycolysis has even been a recent target of new cancer therapies (Ganapathy-Kanniappan and Geschwind, 2013; Sheng and Tang, 2016). Increased glycolysis could drive caspase-3 activity in cancer cells as they proliferate, similar to an effector T cell. Unlike effector T cells however, cancer cells survive for long periods of time, something that can be partially attributed to ROS generated at the ETC (Sabharwal and Schumacker, 2014). It is possible that these ROS induce the inactivation of caspase-3, despite high levels of glycolysis, reducing the likelihood that a tumor cell will die from caspase-3-induced apoptosis. The link between glycolysis and cell death in cancer cells would be valuable to study and might lead to new therapeutic strategies.
4.2.4 Posttranslational modifications as a way to regulate caspase activity

Caspases are not only important for cell death, but also proliferation, cytokine maturation, B cell cycling, and cell maturation and differentiation (Lamkanfi et al., 2007; Misra et al., 2007). Caspases are zymogens and their activity is typically controlled through the cleavage of the pro-caspase into an active enzyme. The identification of the posttranslational regulation of caspase activity has been a significant advance to the understanding of caspase biology. Redox-dependent posttranslational modifications such as S-nitrosylation and glutathionylation are important modifications for caspases because they target the cysteine residues of proteins, such as the active site cysteine on caspases (Huang et al., 2008b; Mitchell and Marletta, 2005b). While ROS play a role in inducing these modifications in T cells, it is not clear which enzymes are responsible for mediating them. Further studies will elucidate the mechanisms of S-nitrosylation and glutathionylation in T cells and provide new ways to modulate caspase activity through the inhibition or activation of the modifying enzymes.

4.3 Future Directions

The work presented in this dissertation successfully addressed the initial aims of the study. While a novel connection between glycolysis, proliferation, and death in T cells has been identified, more questions can be asked to better understand this important connection. Outlined below are several areas of study that have been opened by this work.
4.3.1 Metabolic state, in vivo proliferation, and sensitivity to cell death of T cell subsets ex vivo

The studies in Chapters 2 and 3 are mostly performed in vitro, though it is important to determine if these results can be corroborated in vivo. The results from the flu infection experiment presented in Chapter 2 show that T cells activated by antigen in vivo do display an increase in caspase-3 activity as they proliferate. It would be interesting to study the connection between glycolysis, proliferation, and caspase-3 activity in T cells that are proliferating homeostatically in naïve mice. The T cell subsets of interest include γδ T cells, NKT cells, and Tregs, all of which homeostatically proliferate more rapidly than most other T cells.

Using flow cytometry, it is possible to study this connection in vivo. Bromodeoxyuridine (BrdU), which incorporates into DNA during DNA synthesis, can be used to measure proliferation of cells in vivo. Glucose uptake would be used as a proxy for glycolysis, and can be measured with a fluorescent version of 2-deoxyglucose called 2-NBDG, which has been used in the past to measure glucose uptake in vivo (Chen et al., 2013). These probes, combined with anti-active caspase-3, can be used to determine the level of caspase-3 activity and glucose uptake of the most proliferative T cell subsets by ex vivo flow cytometric analysis.

4.3.2 Heavy carbon labeling and metabolite tracing

Extracellular acidification and oxygen consumption rates are simple readouts for complex metabolic processes. Glycolysis contributes carbons to many different anabolic pathways in the cell. Oxidative phosphorylation is the culmination of any pathway that
might generate NADH. To truly study the metabolism of a cell, more in depth analysis than extracellular acidification and oxygen consumption rates are needed.

IL-2 and IL-15 induce dramatic changes in T cell metabolism, but the full picture of the metabolites used and generated in the cells is still unknown. Using mass spectrometry, it is possible to identify hundreds of metabolites present within a cell. The next step in the study of the metabolic differences between T cells cultured in IL-2 or IL-15 is to use a mass spectrometry approach to determine the differences in individual or classes of metabolites present. However, this approach returns a snapshot of what was present in the cell at the time of the analysis. Using carbon-13 (13C)-labeled reagents, such as glutamine or glucose, it is possible to identify the metabolites generated from those initial fuel sources. This technique, called heavy isotope labeling or metabolite tracing, allows for the identification of the pathways a cell will use to catabolize the labeled metabolite, and then see where the cell incorporates those carbons. These metabolomic studies are in progress and are summarized in Appendix A.

4.3.3 Mechanism of caspase-3 association with lipid rafts

The identification of caspase-3 in lipid rafts was a reasonable expectation considering there was no difference in upstream caspase activity between glycolytic and non-glycolytic T cells. In glycolytic cells, proximity-induced autoactivation of caspase-3 is more likely to occur when the caspase is clustered in a confined region, such as a lipid raft in the cellular membrane. This raises the question of how caspase-3 initially gets to the membrane. Proteins that lack a transmembrane domain often become associated with membranes through a lipid-based posttranslational modification called acylation. Acylation includes modifications with many lipids or fatty acids including palmitate,
which can be generated by glycolytic cells (Levental et al., 2010; Lunt and Vander Heiden, 2011).

Preliminary studies indicate that palmitate is a major fatty acid produced by glycolytic T cells (Fig. A.7). Future studies could investigate the hypothesis that caspase-3 is acylated in glycolytic T cells and to a lesser extent in non-glycolytic T cells. Acylation of caspase-3 can be detected using a biotin switch technique in which the acyl groups on proteins are replaced with a biotin-conjugated compound, and then precipitated and probed for caspase-3. Expression levels of enzymes involved in acylation could also be measured, along with activity levels. If caspase-3 is acylated in glycolytic T cells, then blocking fatty acid production and/or acylation should result in a decrease in caspase-3 localization to lipid rafts and activation. These studies would provide further insight into acylation as a method of regulating caspase activity and would link acylation to the metabolic state of T cells.

4.3.4 Mechanism by which MCJ promotes caspase-3 activity

Glycolysis and MCJ expression are both linked to activation of caspase-3. While glycolysis is known to lead to the localization of caspase-3 in lipid rafts to induce activation, it is not known how MCJ drives caspase-3 activity. It is possible that MCJ acts to prevent the S-nitrosylation and glutathionylation of caspase-3 by reducing complex I activity and subsequently mitochondrial ROS generation. If this is true, then the MCJ-deficient cells should have increased mitochondrial ROS as well as S-nitrosylation and/or glutathionylation of caspase-3. Reducing mitochondrial ROS with mitoQ could then increase caspase activity. It is also possible that the expression of MCJ increases glycolysis, which would promote caspase-3 activity through the glycolysis-related
mechanisms. In this case, the MCJ-deficient cells should have less active caspase-3 present in lipid rafts. Discovering the mechanism of MCJ induction of caspase-3 activity will provide evidence for another non-caspase-mediated method of caspase activation and a better appreciation for the complexity of caspase regulation in T cells.
4.4 References


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APPENDIX A: METABOLOMICS AND METABOLITE TRACING IN T CELLS
A.1 Introduction

Immunometabolism is now recognized as an important factor in understanding the function of immune cells. As was outlined in Chapter 1, different metabolic pathways are important at different points in time during an immune response. This has led to a greater desire for more detail of the levels of actual metabolites in immune cells at various points in an immune response, and for the kinetics and fate of whether these metabolites go in a pathway. Thus, as part of our studies of the metabolism of T cells cultivated in IL-2 versus IL-15, we conducted metabolomics studies in these two T cell populations.

We have shown that IL-2-cultured effector T cells utilize glucose and reduce OXPHOS, whereas IL-15-cultured memory-like T cells manifest the opposite metabolic profile. We thus aimed to better understand how these two T cell populations utilize fuel sources. We initially used mass spectrometry to measure the static differences in metabolites produced in IL-2-cultured versus IL-15-cultured T cells. We then performed kinetic studies using $^{13}$C-glucose or $^{13}$C-glutamine labeling to follow the kinetics and path of carbons from these two fuel sources.

A.2 Results and Discussion

We measured 184 metabolites by mass spectrometry (Table A.1, Fig. A.1). As IL-15-cultured cells are smaller than the IL-2-cultured cells (and the mass spectrometry was performed on an equivalent number of cells), so we reasoned that any metabolites that appeared in a higher amount in the IL-15 cells compared to IL-2, were likely to be significant for IL-15-driven metabolism. Two metabolites were identified: L-glutamine and D-glucose. For most of the metabolites, the IL-2-cultured CD8$^+$ T cells contained the greatest amount of these metabolites.
Given the known higher level of OXPHOS in IL-15-cultured T cells, we hypothesized that the high level of glutamine in the IL-15-cultured T cells was a result of the need to fuel the TCA cycle with glutamine. However, the high level of glucose in the IL-15-cultured T cells was unexpected. This static metabolic picture suggested that IL-15 T cells either took up glucose but did not use it, hence its accumulation, or that it might utilize glucose for pathways other than glycolysis to lactate production (which is low in IL-15 T cells). To better track the kinetics and fate of glucose and glutamine carbons we used $^{13}$C-glucose and $^{13}$C-glutamine labeling. We added the $^{13}$C-glucose or $^{13}$C-glutamine to glucose- or glutamine-free media and cultured the cells for 6 and 24 hours (for the IL-15-cultured CD4$^+$ T cells, only the 6-hour time-point was measurable). Key metabolites in the glycolysis pathway and the TCA cycle, as well as some amino acids, were measured for the presence of $^{13}$C (e.g. M+0 representing no labeled carbons and M+3 representing 3 labeled carbons), indicating the use of either glucose or glutamine to make those metabolites.

In the $^{13}$C-glutamine tracing experiments, the presence of $^{13}$C in the metabolites of the TCA cycle indicated that glutamine is used by both IL-2-cultured and IL-15-cultured T cells to generate glutamate, and subsequently fuel the TCA cycle (Fig. A.2), which is a known use of glutamine in cells. There was a moderate amount of labeling in glutathione and aspartate in both cultures (Fig. A.3). The $^{13}$C-glucose tracing experiments revealed that glucose is catabolized in both the IL-2-cultured cells and the IL-15-cultured cells, though lactate was produced almost exclusively by the IL-2-cultured cells (Fig. A.4). Glucose was used to generate metabolites of the TCA cycle in each of the cell cultures (Fig. A.5), though at a low level. We also found glucose to be used by the IL-2-cultured
T cells to generate certain amino acids such as alanine and serine, but not by the IL-15-cultured T cells. The exception was in cysteine, which was generated from glucose in each culture (Fig. A.6). Most striking was that when tracking the labeled carbons from $^{13}$C-glucose they appeared to disappear in the IL-15-cultured T cells before entry into the TCA cycle. Thus, if glucose was not being metabolized to lactate or the TCA cycle in IL-15 T cells, it suggested that the carbons were being diverted to another pathway. Due to the targeted measurements that were made, we were unable to determine if these carbons entered any pathways that branch off glycolysis, such as the pentose phosphate pathway or incorporation into lipids. These will be pursued in future studies.

In a separate set of analyses, we were interested in the generation of lipids and fatty acids in IL-2-cultured and IL-15-cultured T cells, as IL-15-cultured T cells are known to use fatty acids to fuel the TCA cycle, and IL-2-cultured T cells may use fatty acids such as palmitate to tether caspase-3 to lipid rafts. We measured fatty acids and lipids by mass spectrometry in CD$^4^+$ and CD$^8^+$ T cells cultured in IL-2 or IL-15. We observed that triacylglycerides (TAG) were produced in similar amounts between IL-2-cultured and IL-15-cultured T cells, with the CD$^8^+$ T cells producing the most (Fig. A.7). Palmitate (16:0) and linoleate (18:0) were favored among the medium-chain fatty acids, with the IL-15-cultured cells favoring linoleate with 2 unsaturated double bonds (18:2) (Fig. A.6). Palmitate can be used to modify proteins by acylation, which could be used by glycolytic cells to modify caspase-3 for localization in lipid rafts. Diacylglycerides were found at a higher level in IL-2-cultured cells compared to IL-15 (Fig. A.8). Of the glycerophospholipids, phosphatidylcholine was found at a greater amount in the IL-2-cultured T cells, while cardiolipin was found at a greater amount in CD$^8^+$ T cells,
compared to CD4+ (Fig. A.9). IL-15-cultured T cells contained a greater amount of sphingomyelin compared to IL-2 (Fig. A.10). Together, these data suggest a preference for different lipids between IL-2-cultured T cells and IL-15-cultured T cells. A kinetic approach to determine the metabolites used to generate those lipids will enable us to better understand the role of lipid metabolism in effector function and survival in T cells.
Figure A.1: Heat map of metabolite presences from metabolomics screen
Figure A.2: TCA cycle intermediates from $^{13}$C-glutamine tracing
Figure A.3: Other metabolites detected from $^{13}$C-glutamine tracing
Figure A.4: Glycolysis intermediates from $^{13}$C-glucose tracing
Figure A.5: TCA cycle intermediates from $^{13}$C-glucose tracing
Figure A.6: Other metabolites of interest from $^{13}$C-glucose tracing
Figure A.7: Triacylglycerides detected in lipidomics screen
Figure A.8: Diacylglycerides and MADAGs detected in lipidomics screen
Figure A.9: Glycerophospholipids detected in metabolomics screen
Figure A.10: Other lipids detected in lipidomics screen
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