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Unraveling the Role of DRAK2 in Glycolytic T cell Differentiation

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Kelley Fracchia

Dissertation Committee:
Professor Craig Walsh, Chair
Professor Matt Inlay
Professor Olga Razorenova

2018

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ABSTRACT OF THE DISSERTATION

Unraveling the Role of DRAK2 in Glycolytic T cell Differentiation

By

Kelley Fracchia

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Craig M. Walsh, Chair

DRAK2 is a serine/threonine kinase that is differentially expressed during lymphoid development. *In vivo* mouse models for multiple sclerosis and type 1 diabetes have shown that DRAK2-/- mice display diminished autoimmune susceptibility. Given that DRAK2 acts as a negative regulator of T cell activation, research was been geared toward the mechanism through which it alters autoimmunity. The work in this dissertation was proposed to investigate the mechanism by which DRAK2 alters T cell function and development.

Understanding the mechanisms that control T cell differentiation is crucial to develop novel therapeutic strategies to prevent autoimmune disease. Upon activation, T cells undergo metabolic reprogramming and different T cell subsets utilize selective metabolic pathways. The reason for this reprogramming is that unlike a naïve cell, activated T cells need to generate the necessary biomass and intermediates needed for rapid cell division. Hitherto, the regulating mechanisms promoting the switch from oxidative to glycolytic metabolism have remained elusive. We found that upon SEB stimulation, DRAK2 induces mitochondrial permeability transition pore activity, which

allows T cell proliferation and subsequent glycolytic differentiation. This work provides important mechanistic insight into the transient regulation of the mitochondrial permeability transition pore and is the first to identify that this activity can be modulated during metabolic differentiation. Importantly, we show that loss of DRAK2 prevents the expansion of glycolytic cells and that appropriate signal strength, in the context of CD28 costimulation, is sufficient to overcome the characteristic survival defect. These data suggest that CD28 seems to be an important parallel pathway in terms of survival but not necessarily in terms of metabolism. Overall, this work examines the metabolic dysregulation that occurs following loss of DRAK2 in T cells and provides insight into how proliferation and glycolytic differentiation are initiated following activation.

CHAPTER 1

INTRODUCTION

Abstract

While simultaneously maintaining homeostasis and reducing further harm to the host, the immune system is equipped to eliminate both tumors and pathogenic microorganisms. Bifurcated into cell-mediated and humoral immunity, the adaptive immune system requires a series of complex and coordinated signals to drive the proliferation and differentiation of appropriate subsets. These include signals that modulate cellular metabolism. When first published in the 1920s, “the Warburg effect” was used to describe a phenomenon in which most cancer cells relied on aerobic glycolysis to meet their biosynthetic demands. Despite the early observations of Warburg and his colleagues, targeting cancer cell metabolism for therapeutic purposes still remains theoretical. Notably, many T cells exhibit the same Warburg metabolism as cancer cells and the therapeutic benefit of targeting their metabolic pathways has since been reexamined. Emerging evidence suggests that specific metabolic alterations associated with T cells may be ancillary to their subset differentiation and influential in their inflammatory response. Thus, T cell lymphocyte activation leads to skewing in metabolic plasticity, and issue that will be the subject of this chapter.

T cell Activation

The naïve T cell pool is comprised of diverse repertoire of unique T cell antigen receptors (TCRs), all of which have a discrete affinity for a given MHC plus antigenic peptide. The primary function of these T cells is to survey secondary lymphoid tissues for the presence of stimulatory antigens, antigens that are typically associated with microbial infections or oncogenically transformed tissues. Upon binding to antigen presenting cells presenting stimulatory antigenic peptides, a series of biochemical signaling events occur within the T cell, ultimately culminating in the proliferation and differentiation of effector cells.

Although TCR ligation on its own is able to activate initial signaling and gene expression changes, a second costimulatory signal is putative for an appropriate response [1]. A vital costimulatory signal is provided through CD28, and although once thought to initiate a distinct signaling pathway has since been shown to alter those initiated by TCR activation [1, 2]. Of interest here, CD28 has been shown to increase P13K/Akt signaling, cytosolic free calcium, and activation of a variety of transcription factors including Jnk, Ras, and MAPK [1, 2]. CD28 has also been shown to impact the production of various effector cytokines such as IFN-gamma and IL-4, thus influencing Th1 and Th2 subsets, respectively [3-5].

Following T cell engagement in the context of proper costimulation, T cells are induced to differentiate, proliferate, and acquire specialized effector functions. These activities require energy, both in the form of growth but also for the engagement of biosynthetic machinery necessary for survival. To fulfill the biosynthetic and metabolic demands necessary for clonal expansion, thymocytes undergo extensive reprogramming; those unable to meet these energetic demands succumb to apoptosis. Emerging evidence

over the past several years reveal specific signal transduction pathways downstream of the TCR that couple biomass synthesis to metabolic regulation. Resting T cells are essentially quiescent, and maintain a basal level of nutrient uptake, a process that prevents cell death [6-8]. Basal glucose uptake is split between various metabolic pathways including oxidation, lactate production, and glycosylation. Interestingly, resting T cells are able to sustain majority of their ATP production from the TCA cycle but following activation shift towards glycolysis. In this sense, resting T cells employ a process that results in highly efficient energy utilization, thus providing a means to maintain a very large naïve T cell pool.

Proliferating T cells exhibit considerably different metabolic requirements to their respective quiescent or resting precursors. For example, in order to support their high rates of growth and proliferation, T cells must consume additional nutrients and divert metabolites into macromolecular synthesis pathways. Metabolic pathways are therefore rewired in a fashion that helps to maintain a balance between sufficient ATP production and biomass processes. With T cell activation, glucose serves as a critical metabolite and primary substrate for ATP generation. It has been documented in many studies that removing glucose, even when other metabolic substrates are available, is sufficient to inhibit T cell proliferation and cytokine production [9]. The high dependence on glucose uptake and usage in activated T cells is also important for their eventual conversion into effector cells [10]. While this high dependence on glucose in activated effector T cells may seem energetically wasteful, the need to control rapidly emerging microbial infections places significant energy demands on clonal expansion and effector cell differentiation. This may explain why T cells needs are known to divide at an extremely high rate, with

roughly a single cell division occurring on the order of every 4-6 hours[11]. Here, we review the recent progress supporting the differential metabolic phenotypes within effector T cell lineages. We also discuss the overlap between specific metabolic enzyme targets and how they might be influenced by T cell activation. Although many questions remain to be answered, the substantial recent progress in our understanding of these molecular events holds tantalizing therapeutic targets that have yet to be exploited.

Glucose Transport & Secondary Signals influencing Transporter Expression

Glucose Transport

In order to sustain the demands associated with T cell activation, T cells must engender increased transport of glucose. Facilitative glucose transporters (GLUTs) allow for the diffusion of glucose, down its concentration gradient, across the lipid bilayer. To date, 14 members of facilitative glucose transporters have been identified all of which belong to the solute-linked carrier gene family SLC2A [12, 13]. The first to be identified, GLUT1, is the most ubiquitous vertebrate glucose transporter, and is also the primary glucose transporter found on lymphocytes [8, 14]. In T lymphocytes, the rate-limiting step for glucose metabolism is often the transport of glucose via GLUT1, since GLUT1 transporters are saturated at or near physiological glucose concentrations [15]. As a result, understanding the molecular events that regulate GLUT1 cell surface expression is necessary to fully appreciate the alteration of metabolism following naïve T cell stimulation. Because activated T lymphocytes do not store large quantities of carbon substrates, they must import glucose through GLUT1 to accommodate their metabolic demands [16]. In resting lymphocytes, GLUT1 cell surface expression is undetectable, but

upon T cell stimulation, GLUT1 quickly relocates to the surface and serves as a marker of lymphocyte activation [8, 17, 18]. One factor known to have a major role in regulating glycolysis in T cells is Myc. Myc promotes the up-regulation of GLUT1 and stimulates glycolysis through the expression of various glycolytic enzymes, including hexokinase 2, lactate dehydrogenase A (LDHA) and phosphofructokinase [19]. However, in order to achieve maximal glucose uptake, T cells must receive a co-stimulatory signal or else they will become anergic, a quiescent state refractory to subsequent antigenic stimulation [9]. If a CD28 costimulatory signal is encountered, then increased GLUT1 expression results via the induction of the PI3K/Akt pathway [9]. As seen in Glut1/mAkt double transgenic mice, CD4+ T cells with constitutively active Akt displayed enhanced surface GLUT1, and the resulting peripheral T cells had a marked increase in cell size, glucose uptake and activation marker expression relative to single-transgenic mice [9].

Glucose transporter localization and expression is influenced in a variety of ways depending on the cell type and stimulus encountered. Upon its entrance into the cytoplasm, glucose is phosphorylated by hexokinase and the resulting glucose-6-phosphate (G6P) is then processed through a variety of metabolic pathways, including the pentose phosphate pathway (PPP) or glycolysis. Given the intricate maintenance involved in glucose metabolism and the implication in the regulation of cell survival, researchers alike have begun to examine transporter expression in response to hormones and insulin release.

GLUT1 expression in response to insulin

Similar to GLUT1 expression, resting CD4+ and CD8+ T cells do not express the insulin receptor and as a result, are insensitive to fluctuating insulin, although this is reversed

following T cell activation [20, 21]. Upon expression and phosphorylation of the insulin receptor substrate-1 (IRS-1), T cells increase glycolytic and pyruvate flux and increase their uptake of glucose and amino acids [20, 22, 23]. Viardot and colleagues found that insulin signaling specifically promoted the differentiation of Th2 CD4+ T cells (20). Although there had been no evidence for a role of insulin signaling regulating T cell differentiation prior to this report, it had been well documented that insulin can activate some MAPKs including ERK [24]. Given that ERK serves as an important regulator in Th2 T cell differentiation, Viardot and colleagues were interested to determine if altered T cell polarization is associated with increased ERK phosphorylation [25, 26]. They attributed the phenotypic switch towards the Th2 lineage as an important immunomodulatory effect, which is in accord with the known anti-inflammatory effects of insulin [27-29]. It is important to note that the T cell metabolism is modulated not only by TCR stimulation and insulin but also by other signals including systemic hormones and cues from the local microenvironment.

T cell function in response to leptin

Leptin is a well-established adipokine influencing appetite control and energy expenditure, primarily through its actions within the hypothalamus [30, 31]. Leptin has been found to impact glucose homeostasis in T cells, with recent studies demonstrating that fasting induced leptin expression led to diminished T cell numbers in the periphery relative to the mice receiving a normal feeding schedule [21]. Of relevance here, leptin promotes Th1 and Th17 lineage differentiation while simultaneously inhibiting Treg expansion [21, 32, 33]. As shown in both mouse and human studies, leptin deficiency has been associated with decreased immune reactivity, suggesting the hypothesis that leptin has a dual role in

regulating not only overall systemic immunity but also T cell homeostasis and function [34, 35]. While little is yet understood with respect to leptin and adaptive immunity, linking these will likely be important for improving therapeutic approaches that target inflammation.

Relationship between the metabolic state & activity of T cells

Glycolysis

Upon entering the cytoplasm, glucose can either be metabolized through the PPP or via glycolysis. Glycolysis is the process whereby glucose is broken down into two units of pyruvate, which can be further metabolized. In the presence of oxygen, the majority of differentiated cell types utilize the metabolism of glucose through glycolysis and the resulting pyruvate is further oxidized in the TCA cycle [36]. However, when oxygen is limited, mitochondrial oxidative metabolism is restricted and the pyruvate is preferentially converted into lactate. It is interesting that both tumor cells and proliferating T cells adopt aerobic glycolysis, an unusual metabolic profile in which cells preferentially utilize glycolysis to generate ATP, even when oxygen is not limiting [37, 38].

Following its initial description in 1928 by Otto Warburg [39, 40], the term the Warburg effect (e.g. aerobic glycolysis) has been used to describe the upregulation of glycolysis even in the presence of oxygen [36]. Despite the more rapid turnover of ATP via glycolysis when compared to oxidative phosphorylation, as depicted in Figure 1.1, aerobic glycolysis is much less efficient in terms of the amount of ATP generated per molecule of glucose consumed. As a result, a shift from respiration to aerobic glycolysis ensures that activated T cells meet their increased energy and biosynthetic demands during clonal

expansion [41]. Although less efficient, glycolysis does allow glucose to be metabolized into biosynthetic precursors [36]. Given that the cell requires an extensive supply of these precursors to quickly replicate its entire biomass, it is not surprising that the glucose molecules can be diverted into additional pathways such as the PPP to produce the necessary co-factors such as acetyl-CoA and NADPH [42, 43].

The Pentose Phosphate Pathway (PPP)

The PPP is crucial for cell survival as it helps to synthesize reducing equivalents and it also provides the metabolic precursors for amino, nucleic, and fatty acid synthesis. During the oxidative portion of the PPP, G6P is converted to ribulose-5-phosphate (R5P), with the production of two molecules of NADPH. Although other metabolites are generated during the PPP, including ribose, which itself can be broken down to generate nucleotides, the production of NAPDH is considered to be the most important byproduct. Not only does NADPH aid in anabolic biosynthesis reactions, particularly through the promotion of fatty acid chain elongation, but it also acts a crucial antioxidant and aids in scavenging ROS produced during cellular expansion. In this respect, NADPH helps to reduce both glutathione (GSH) and thioredoxin (TRX), both of which scavenge ROS and help repair DNA damage induced by reactive oxygen species (ROS) [44]. If glucose is readily available for metabolism, then undoubtedly some of it is redirected towards the PPP in order to sustain increased amino acid, lipid and nucleotide synthesis. It is important to note that alterations in T cell metabolism are not unidirectional or permanent. Indeed, the changes are quite reversible, as is seen in memory T cells that revert from a glycolytic metabolism towards respiration [45, 46]. If glucose is not readily available, T cells prioritize the production of

ATP and biomass synthesis as seen in the preferential use of glycolysis and TCA cycle over the PPP [16].

The tricarboxylic acid (TCA) cycle

The mitochondrion has long been identified as a source of cell survival, energy, and the site of oxidative phosphorylation. The generation of ATP is promoted by an electrochemical gradient across the inner mitochondrial membrane and is sustained through the electron transport chain (ETC) [47]. The TCA cycle serves to rearrange the carbon backbone of pyruvate and concomitantly releases ATP, CO2, FADH2, GTP, NADH, and various metabolites. However the maximum production of ATP does come at a cost, and cost is in the form of free radicals released by the ETC that react oxygen and form ROS. Although ROS have long been considered to be toxic, accumulating evidence suggests their role in signal-transduction pathways, including calcium signaling and apoptosis [43, 48, 49].

Given the multifaceted function of the TCA cycle and its byproducts, many questions have arisen as to how the cell controls its metabolic phenotype. Inhibition of oxidative phosphorylation, using either cyanide or carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) has been shown in both epithelial and cardiac cells to increase GLUT1 mediated transport independent of GLUT1 gene expression, localization or protein level [50, 51]. In addition to its potential role in glucose transport, oxidative phosphorylation (OXPHOS) has been found to be important for T cell activation. Chang and colleagues demonstrated that upon treatment with oligomycin, an inhibitor of ATP synthase, T cells failed to express activation markers and experienced reduced

proliferation [52]. This observation suggests that T cell activation and optimal proliferation require ATP derived from mitochondrial OXPHOS.

Glutaminolysis

In addition to glucose metabolism, T cells utilize glutaminolysis to meet their increased bioenergetic and biosynthetic demands [53, 54]. Although the mitochondrial pool of acetyl-CoA is mainly derived from the breakdown of glucose, glutaminolysis serves to replenish intermediates of the TCA cycle that are redirected into biosynthetic reactions, a process known as anaplerosis [55]. In order to accomplish this, the cell allows glutamine to be enzymatically converted into glutamate, which can be directly processed into GSH or to α-ketoglutarate (αKG) and can then enter the TCA cycle [56]. One transcription factor known to indirectly increases the level of glutaminase 1 (GLS1), the first enzyme of glutaminolysis, is Myc [57].

The sustained upregulation of Myc following TCR ligation, has been shown to be dependent on TORC1, and the associated increase in metabolic enzymes can further influence the AMPK-mTOR signaling pathway in a feed-forward manner [54]. In Myc-deficient T cells, both glycolysis and glutaminolysis are markedly decreased and the addition of nucleotides and polyamines to these cells only promotes a partial rescue [54]. Interestingly, when CD4+ T cells are treated with the OXPHOS inhibitor myxothiazol and simultaneously stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, there appears to be no inhibition of proliferation or cytokine production [58]. Collectively, these results suggest that glutaminolysis is vital for activated T cells, perhaps by facilitating the synthesis of biomass precursors rather than for oxidative ATP production. The exact

mechanisms regulating the fate of glutamine in T lymphocytes are not yet fully understood, but it is plausible that microenvironmental factors have an influence.

Microenvironment and associated effects on T cell metabolism

In addition to the genetic changes that alter T cell metabolism, the immune microenvironment plays a role in determining the metabolic phenotype of effector T cells. For example, glucose starvation has been shown to inhibit IFN-gamma, but not IL-2 production; these findings support the notion that while T cells are able to grow and proliferate without glucose, their acquisition of effector functions may be limited [59]. Given that T cells need to migrate to sites of inflammation that vary in abundance of nutrients and free oxygen, metabolic plasticity is paramount if the cell is to survive.

ROS and Oxidative Stress

Upon TCR cross-linking, mitochondrial ROS are generated within 15 minutes [60, 61]. As a result, the increased biosynthetic activity of activated T cells requires not only enhanced uptake of glucose, but also a complementary increase in NADPH production to maintain cellular redox balance. At low levels, ROS have been associated with increased cell proliferation and survival, primarily via their ability to cause post-translational modifications on kinases and phosphatases [62, 63]. These low levels of ROS are in part maintained by NADPH, NADPH oxidase and antioxidants, but upon accumulation, these ROS influence gene expression and can lead to DNA damage, permeabilization of mitochondrial membranes and apoptosis. The superoxide generated from the mitochondrial electron transport chain [47] through complexes I, II, and III is one of the

primary sources of free radicals generated in cell [64]. Upon imbalance between these mitochondrial produced superoxide and insufficient clearing of ROS oxidative stress is provoked in the cell.

HIF-1α and Hypoxia

The hypoxia inducible factors (HIFs) play a well-established role in regulating oxygen homeostasis and of these, HIF-1α is considered to be one of the master transcription factors regulating the expression of glycolytic enzymes. As outlined in Figure 1.2, the induction of HIF-1α transcription is attributed to the activation of STAT3 by IL-6 [54]. HIF-1α acts to repress mitochondrial function and oxygen consumption by inducing pyruvate dehydrogenase kinase 1 (PDK1), a molecule that promotes inhibitory phosphorylation of pyruvate dehydrogenase (PDH) [65]. Under normoxic conditions, HIF-1α is targeted for proteosomal degradation through interactions with Von Hippel Lindau (VHL) [66]. However, VHL fails to bind to and promote the degradation of HIF-1α with hypoxia, and upon translocation to the nucleus, promotes the upregulation of glycolytic genes. Importantly, activation of CD4+ and CD8+ T cells leads to a strong transient induction of HIF-1α [67]. HIF-1α is also known to promote the function of RORγt while simultaneously binding Foxp3 and targeting it for proteosomal degradation [68]. Since Foxp3 is a key transcription factor for regulatory T cell (Treg) induction, the degradation of Foxp3 mediated by HIF-1α binding likely influences subsequent effector lineage differentiation [67, 69]. Thus, it is thought that Th17 T cells result from strong induction of HIF-1α, whereas Treg differentiation is favored in cells with low HIF-1α expression [70, 71]. mTORC1 also influences HIF-1α activity by promoting the translation of the respective

messenger RNA [72]. As described in greater detail below, mTORC1 signaling is intimately involved in glycolytic function, as well as in effector T cell differentiation. The relative contributions of mTORC1 and HIF-1α to T cell metabolism and differentiation are currently an area of intense focus.

PI3K/Akt

Crucial for the upregulation of glycolysis, the PI3K/Akt signaling pathway has been extensively studied in lymphocytes [73]. The establishment of a cellular contact between antigen/MHC to the TCR complex results in the rapid elevation of PI(3,4,5)P3, subsequent stimulation of PI3K, and recruitment of key signaling intermediates which are themselves activated [74]. Akt, is one such key effector molecule that is activated in this manner and upon interaction with the PH domain of Akt, PI(3,4,5)P3 has been shown to induce a conformational change that allows Akt to be phosphorylated on threonine 308 and serine 473 by phosphoinositide- PDK1 and mTORC2 [75].

PI3K and Akt have both been implicated in the upregulation of glucose metabolism via the induction glycolytic enzymes, in part due to TCR-mediated activation of mTORC1 and Myc expression [16, 41, 54, 76, 77]. Through sustained activation of Akt, T cells upregulate surface expression of GLUT1 and increase the activity of mitochondria-associated hexokinase [78]. In the absence of PI3K, constitutively active Akt enhances glucose metabolism, supporting the hypothesis that Akt activation downstream of PI3K is a crucial determinant in the enhanced glycolytic flux observed in activated T cells [16]. Lastly, in mice expressing a catalytically inactive subunit of PI3K, it has been noted that although there is a minor impairment in the immune response, PI3K deficient T cells have

only a mild defect in their growth and proliferation [79]. In contrast to the above findings, when treated with PI3K inhibitors T cells are prevented from up-regulating glycolysis [41]. However, due to the complexities of pharmacokinetics and the use of the PI3K inhibitor LY29002 (recently found to be rather non-specific), the direct connection between PI3K signaling and glycolytic upregulation has been called into question [80, 81]. Collectively, these results suggest that PI3K is not solely responsible for metabolic control mediated by Akt following antigenic stimulation of T cells. While the specific means by which PI3K and Akt influence glucose metabolism is still being explored, loss of PI3K signaling has revealed rather unanticipated results, the relative involvement of PI3K, mTORC1 and Akt in this process remains an important question.

mTOR

Both mTORC1 and mTORC2 are activated within minutes of TCR stimulation, with the magnitude of mTOR activation directly correlated to the duration of TCR engagement and TCR:MHC-peptide binding kinetics [82, 83]. The activity of mTOR is further modulated by CD28, as this costimulatory signal activates the PI3K-Akt pathway [84-86]. Amongst the plethora of functions regulated by mTOR, it plays a pivotal role in orchestrating cellular metabolism. Emerging evidence suggests that mTOR acts as a crucial environmental sensor, and serves to limit and/or defer signaling depending on the availability of nutrients and amino acids [87]. As the role of mTOR in metabolism has been extensively detailed in recent reviews, the authors wish to redirect the reader to such resources [88-91]. Of interest to this dissertation, mTORC1 has roles in regulating glucose metabolism independent of PI3K and Akt within specific effector subsets [92, 93].

Effector and memory T cells perform different functions

As essential players in the adaptive immune response, T lymphocytes have evolved to rapidly combat invading pathogens. Upon infection, responding T lymphocytes give rise to two distinct classes of cellular progeny, effector cells and long-lived memory cells. Each with unique functions, these T cells mediate acute host defense and provide long-term immunity. The balance between protective immunity and suppression of autoreactivity and chronic inflammation requires that T cells appropriately differentiate into effector and regulatory subsets. However, the provenance of these distinct T cell subsets remains an active area of investigation.

CD4+ T Cells

As previously mentioned, T cells acquire their own specific homing and cytokine secretion profiles depending on the environmental signals each receive. While once thought to be immutable, CD4+ T cell differentiation is now considered to be quite dynamic and T cells are able to re-differentiate upon environmental stimuli [94]. Following activation and differentiation, the pro-inflammatory CD4+ T cell subsets are currently categorized as Th1, Th2 or Th17 while the anti-inflammatory subset is referred to as induced Treg (iTreg). As highlighted in Figure 1.3, each of these subsets bears an transcription factor, and of recent appreciation, metabolic signature. Given these trends, research has focused on the hypothesis that a hallmark of inflammatory cells might indeed be a shift towards high glycolysis [95].

Although it is unclear whether cellular metabolism controls T cell lineage decisions, it has been reported that the glycolytic pathway is regulated during Th17 and Treg

differentiation [70]. Shi and colleagues demonstrated that upon skewing towards the Th17 lineage, strong up-regulation glycolytic activity was observed, and this was absent under Treg skewing conditions. Glycolytic blockade imposed on Th17 cells via 2-deoxyglucose (2DG) treatment led to enhanced generation of Tregs and this apparent lineage switch could be inhibited by treatment of the fatty acid oxidation (FAO) inhibitor etomoxir [67]. This inherent plasticity of T cells serves as a noteable result and suggests that the metabolic cues can perhaps be manipulated to favor specific effector lineage differentiation.

Spurred on by these findings and others, Powell and colleagues reported that mTOR activation is essential for the differentiation of unique effector T cell lineages. Using a T cell specific mTOR knockout model, they observed a defect in Th17 development and a concomitant bias towards the Treg differentiation [96]. Similarly, upon treatment using the mTOR inhibitor, rapamycin, Peng et al. [97] demonstrated how T cells up-regulated genes involved in β-oxidation and down-regulated those associated with lipid, nucleotide and protein synthesis. Given that rapamycin has long been used to restrict the progression of allograft rejection [47], these findings are consistent with previous work demonstrating how this powerful immunosuppressant facilitates the generation of CD4+/CD25+/Foxp3+T cells [98]. It has also been proposed that mTOR utilizes two distinct signaling pathways when dictating T helper cell differentiation, TORC1 for Th1 and Th17, and TORC2 for Th2 cell differentiation [99]. These studies collectively provide a critical link between T cell differentiation and metabolism.

Given these findings, it is proposed that the relative fraction of glucose versus fatty acid substrates within the inflammatory microenvironment may help to shape the immune

response. These microenvironments are likely acting as to prevent the over consumption of metabolites during immune activation, although an alternative view is such signals may also serve to limit chronic inflammation and the associated tissue damage [100].

CD8+ T cells

Memory T cells (Tmem), unlike effector T cells (Teff), are quiescent and do not enter the cell cycle. However, TCR mediated recognition of antigen results in an exit from quiescence, and the cells rapidly undergo clonal expansion and acquire effector functions. The establishment of a memory population is a key feature of the adaptive immune system, as it provides a means to develop a rapid and effective recall response against microbial pathogens commonly encountered in the environment. Although it remains unclear what drives the differentiation of Teff vs. Tmem, emerging evidence suggests that mTOR partly influences the metabolism and trafficking of these cells.

Like Tregs, memory CD8+ T cells depend on AMPK-mediated FAO to meet their bioenergetic demands [46, 101]. Pearce and colleagues compared the metabolism in CD8+ Teff vs. Tmem cells and found that the memory population possesses greater mitochondrial spare respiratory capacity. They attributed the metabolic transition towards oxidative ATP production as a result of the pro-memory cytokine IL-15, which has a well-established role in mitochondrial biogenesis and expression of carnitine palmitoyl transferase-1 (CPT1). In another study conducted by Araki *et al*., it was found that rapamycin treatment during the course of acute infection with lymphocytic choriomeningitis virus (LCMV) promoted the generation of CD8+ Tmem cells [102]. To further ensure that mTORC1 was responsible for regulating memory T cell differentiation the authors targeted Raptor expression using

RNAi and again found higher expression of memory T-cell markers. Collectively, these studies suggest mTORC1-signaling plays a vital role for the differentiation and function of Tmem and Teff subsets.

The switch from anabolic metabolism to oxidative phosphorylation is indicative of a quiescent phenotype in Tmem. Recent studies attribute the differentiation of CD8+ Tmem cells to the regulation of FAO. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is required for this transition, as TRAF6-deficient T cells are unable to switch from glycolysis to FAO [46]. Pearce et al. reported that upon cytokine withdrawal, AMPK activation is diminished in TRAF6-deficient T cells, suggesting a role for AMPK in TRAF6-dependent FAO metabolism. Moreover, the inhibition of mTOR by rapamycin or activation of AMPK by metformin restores the generation of CD8+ Tmem in TRAF6-deficient mice. Taken together, these results support the hypothesis that the regulation of FAO and mitochondrial spare respiratory capacity contributes to the differentiation of effector and memory T cells.

During CD8+ T cell differentiation, the choice to differentiate into cytotoxic T cells (CTLs) is in large part driven by IL-2[103]. As described above, the PI3K/Akt signaling pathway influences metabolic reprograming. However, in CTLs, the activation of this signaling pathway is not required for the initializing glucose uptake [93]. Instead, CTLs appear to utilize an mTORC1:HIF-1α pathway that promotes glycolytic metabolism and the expression of cytotoxic effector molecules [104]. Considering that HIF1β-deficient $CD8^+$ Teff have diminished glycolysis, increased trafficking molecules, and induction of perforin and granzymes, there is strong rationale supporting the critical necessity of HIF-1α in activated T cell metabolism. However, it remains to be established how this mTORC1:HIF-

1α pathway initiates an appropriate immune response and coordinates with other signaling pathways to drive $CD8^+$ T cell differentiation.

Concluding Remarks

Upon activation, naïve T cells initiate a diverse array of transcriptional and translational responses, which ultimately results in their clonal expansion and differentiation into distinct effector subset populations. The advent of technologies over the last several decades has not only allowed researchers to measure specific cytokine expression on a per-cell basis, but has also allowed the determination of unique metabolic programs associated with distinct T cell subsets. Despite these advances, the extent to which T cells regulate their metabolic processes remains a contentious issue. As described, the bioenergetic profile adopted by specific T cell subsets depends on a variety of factors including the source of activation, the availability of nutrients, and the cytokine and metabolic substrate availability within the microenvironment. Moreover, T cell metabolic regulation involves a network of signaling molecules including mTOR, Akt, HIF-1α and Myc.

Understanding the dynamic interplay between the metabolic machinery and cellular signaling within T cells is without a doubt becoming an important topic in the immunology field. Current studies have focused on the metabolic reprogramming of T lymphocytes primarily in the context of activation and have demonstrated how particular T cell subsets tend to preferentially adopt a specific metabolic phenotype. Clearly, the development and maintenance of specific subsets of effector T cells is highly dependent on metabolic plasticity. This is perhaps most evident in the case of GLUT1 surface expression, in which

signals delivered from the antigen receptor and costimulatory receptors strongly influence GLUT1 expression and glucose uptake [9].

Although a constant supply of energy is required to maintain basal metabolic homeostasis, metabolites can be diverted within unique effector subsets in response to exogenous signals. There is some debate as to the importance behind the selective metabolic profiles these distinct subsets appear to possess, and more work will be required to discern the relative influence of metabolic signaling in these different T cell subpopulations. Given that each effector T cell subset appears to have a distinct metabolic profile, modulation of such metabolic pathways may yield selective therapies to control immune responsiveness. However, because normal proliferating and differentiating cells in other organ systems possess similar metabolic requirements as specific Teff subsets, teasing out such a therapeutic strategy remains a major challenge. Moreover, the question as to why oxidative phosphorylation is associated with the production of anti-inflammatory cytokines, whereas glycolysis is associated with production of pro-inflammatory cytokines remains unanswered. The continued expansion of our understanding of metabolic signaling in distinct T cell subsets remains an important area of interest. Despite challenges, understanding the signaling pathways that lead to altered cellular metabolism within T cells will likely yield important targets for immune therapies.

As discussed in the coming chapter, the death-associated protein-kinase-related-2 (DRAK2) not only acts as a rheostat in calcium signaling following TCR signaling but also acts as a negative regulator of T cell activation. Paradoxically, loss of DRAK2 in mice leads to diminished autoimmune susceptibility, with reduced clinical severity observed in animal autoimmune models for multiple sclerosis (experimental autoimmune encephalomyelitis;

EAE) and type I diabetes-prone non-obese diabetic (NOD) mice [109-112]. Although these data indicate that DRAK2 plays a role in autoimmune, the precise mechanisms through which it alters autoimmunity remains unclear. The work in this dissertation was proposed to investigate the mechanism by which DRAK2 alters T cell function and development. Previous studies have linked DRAK2 deficiency with heightened CRAC channel activity [113], which as discussed in Chapter 2, may have profound consequences on metabolic signaling. Therefore we explored whether the enhanced calcium mobilization within DRAK2-/- T cells increased mitochondrial metabolism and/or altered T cell development. Collectively, this thesis provides evidence that DRAK2 may be suitable as a novel therapeutic target given its selective inhibition of glycolytic differentiation.

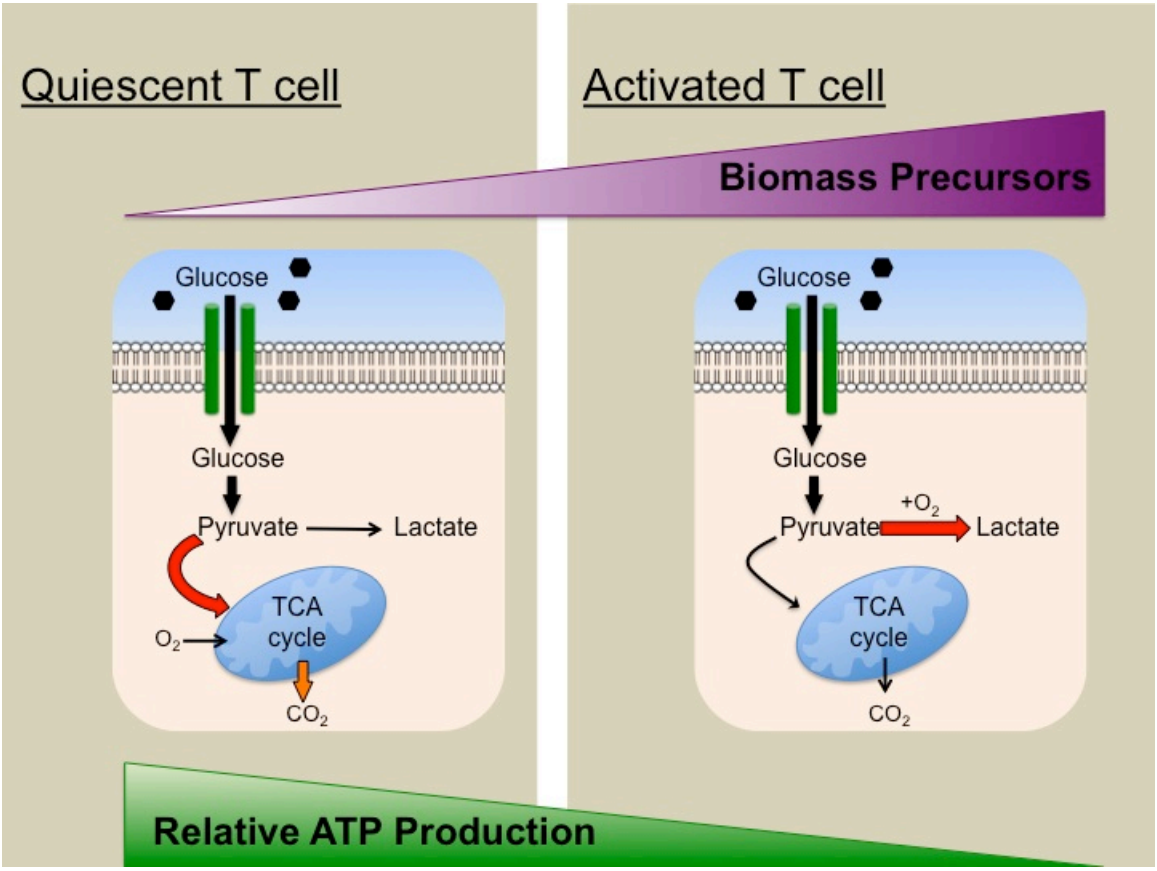


Figure 1.1: Metabolic differences between quiescent and activated T cells. Schematic representation highlights the metabolic differences between quiescent and activated T cells. Relative to quiescent T cells (left hand panel), the shift to aerobic glycolysis in activated T cells (right hand panel) is accompanied by an increase in biomass precursors and simultaneous decrease in ATP production per glucose molecule. Quiescent T cells primarily metabolize glucose to pyruvate, followed by its oxidation to CO2 through the TCA cycle yielding 36 ATPs per glucose. In contrast, activated T cells redirect the pyruvate away from oxidative phosphorylation by generating lactate regardless of oxygen availability (Warburg effect). This generation of lactate is not only associated with increased precursors that can be used for biomass replication, but it also results in decreased ATP production per molecule of glucose (relative ATP production).

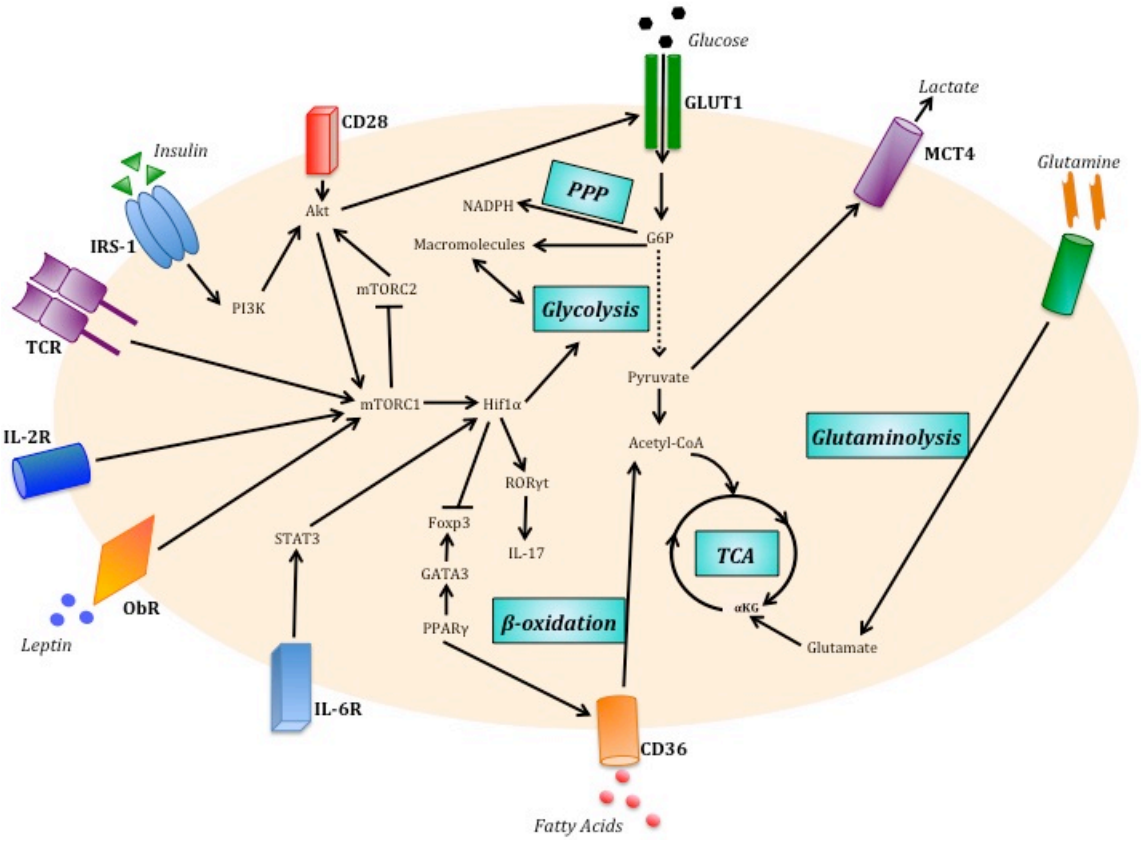


Figure 1.2: Schematic illustrating prominent metabolic pathways in T cells. Glycolysis, PPP, TCA cycle, β-oxidation, and glutaminolysis (all respectively highlighted in teal boxes) are all separate yet connected biochemical pathways that function to generate ATP in addition to biomass precursors. Upon entering the cell, glucose either shunted to the PPP or it is broken down to pyruvate by glycolysis, which can be further oxidized by the TCA cycle in the mitochondrion. Additional metabolic pathways, such as glutaminolysis or β-oxidation, can directly feed into the TCA cycle and further enhance the output. Not highlighted in this figure are the biomass products for amino, nucleic, and fatty acid synthesis; for example the acetyl-CoA generated from citrate in the TCA cycle can be used for lipid biosynthesis. Transporter abbreviations:GLUT1, glucose transporter 1; MCT4, monocarboxylate transporter 4; CD36, cluster of differentiation 36; IL-6R, interleukin-6 receptor; Obr, Astrocyte leptin receptor; IL-2R, interleukin-2 receptor; TCR, T-cell receptor; IRS-1, insulin receptor substrate 1; CD28, cluster of differentiation 28.

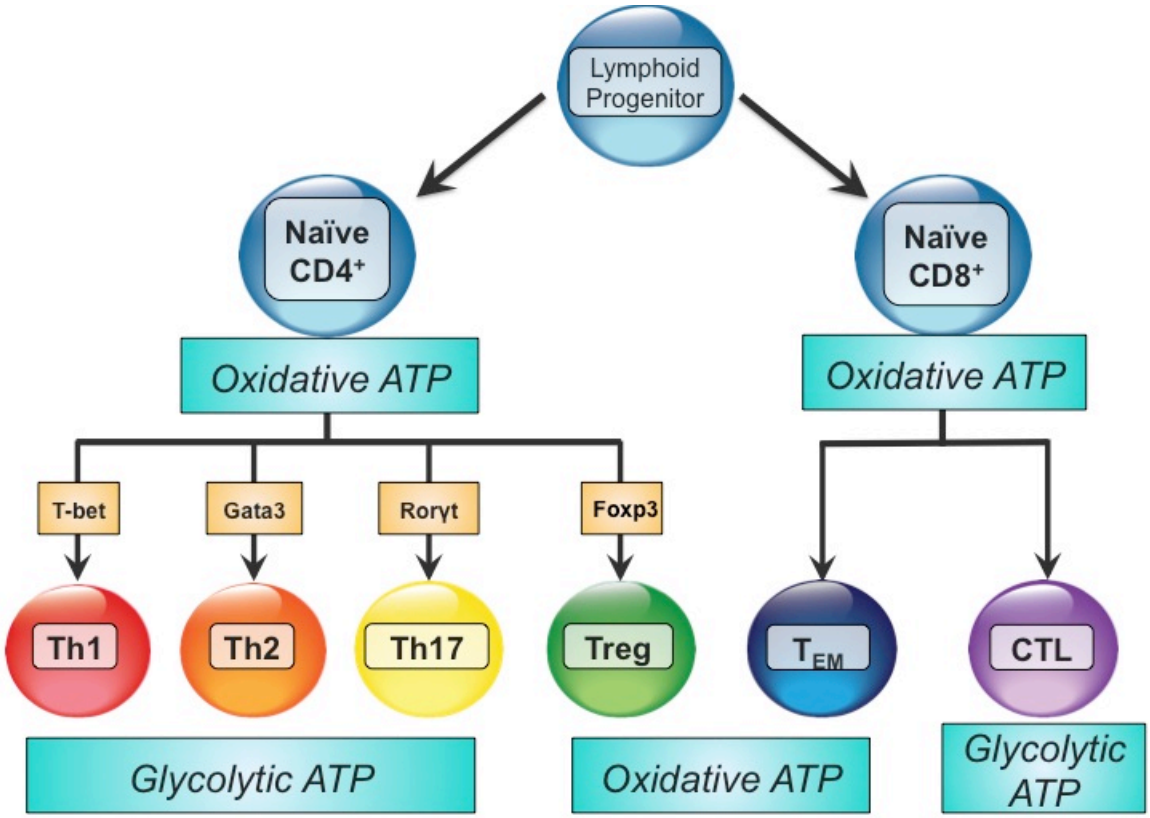


Figure 1.3: Metabolic reprogramming of effector T cells. Upon recognition of MHC-peptide complexes on the APC and following T cell engagement naïve T cells are induced to differentiate, proliferate, and acquire specialized effector functions. Concordantly, T lymphocytes undergo metabolic reprogramming shifting away from utilizing oxidative metabolism as their primary source of ATP generation, to a more glycolytic profile. As the cells continue to differentiate into effector subtypes including Th1, Th2, Th17, Treg, T-memory, and CTLs they undergo changes in metabolism as highlighted by teal boxes. Accordingly, some of these subsets express lineage-defining transcription factors and have been highlighted in orange boxes. Additionally, due to the migratory patterns of these cells, T cells display an inherent metabolic plasticity that allows them to adapt under various microenvironments. Taken together, these predominant highlighted pathways are not the sole metabolic signaling these subsets utilize.

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CHAPTER 2

Modulation of T cell metabolism through calcium signaling

Abstract

As a vital second messenger in the activation of lymphocytes, the divalent cation calcium (Ca2+) plays numerous roles in adaptive immune responses. Importantly, Ca2+ signaling is essential for T cell activation, tolerance of self-antigens, and homeostasis. Supporting the essential role of Ca2+ signaling in T cell biology, the Ca2+ regulated protein phosphatase calcineurin is a key target of pharmacologic inhibition for preventing allograft rejection and for autoimmune therapy. While Ca2+ is known to modulate T cell activation via effects on calcineurin and its target, nuclear factor of activated T cells (NFAT), this second messenger also regulates other pathways, including protein kinase C, calmodulin kinases, and cytoskeletal proteins. Ca2+ also modulates the unique metabolic changes that occur during in distinct T cell stages and subsets. In this chapter, the means by which Ca2+ mobilization modulates cellular metabolism following T cell receptor ligation is discussed. Additionally the crosstalk between mitochondrial metabolism, ROS generation, and CRAC channel activity is highlighted. As a target of mitochondrial ROS and Ca2+ regulation, we describe the involvement of the serine/threonine kinase DRAK2 in the context of these processes. Given the important roles for Ca2+ dependent signaling and cellular metabolism in

adaptive immune responses, the crosstalk between these pathways is likely to be important for the regulation of T cell activation, tolerance, and homeostasis.

Introduction

A key mediator of T cell signaling is the divalent cation Ca2+. Indeed, this second messenger is so important to T cell biology that its downstream signaling serves as a vital pharmacological target for the treatment of autoimmune disease and the prevention of chronic allograft rejection [1]. As with many cell types, ligation of surface molecules that induce phospholipase C leads to mobilization of intracellular calcium, with depletion of Ca2+ from the endoplasmic reticulum (ER). However, it has long been known that for sustained signaling downstream of Ca2+ release, T cells bear special plasma membrane Ca2+ channels that promote the influx of extracellular Ca2+ [2]. Work in several laboratories has revealed that T cells and a select few other cell types, express plasma membrane channels that are activated in response to depletion of intracellular stores of Ca2+ [3]. These calcium release activated calcium (CRAC) channels are activated by Stim1, an ER membrane protein that itself is induced upon depletion of Ca2+ stores in ER. Major efforts are currently underway to understand the biophysics of CRAC activation, and importantly, to determine if manipulation of CRAC signaling may be of therapeutic benefit.

Ca2+ modulates a variety of signaling cascades downstream of the TCR and costimulatory receptors, many of which impinge upon bioenergetic and biosynthetic pathways needed for T cell clonal expansion, differentiation and homeostasis. Moreover, the crosstalk between mitochondrial metabolism and CRAC activity suggests an influence of the metabolic status of a given T cell on Ca2+ mobilization and signaling. The impact of the release of mitochondrial ROS on Ca2+ signaling will also be addressed, with a focus on the immunoregulatory serine/threonine kinase DRAK2 in this process.

Calcium mobilization following TCR ligation

Engagement of the TCR initiates a wide range of biochemical signaling events, resulting in cellular activation and proliferation. One such molecule that is activated is phospholipase Cγ1 (PLCγ1), which results in the hydrolysis of phosphatidylinositol-3,4-bisphospahte (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Ca2+ release from ER stores is triggered upon the binding of IP_3 to inositol trisphosphate receptors (IP_3R) found on the ER membrane. The two major ion channels that are known to participate in Ca2+ release from the ER in response to agonist stimulation are IP_3Rs [4] and ryanodine receptors (RyRs) [5]. Mobilization of intracellular Ca2+ triggers store-operated Ca2+ entry (SOCE), primarily through CRAC channels [6]. The binding of antigen/MHC to the TCR complex results in the phosphorylation and activation of PLCγ1, which then hydrolyzes phospholipids at the plasma membrane to produce DAG and IP3. IP3 then binds to its respective receptors in the ER, triggering calcium release into the cytosol. This secondarily activates calcium release activated calcium (CRAC) channels within the plasma membrane, causing a rapid influx of extracellular calcium that sustains high calcium levels required for T cell activation. Cytosolic calcium binds calmodulin, a major transducer of calcium signaling, and induces a conformational change, thereby enabling the activation of phosphatase calcineurin [7]. Calcineurin dephosphorylates the cytoplasmic subunits (NFATc) of NFAT transcription complexes, exposing a nuclear localization sequence that results in their import into the nucleus [8].

Cytosolic calcium is a universal second messenger effecting a variety of cellular processes extending from short-term to long term responses in immune cells [9]. Activation of immune receptors initiates a signaling cascade culminating in the depletion of

intracellular Ca2+ stores. Upon depletion, store-operated calcium channels are activated, allowing Ca2+ to enter into the cell. The best-characterized ion channel is the CRAC channel. Ca2+ ions have long been known to act as signal transduction elements within intracellular signaling. Unlike other intracellular messengers, Ca2+ is neither synthesized nor metabolized. Rather, Ca2+ storage and release are intimately controlled by a range of channels and pumps to maintain precise concentrations across cellular compartments. The increase in intracellular free calcium is essential for lymphocyte activation given that the transcription factor families NFAT, NF-kB and AP-1 are all targets of Ca2+ mediated signaling. T cell proliferation and transcriptional activity are, in large part, regulated by Ca2+ oscillations through store-operated CRAC channels. Remarkably, 75% of all activation-regulated genes in T cells show a dependence on Ca2+ influx [10]. Store depletion of Ca2+ dramatically reduces, and often prevents, T cell activation and proliferation [10-12].

Store-Operated Ca2+ channels

CRAC channels are a widespread and highly conserved Ca2+ entry pathway and are activated following the depletion of Ca2+ within the endoplasmic reticulum (ER). This phenomenon is referred to as store-operated Ca2+ entry (SOCE) and CRAC activity is proportional to the amount of Ca2+ depletion within the ER. Over the last decade, the key components of the CRAC channel machinery were identified as STIM1, the ER Ca2+ sensor and Orai1, the CRAC channel's pore forming subunit [13]. Depletion of Ca2+ within the ER is detected by the Ca2+ sensors STIM1 and STIM2, resulting in the activation of store operated calcium channels. STIM1 and STIM2 are EF-hand containing single-pass

transmembrane proteins with both EF hands capable of binding Ca2+. STIM2 is thought to have a lower affinity for Ca2+ since STIM1-deficient mouse T cells and fibroblasts bear a severe impairment in store-operated Ca2+ influx whereas a deficiency in STIM2 has a less dramatic effect [14].

The luminal EF-hand of STIM1 senses Ca2+ depletion within the ER and subsequently causes its oligomerization and translocation towards the plasma membrane [15]. STIM1 binds through specialized ER-PM junctions, located within 25nm of the plasma membrane, to a component of the CRAC channel known as Orai1 [16]. Once activated, CRAC channels have a remarkably selective single channel conductance for Ca2+ and sensitive Ca2+ dependent feedback regulation [17]. Association of STIM1 and Orai1 depends on the involvement of STIM1 with the phosphoinositides [18] of Orai1. Within STIM1, the cytosolic SOAR/CAD domain contains an alpha-helical domain necessary for Orai1 binding [19, 20]. Deletion of the SOAR/CAD domain abolishes store depletion-induced clustering of Orai1 monomers and dramatically reduces SOCE, suggesting this domain is required for the assembly of the SOCE complex at ER-PM junctions [19, 20].

Mitochondrial calcium buffering and homeostasis

Mitochondria are recognized as essential calcium signaling organelles. Through calcium buffering and maintenance of sustained calcium flux, mitochondria influence CRAC channel activity [21]. With their ability to sense Ca2+ microdomains, mitochondria are able to translocate to the immunological synapse upon Ca2+ influx, leading to maximal Ca2+ uptake. The positioning of mitochondria correlates with the magnitude of local calcium

entry via the reduction of Ca2+ dependent channel inactivation [22]. By co-localizing near ER, mitochondria are better suited to buffer cytosolic Ca2+.

Isolated mitochondria take up Ca2+ when supported by exogenous ETC substrates [23]. Although it has been well known that mitochondria are endowed with a complex array of Ca2+ transporters, their role was not well appreciated until the early 1990s when Pozzan, Rizzuto and colleagues examined aequorin, a Ca2+ sensitive bioluminescent protein. They found that agonist-stimulated elevation of cytosolic free Ca2+ results in a rapid and transient increase in mitochondrial Ca2+, which is blocked by pretreatment with a mitochondrial uncoupler [24]. Prior to this discovery, it was believed that mitochondria failed to release much Ca2+ into the cytosol and had little effect on IP_3-mediated Ca2+ release. Since then, its has become appreciated that mitochondrial matrix Ca2+ concentrations are crucial regulators of the Ca2+ sensitive enzymes of the TCA cycle [25]. Mitochondria also play a crucial role in the gating of CRAC channels and do so through Ca2+ buffering [26].

Mitochondria act as Ca2+ buffers in the sense that the Ca2+ released by IP_3R on the ER, is rapidly taken up by mitochondria in the vicinity. Mitochondria accomplish this via their ability to detect changes in Ca2+ microdomains [27]. Ca2+ uptake by the mitochondria is accomplished by mitochondrial Ca2+ uniporter (MCU) activity across the inner mitochondrial membrane [28]. This Ca2+ buffering is functionally significant as it modifies the Ca2+-dependent inactivation of IP_3R, ultimately leading to greater Ca2+ mobilization. Secondly, mitochondria serve to deplete Ca2+ adjacent to the ER, resulting in less Ca2+ available for transport into the ER mediated by SERCA pumps. Taken together, these effects result in a robust activation of CRAC channels [29, 30]. It has been estimated

that between 25 and 50% of the Ca2+ released from ER is taken up by mitochondria, dependent on cell type and the Ca2+ release channel involved [31]. This Ca2+ uptake influences mitochondrial metabolic processes, as three rate-limiting enzymes of the Krebs cycle are activated by a rise in Ca2+ concentration, subsequently causing an increase in mitochondrial ATP generation [32] (Figure 2.1). These enzymes include pyruvate dehydrogenase (PDH), NAD+-isocitrate dehydrogenase (NAD-IDH) and 2-oxoglutarate dehydrogenase [25].

In addition to influencing CRAC channel gating, mitochondria also influence STIM1 trafficking [33]. Formation of STIM1 puncta below the plasma membrane is diminished, independently of its oligomerization, upon strong mitochondrial depolarization. This is accomplished by the mitochondrial protein mitofusin 2 (MFN2), which couples the mitochondria's energized state to STIM1 migration [33]. In MFN2-deficient cells, mitochondrial depolarization fails to influence STIM1 trafficking and Ca2+ entry, suggesting that MFN2 confers sensitivity to mitochondrial depolarization. MFN2 assists in tethering mitochondria to the ER and is abundant in the contact sites between the two organelles, though the exact mechanism through which these two organelles interact remains unclear [34]. It is speculated that the most likely function of the tethering is to allow the mitochondria to localize in the immediate vicinity of Ca2+ release from the ER. However the question as to why STIM1 overexpression partially rescues Ca2+ entry in mitofusin 2 –overexpressing cells, has yet to be answered.

Mitochondria are functionally distinct from the ER in terms of Ca2 + transport mechanisms. First, mitochondria have a large electrical gradient across their inner membrane, referred to as the mitochondrial membrane potential ($\Delta\Psi m$), which acts as a

driving force for the uptake of Ca2+ [35]. Unlike with ER, mitochondrial Ca2+ release occurs via ion exchangers, and Ca2+ uptake is mediated via a selective ion channel [36]. High calcium levels, in combination with other signals, have been known to trigger the opening of channels within the inner mitochondrial membrane, ultimately resulting in cell death [37]. The combined action of these mechanisms allows for the rapid cycling of Ca2+ across the inner mitochondrial membrane [38].

Regulation of STIM1/Orai signaling by oxidative stress

Numerous physiological processes, including proliferation, and cell death, are influenced by ROS [39, 40]. These chemically reactive molecules are a byproduct of redox-active proteins and are found in both intracellular and extracellular compartments. To prevent oxidative stress, the cell must maintain a balance between superoxide production and sufficient clearance of ROS; this is achieved through circulating antioxidants that act as ROS scavengers. Out of the ~20 types of ROS, hydrogen peroxide (H_2O_2) appears to be the major contributor to oxidative damage [39]. Of interest here, it has been found that oxidative stress regulates STIM1/Orai1 signaling [41-43]. CRAC channels, under the action of STIM1, are stimulated by micromolar concentrations of H_2O_2 [41-43]. Altered calcium signaling during oxidant stress is attributed to a reduction in the Ca2+ binding affinity of STIM1 due to a modification involving its cysteine 56, ultimately resulting in constitutive CRAC activation [43]. Upon exposure to ROS, STIM1 becomes S-glutathionylated, thereby triggering its oligomerization and translocation to the plasma membrane [43]. Alternatively, Grupe and colleagues demonstrated that H_2O_2 enhanced the CRAC current, I_{CRAC}-mediated Ca2+ influx by activating IP_3R independent of Orai1 [42]. Regardless of the

route of entry, both authors support that an increase an ROS leads to enhanced Ca2+ entry into the cell. While several studies support the hypothesis that ROS positively stimulate CRAC channel activity by triggering the oligomerization of STIM1, Bogeski and colleagues report that oxidation via H_2O_2 specifically blocks the activation of Orai1 channels in human T helper lymphocytes [41]. It is speculated that the oxidation of the cysteine, found within the extracellular loop of Orai1, may lock the channel in the closed conformation. Nevertheless, cells experiencing SOCE deficiency are more susceptible to oxidative stress. Despite having an up-regulated basal antioxidant response, STIM1-deficient mouse embryonic fibroblasts experience an imbalance of ROS produciton and detoxificaiton upon additional exogenous oxidants [44]. Henke and collegues conclude that functional SOCE machinery is required to balance ROS production and the cellular antioxidant defense system. Collectively these studies suggest that oxidative stress is influenced through a dynamic interplay between the levels of ROS and Ca2+ influx. The counteracting stimulatory or inhibitory effects brought on by oxidative stress need further study in order to understand the complex mechanisms at hand.

Oxidant dependence of T cell activation

Oxidative stress is provoked by an imbalance between the production of mitochondrial superoxide and insufficient scavenging of ROS, ultimately resulting in a wide range of pathological conditions. The dynamics of ROS production and scavenging can be detected using several fluorescent dyes, including reduced forms of ethidium bromide (DHE) [45], fluorescein (DCFDA) [46, 47], rhodamine (DHR) [48], and hydroethidine (HE) [49]. These dyes remain relatively non-fluorescent until they are oxidized by ROS, and their

unquenched fluorescence correlates with increased oxidation. These intracellular dyes provide sensitive probes for multiple reactive species and allow for the detection of fluorescent products using fluorimetry and flow cytometry. In T cells, DCFDA, DHR, and DHE have been utilized to examine ROS production from a variety of stimuli including mitogens [50], superantigen [51], and TCR peptide agonists [52, 53]. Stimulation of cognate receptors on T cells induces intracellular production of ROS from various ligands including TGF-β, insulin, angiotensin II and epidermal growth factor (EGF) [54-57]. Collectively these studies suggest that ROS function as secondary messengers necessary for complete T cell activation. Superoxide is generated from the mitochondrial ETC through complexes I, II, and III [58]. Complexes I and II emit superoxide into the mitochondrial matrix, upon which superoxide dismutase 2 (SOD2) converts it into hydrogen peroxide (H_2O_2). In contrast, complex III emits superoxide into both the matrix and intermembrane spaces. Intermembrane-space superoxide is capable of reaching the cytosol through voltage-dependent anion channels (VDAC) without conversion into H_2O_2 [59, 60].

Recently, Sena *el al*. reported that blocking mitochondrial ROS, specifically generated at complex III, results in the inhibition of CD3/CD28 induced IL-2 expression. Additionally, extracellular calcium influx and subsequent Ca2+ update by mitochondria were both required for mitochondrial ROS production [61]. Collectively, these data support the notion complex III produced mitochondrial ROS, a byproduct of mitochondrial metabolism, are required to support T cell activation and functionality. However, there is still conflicting evidence surrounding the role of ROS in T cell function. Specifically, the mechanism through which oxidants may regulate T cell responses upon receptor stimulated ROS generation remain poorly defined. Various approaches have been used to

study the interplay, including studies with cells stimulated in the presence of antioxidants, or treated with oxidants. While providing useful information, caution needs to be taken when interpreting the results from studies using exposure to extracellular oxidants vs. receptor-stimulated intracellular ROS, as those are not comparable. It is quite likely that time and place where occur within the cell are of greater influence in cellular responses than the overall redox balance [62-64].

DRAK2 as a an intermediary in calcium and metabolic signaling in T cells

DRAK2 is a serine/threonine kinase of the death associated protein kinase (DAPK) family [65], which is comprised of five known members (DAPK1, DAPK2, DAPK3, DRAK1 and DRAK2). All of these kinases are capable of inducing cell death when ectopically expressed in cells, and the prototype DAPK1 possesses a calmodulin regulatory domain that, through binding to calmodulin/Ca2+, regulates its catalytic activity [66]. While DRAK2 lacks a calmodulin binding domain, like other DAPK family members, DRAK2 has been shown to induce apoptosis upon overexpression in cell lines [67], and its phosphorylation and nuclear translocation participates in ultraviolet light induced cell death [68].

Although broadly expressed at low levels [69], DRAK2 expression is enriched in cells of hematopoietic origin [70]. Loss of expression by virtue of a germline deletion of the Drak2 gene leads to hyperactive Ca2+ mobilization in T cells, especially under suboptimal TCR stimulus conditions [70, 71], supporting the hypothesis that DRAK2 acts as a negative regulator of TCR signaling [72]. The notion that DRAK2 serves as a rheostat in calcium signaling following TCR signaling is supported by the finding that it is itself activated by calcium mobilization, and that its ectopic expression in double positive thymocytes raises

the threshold for both negative and positive selection [73, 74]. Enigmatically, loss of the Drak2 gene leads to diminished autoimmune susceptibility, with reduced clinical severity observed in animal autoimmune models for multiple sclerosis (experimental autoimmune encephalomyelitis) and type I diabetes-prone NOD mice [71, 75-77]. The reduced EAE susceptibility is due, at least in part, to diminished survival of activated effector T cells, perhaps a consequence of impaired calcium homeostasis [75-77].

Using a mass spectrometry based approach, we determined that DRAK2 possesses several autophosphorylation sites, most notably Ser10, Ser12 and Ser351. Using antibodies specific for phosphorylation at Ser12, TCR stimulation induces DRAK2 Ser12 autophosphorylation [73], and this requires entry of extracellular Ca2+ into T cells. The SERCA inhibitor thapsigargin was also found to potently induce DRAK2 autophosphorylation on Ser12, supporting the hypothesis that ER store operated calcium entry modulates DRAK2 catalytic activity. Recent studies have revealed that TCR induced DRAK2 activation is highly dependent on CRAC function, as DRAK2 was poorly activated following antigenic stimulation in Orai2-deficient T cells [78]. Using a panel of small molecule inhibitors and RNA interference approaches, it was found that DRAK2 activation by antigenic stimulation requires protein kinase D (PKD).

Curiously, a DRAK2 catalytic activity in activated T cells also depended on mitochondrial ROS, as antioxidants blocked Ser12 autophosphorylation [78]. Since the electron transport complex III inhibitor FCCP led to Ser12 phosphorylation independent of antigenic stimulation of T cells, these findings demonstrate that ROS are both necessary and sufficient to promote DRAK2 catalytic activity. PKD activity is itself subject to activation by mitochondrial ROS, and thus ROS induced by enhanced mitochondrial

respiration that results from TCR stimulation likely participates in PKD-mediated DRAK2 activation. The specific nature of the interaction between mitochondrial ROS and DRAK2's substrates is poorly understood. However, as Drak2-deficient T cells bear enhanced CRAC activity [78], and Drak2-transgenic thymocytes have diminished Ca2+ mobilization following TCR stimulation [74], it is likely that DRAK2 may phosphorylate an intermediate in CRAC signaling. DRAK2 has been shown to phosphorylate S6K1 [79], an important target of mTOR and of TCR signaling that impacts cellular metabolism [80-82]. Thus, DRAK2 may itself serve as an important link between calcium and mTOR signaling, impacting the differentiation and survival of T cell subsets selectively required for immune responsiveness.

Conclusion

Research into the regulation of CRAC activity, and the unique involvement of metabolic signaling in T cells, has been intense over the last several years. It is becoming quite evident that both signaling paradigms are highly regulated and vital to T cell activation, differentiation and homeostasis. These pathways also appear to play selective roles in distinct T cell subsets, with the potential that manipulation of such pathways could influence the balance between immunity vs. tolerance in different disease states. A significant challenge to immunologists is to develop selective therapies to influence this balance. In the case of autoimmune disorders, the difficulty is to block autoreactive T cell function without promoting general immunosuppression. In the case of chronic microbial pathogen infections, therapeutic approaches to reinvigorate antimicrobial T cell responsiveness are highly sought after. It is thus imperative that novel methods are

considered that influence the appropriate immune response. Given the unique crosstalk between calcium mobilization and bioenergetic metabolism, there may be an opportunity to uniquely affect the outcome of immune therapies by targeting the molecules that mediate this crosstalk.

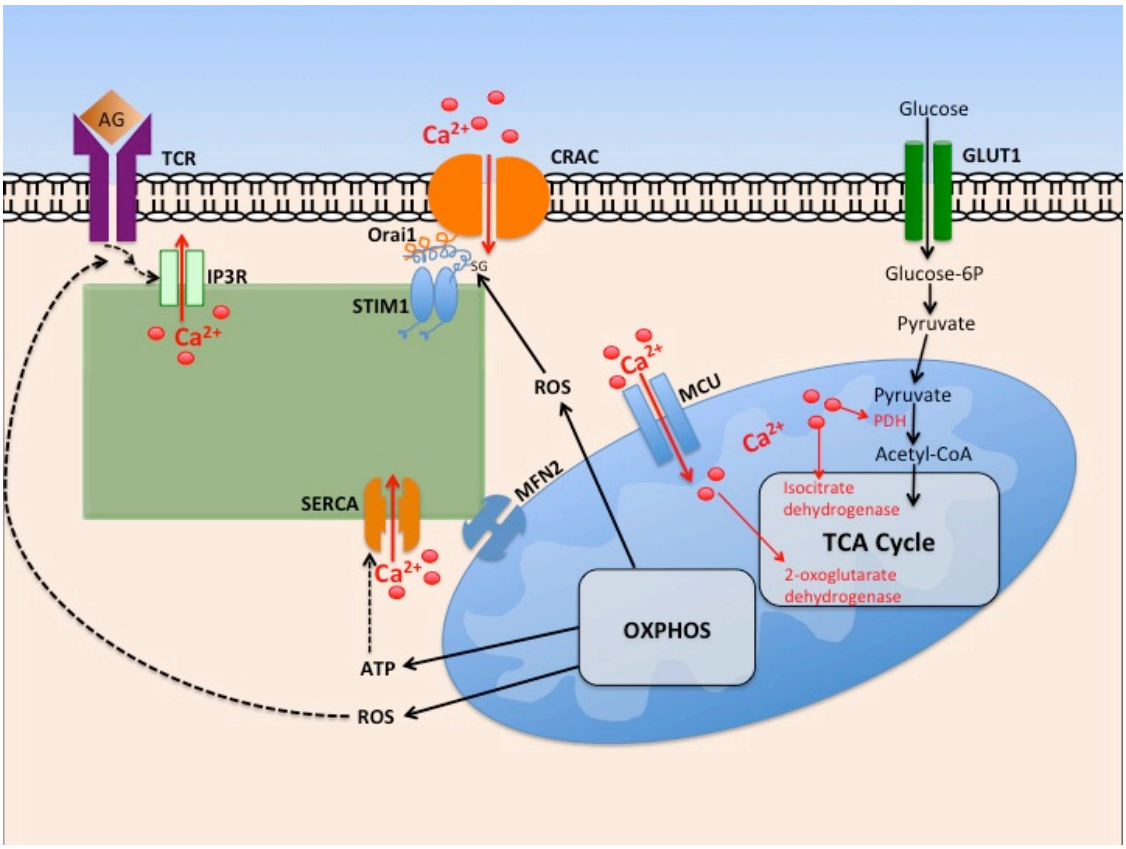


Figure 2.1: The interplay between intracellular calcium mobilization and cellular metabolism in T cells. Ligation of the T cell receptor (TCR) by antigen (Ag) promotes opening of IP_3R and depletion of ER Ca2+ via IP3, itself generated by phospholipase Cγ1. Depletion of ER Ca2+ stores promotes STIM1 interaction with Orai1, promoting oligomerization of Orai monomers, thus forming CRAC channels. The resultant increase in cytosolic Ca2+ promotes its entry into the mitochondrial matrix via the mitochondrial calcium uniporter (MCU). Elevated matrix Ca2+enhances the activity of key tricarboxylic acid cycle (TCA) enzymes, leading to elevated oxidative phosphorylation (OXPHOS), and ATP generation. Reactive oxygen species (ROS) byproducts of OXPHOS enhance TCR proximal signaling by inhibiting tyrosine phosphatase activity. Elevated cytosolic ROS governs CRAC activity by ROS mediated modification of cysteine residues exposed on Orai1.

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CHAPTER 3

DRAK2 deficiency alters metabolic efficiency upon anti-CD3 stimulation

Abstract

Metabolic programming in T cells following activation not only affects the ability of Teff to survive, but can also regulates the development of different T cell subsets. Here we show that loss of DRAK2 leads to metabolic dysfunction upon anti-CD3 stimulation. Specifically, DRAK2-/- T cells exhibited elevated glycolysis, glucose uptake and glucose transporter expression following stimulation. Moreover, DRAK2-/- T cells were more resistant to nutrient withdrawal and pharmacologic perturbations. The altered metabolism within DRAK2-/- T cells likely causes an imbalance in the effector subset distribution, but further studies are needed. Although DRAK2 deficiency leads to enhanced metabolism, activated T cells do not display a survival phenotype when activated with anti-CD3/28. Therefore, the research performed in Chapter 4 was conducted using a stimulation modality that induced differential survival between wildtype and DRAK2-/- T cells; these studies propose that the need for DRAK2 can be overcome with costimulation. Collectively, these studies suggest the importance of DRAK2 in more physiologic or weaker stimulations conditions that are not necessary for high signaling activation including antiviral immune responsiveness. However, even under conditions where no survival defect occurs, DRAK2-/- T cells display an inherent dysregulation in their metabolism, which supports the correlation between DRAK2-deficiency and metabolic signaling.

Introduction

T cell activation initiates an intra-cellular signaling cascade that results in proliferation and acquisition of effector functions. While DRAK2 does not appear to function as a pro-apoptotic protein, several studies suggest that DRAK2 functions as a negative regulator of TCR signaling. In response to suboptimal stimulation, DRAK2-/- peripheral T cells exhibit hyperproliferation, enhanced cytokine production and increased calcium mobilization [1, 2]. Similarly, loss of DRAK2 leads to an enhanced proportion of apoptotic cells *in vitro* [1, 3]. However the exact mechanism for enhanced apoptosis has yet to be determined.

Originally identified as a serine/threonine kinase involved in apoptosis, DRAK2 has since been described as a unique regulator of T cell activation [4]. Unlike other negative regulators that prevent autoimmunity, DRAK2-/- mice are resistant to autoimmune disease in mouse models of T1D and MS [1, 5]. This resistance is due to reduced accumulation of autoreactive T cells in the pancreas and central nervous system (CNS) compared to wildtype mice. Similarly, ectopic expression of Bcl-xL in T cells restored EAE susceptibility, not only implicating apoptotic hypersensitivity as a mechanism of resistance of Drak2 mice to EAE, but also demonstrating that this resistance is T cell intrinsic. Furthermore, DRAK2-/- T cells cannot reject allogeneic tumors *in vivo* due to enhanced effector cell death [6]. Thus, DRAK2 plays a role in promoting T cell survival rather than apoptosis.

Concomitant with T cell activation is the engagement of aerobic metabolism, a metabolic phenotype shared by numerous cancer cells [7-9]. Upon T cell activation, cells must build biosynthetic precursors for biomass thus causing a preferential conversion of pyruvate to lactate in the cytoplasm rather than oxidation in the TCA cycle. Failure to engage specific metabolic profiles impairs the differentiation of T cells [10]. Since we

previously determined that DRAK2 is required for effector cell survival following activation with allogeneic splenocytes [6], we sought to determine whether the enhanced death was due to an inability to undergo proper metabolic reprogramming.

Upon import into the cell through glucose transporters, glycolysis converts each molecule of glucose into 2 molecules of pyruvate and 2 units of ATP. In oxidative metabolism, the pyruvate is converted into acetyl-CoA by mitochondrial PDH before entering into the Krebs cycle, which generates NADH. NADH is oxidized at complex I of the electron transport chain, passing 2 electrons to ubiquinone and pumping 4 H^+ ions into the intermembrane space. As the electrons travel across the ETC to complex 4, electrons are pumped into the intermembrane space. This continuous pumping of H^+ ions into the IMS causes the generation of a higher concentration of H^+ ions into the IMS compared to the matrix. This generates an electrochemical gradient whose energy is harnessed by the ATP synthase, which couples the synthesis and hydrolysis of ATP to the movement of protons down their electrochemical proton gradient.

Work described in this chapter addresses the *in vitro* role of DRAK2 in the regulation of glucose metabolism. Loss of DRAK2 signaling under anti-CD3/28 stimulation resulted in no appreciable survival deficit. Yet, pharmacologic perturbations following stimulation yielded differential metabolism depending on the presence of DRAK2. This study, therefore, identifies a role for DRAK2 in limiting the magnitude of metabolic activity following stimulation with anti-CD3/28.

Materials and Methods

Mice and Reagents

As previously described, DRAK2-/- mice were backcrossed onto the C57BL/6J for more than 10 generations [1]. Four to eight week old DRAK2-/- mice were used for all experiments unless otherwise indicated. In all cases, age-matched littermates were used as controls. Reagents used for flow cytometry included anti-CD4, anti-CD8 (Tonbo Biosciences, San Diego), 7-Aminoactinomycin D (7AAD), and mitoSOX (Molecular Probes, Invitrogen, Carlsbad).

T cell purification and Stimulation

Naïve T cell populations were isolated from splenocytes by negative selection using Dynal Negative Selection T cell Kit (Invitrogen, Carlsbad, CA). Naïve T cells were stimulated with plate bound anti-CD3 (200ng/ml) and soluble anti-CD28 (200ng/ml, eBioscience) for the indicated time points.

CFSE labeling

Following isolation, purified C5BL/6 or DRAK2-/- T cells were labeled with 5μM 5,6-carboxyfluorescein succinimidyl ester (CFSE) for 8 minutes at 37°C. Cell purification was assayed by flow cytometry following stimulation.

Calcium Mobilization

To assess CRAC activity, purified T cells were labeled for 1hr at 37°C with Fluo-3 (3.4μM) and Fura-Red (4.6μM) in the presence of 0.02% pluronic (Molecular Probes). Following labeling, cells were washed with cold RPMI and kept on ice. Prior to analyses, cells were warmed to 37°C. Baseline data was collected for 60 sec followed by the addition of thapsigargin (1mM) to induce release of ER calcium under Ca2+ free media and after 4 min Ca2+ (2mM) was supplemented to induce CRAC-dependent calcium uptake. Ca2+ influx kinetics were calculated by Flowjo based on median fluorescence data and smoothing.

Immunoblotting

Whole cell lysates were prepared by lysis in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, with phosphatase and protease inhibitors (Sigma). Protein concentrations were calculated using a BCA Protein Assay (Thermo Scientific). 50μg of whole cell protein was loaded per condition, separated by SDS-PAGE, and transferred to PVDF membranes for overnight probing with various antibodies in 5% BSA at 4°C. Bands were detected by incubating blots with HRP conjugated secondary antibodies for one hour at room temperature followed by ECL detection. Densitometry was performed on scanned immunoblot images using the ImageJ.

Metabolic Assays

Glycolysis and glucose uptake assays using ^{3}H-glucose or ^{3}H-2-deoxyglucose were performed as described previously [11, 12].

Analysis of Mitochondrial Membrane Potential.

In order to analyze mitochondrial membrane potentials (ΔΨm) cells were labeled with either 2μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes) or 0.04μM DiOC6 (Molecular Probes) in PBS for 15 min at 37°C. After washing with PBS, cells were further processed for analysis using flow cytometry. JC-1 selectively enters the mitochondria as monomers and it reversibly forms aggregates upon membrane polarization causing a shit in light emission.

Metabolic manipulation

In order to limit metabolism fueled by glucose, cells were cultured in full serum media without glucose. Alternatively, pharmacologic inhibitions were done with DCA and lonidamine to either promote oxidative phosphorylation or inhibit aerobic glycolysis, respectively.

Results

DRAK2-/- T cells exhibit enhanced Ca2+ mobilization following stimulation

We previously reported that DRAK2-deficient T cells display enhanced CRAC activity and suggested that DRAK2 may phosphorylate an intermediate in CRAC signaling [13, 14]. These observations prompted us to establish whether the enhanced calcium mobilization found within DRAK2-/- T cells was preserved following *in vitro* stimulation. Using the ratiometric dyes Fluo-3 and Fura red, SOCE was evaluated in resting and activated T cells (Fig 3.1). Following 2 days of stimulation with anti-CD3/28, T cells were isolated and calcium flux was compared against naïve cells. After treating the cells with thapsigargin, a pharmacological agent that empties Ca2+ stores within the ER, activated CD4+ and CD8+ DRAK2-deficient T cells exhibited elevated calcium flux relative to WT controls. These results are significant because they show that the enhanced CRAC phenotype within DRAK2-/- T cells is preserved following *in vitro* stimulation.

In contrast to previously reported findings [1], CRAC activity did not differ between naïve CD4+ and CD8+ T cell subsets. However these previous investigations stimulated calcium flux by TCR activation using biotin anti-CD3 and streptavidin. Unlike thapsigargin, which directly promotes the depletion of Ca2+ from the ER, TCR stimulation engages numerous signaling cascades that support calcium flux. One such molecule that is activated is PLCγ1, which mediates the hydrolysis of PIP_2 into IP_3 and DAG. Ca2+ release from ER stores is triggered upon the binding of IP_3 to IP_3R's found on the ER membrane. To establish whether DRAK2 phosphorylated an intermediate in CRAC signaling before Ca2+ efflux from the ER, titrating anti-CD3 conditions were generated (Fig 3.2). While we were able to recapitulate the previous report, the phenotype was found under very specific

conditions, supporting the hypothesis that it was a threshold effect. Taken together, these data support a function of DRAK2 in controlling the threshold for calcium mobilization and suggest that this phenotype is preserved following *in vitro* stimulation.

DRAK2-/- T cells exhibit elevated metabolism

By enhancing the activity of rate limiting enzymes in the mitochondrial matrix, Ca2+ plays an important role in energy metabolism [15, 16]. By promoting the activity of mitochondrial dehydrogenases, rising Ca2+ concentrations lead to increased oxidative phosphorylation and ATP production. In order to support the enhanced metabolic demands, T cells up-regulate glucose transporters, including GLUT1 [17, 18].

As previously demonstrated, murine T cells display an increase in glucose uptake following stimulation with anti-CD28 [19]. Consistent with this finding, 24 hour activated T cells displayed enhanced glycolysis and glucose uptake compared to naïve precursors (Fig 3.3). Densitometric analysis indicated a 1.3 fold increase in GLUT1 expression in DRAK2-/- T cells compared to wildtype control following stimulation (Fig 3.3A). Moreover DRAK2-deficient T cells had elevated rates of glycolysis and glucose uptake relative to wildtype control (Fig 3.3B,C). These data demonstrate while activation alone induces enhanced metabolism, feedback from DRAK2 limits the magnitude.

Since activated DRAK2-/- T cells had elevated glycolytic activity compared to wildtype T cells, we wanted to determine if they were more or less sensitive to glucose restriction. To determine the requirement for glucose metabolism to induce proliferation, T cells were cultured under glucose-limited media. As expected, T cells recovered from wildtype stimulated cultures exhibited reduced proliferation, demonstrating a requirement

of glucose for proliferation (Fig 3.4). In contrast, DRAK2-/- T cells maintained their proliferative capacity even under glucose free media supplemented with serum. These data suggest that increased expression of GLUT1 is sufficient to augment both metabolism and proliferation, demonstrating that glucose uptake is a limiting factor in T cell activation. However upon loss of DRAK2, T cells are able to survive under glucose restrictive conditions due to their enhanced glucose uptake and glycolytic capacity. As an alternative explanation, these data support that DRAK2-/- T cells are less reliant on glucose as a substrate for fuel utilization and suggest that perhaps these cells utilize alternative sources of carbon catabolism.

To explore the metabolic changes in DRAK2-/- T cells, we evaluated the expression of genes involved in metabolic reprogramming by western blot (Fig 3.5). Consistent with a role for metabolic reprograming, HIF-1α, HK2 and enolase-1 were all upregulated following activation with anti-CD3/28. Surprisingly, protein expression of glycolytic-associated genes was not significantly different between DRAK2-deficient and wildtype T cells. In addition to its stability, HIF-1α's transcriptional activity can be altered via ubiquitination, acetylation, sumoylation, hydroxylation and phosphorylation [20]. This would support the hypothesis that post-translational modifications and secondary messengers may be differentially modulating the activity of metabolism-associated genes. Although aerobic glycolysis has been described as the primary energy source following activation, T cells still utilize oxidative metabolism. Therefore, we next sought to determine whether loss of DRAK2 led to altered oxidative metabolism.

ETC

Mitochondria are considered the “power house” of the cell wherein energy is derived from the transfer of electrons down the ETC. The ETC is present in the inner mitochondrial membrane and contains five complexes responsible for shuttling electrons, forming a ΔΨm and generating ATP (Fig 3.6). The electrochemical potential across the inner mitochondrial membrane supports Ca2+ uptake, detoxification of ROS and ATP generation [21]. As the ETC is responsible for the generation of ATP, it is plausible to consider the role of the mitochondria in abnormal energy metabolism.

We therefore asked whether DRAK2-deficiency alters ETC functionality. We subjected stimulated T cells to 1hour perturbations with direct inhibitors of the ETC complexes (Fig 3.7A). As expected, oligomycin treatment prevented the exchange of protons across the gradient, leading to an increase in the ΔΨm. Similarly, treatment with antimycin A and rotenone prevented consumption of oxygen by the ETC, resulting in a decrease in ΔΨm. As a control, the proton ionophore, CCCP was included to allow uninhibited movement of protons across the membrane, which depolarized the cells. Given that acute perturbations did not elicit differential mitochondrial ETC activity upon loss of DRAK2, we next sought to determine the requirement for functional ETC activity upon stimulation. To test the dependence of T cells on ETC for activation-induced proliferation, T cells were labeled with the proliferation dye CFSE and treated with inhibitors for 3 days (Fig 3.7B). Neither wildtype nor DRAK2-/- T cells were able to proliferate; indicating that maintenance of the mitochondrial membrane potential is a prerequisite for proliferation. Collectively these data suggest that loss of DRAK2 does not protect against the induction of bioenergetic dysfunction in mitochondria.

Inhibition of aerobic glycolysis sensitizes DRAK2+/+ T cells

As described, DRAK2 serves to limit the magnitude of glycolytic metabolism upon T cell activation. To access the selective metabolic dependence of DRAK2-deficient T cells on glycolysis we sought to rewire glycolytic flux by stimulating glucose oxidation. By inhibiting PDK in the mitochondria, dichloroacetate (DCA) rewires cellular energy metabolism away from lactate production and towards oxidative phosphorylation [22]. As a result, the Krebs cycle generates NADH, which is used to fuel the ETC and promote membrane polarization.

To determine the effect of PDK inhibition on T cell fate and function, T cells were stimulated in the presence of DCA (Fig 3.8). DCA treatment did not affect the polarization state of DRAK2-/- T cells, as DiOC6 staining was similar regardless of treatment. This is likely due to the oxidative machinery operating at maximum capacity; therefore the additional stimulus had no affect. In contrast, DRAK2+/+ T cells experienced a dose dependent mitochondrial membrane depolarization, as evidenced by decreasing DiOC6 staining. While contrary to our hypothesis, that increased TCA activity would support a higher membrane potential; studies have found that increased ROS promote mitochondrial depolarization through the opening of the mitochondrial permeability transition pore (MPTP) [23, 24]. Interestingly, examination of the recovered viable cells revealed that DCA treatment actually increased the number of viable wildtype cells, whereas the DRAK2-/- T cells displayed a modest dose dependent decrease in cell number (Fig 3.8B). Collectively these data support that loss of DRAK2 leads to metabolic dysfunction. However due to the inability of DCA to cause an increase in $\Delta\Psi m$, we next sought to alter glycolytic metabolism.

Glycolytic metabolism is intimately connected to the conversion of glucose into glucose-6-phosphate mediated by hexokinase activity. Lonidamine was originally

developed as an antispermatogenic reagent [25]. However more recent applications have focused on targeting cells with high metabolic needs, given its ability to selectively inhibit mitochondrial bound hexokinase [26, 27]. Because lonidamine is suggested to inhibit mitochondrial bound hexokinase, one would expect it to affect glycolytic metabolism by reducing the efficiency of converting glucose into glucose-6-phosphate. DRAK2 sufficient- and deficient-T cells were exposed to increasing concentrations of lonidamine (20-100μM) for 72 hours in a dose range previously described by others [28, 29]. The comparative recovery of CD4+ and CD8+ T cells is presented in Figure 3.9A,B. While no significant difference was found in the absolute number of cells, the absolute fraction of dividing cells treated with lonidamine was strikingly different (Fig 3.9C). While both DRAK2+/+ and DRAK2-/- T cells slowed their proliferation upon treatment, the wildtype cells exhibited enhanced sensitivity, as evidenced by greater CFSE retention. Overall these data suggest that DRAK2-/- T cells are more resilient to glycolytic inhibition. Given that anti-CD3/28 stimulation does not elicit a survival defect, we next sought to confirm that withdrawal of costimulation would be sufficient to demonstrate inappropriate proliferation in DRAK2-/- T cells.

Altering signal strength reveals DRAK2-/- T cells do not experience a survival deficit upon anti-CD3 stimulation

Although DRAK2-deficeint T cells exhibited an altered metabolic profile, we did not observe altered survival. Strength of T cell stimulation is altered by various factors including: the presence or absence of co-stimulation, the duration of interaction between MHC:TCR and the concentration and affinity for a given antigen [30]. To determine if TCR

signal strength was overcoming the characteristic survival defect, T cells were activated without anti-CD28 in the presence of titrating anti-CD3 (Fig 3.10). At the lowest dose of anti-CD3 a greater number of DRAK2-/- T cells were recovered but these results were variable. Furthermore, previous studies demonstrated that exogenous anti-CD28 was able to restore the levels of costimulatory markers and the observed hyperproliferation of activated DRAK2 T cells back to wildtype levels [1, 3]. Collectively, these data suggest that anti-CD3/28 stimulation is sufficient to overcome the survival deficit and promote augmented metabolism.

Discussion

The metabolic demands of activated T cells must be assuaged in order to promote activation, proliferation and differentiation. Regulation of calcium signaling during T cell activation is intimately linked with the regulation of metabolism. Thus, the stability of the crosstalk between Ca2+ signaling and energy metabolism is essential for the proper differentiation of T cells. Disturbance of this interplay causes Ca2+ dyshomeostasis and energy metabolism dysregulation, which may contribute to increased cell death. Therefore, the frequency, duration and the topography of calcium signaling constitute indirect indications of cell status. This chapter has been devoted towards the examination of acute metabolic alterations following anti-CD3 stimulation paired with pharmacologic metabolic perturbation.

DRAK2 has been characterized as a negative regulator of T cell activation and in the absence of DRAK2 T cells exhibit increased calcium flux and CRAC channel activity [13, 31]. DRAK2 also redistributes to the immunological synapse and is activated by ROS production following activation in T cells [13]. Given these results, we sought to determine whether the enhanced calcium phenotype would be preserved across stimulation and not just an acute phenotype. We investigated the effect of DRAK2 on T cell function using primary T cells isolated from splenocytes from DRAK2-/- mice. While we report that following 48 hours of anti-CD3/28 activation, DRAK2-/- T cells exhibit enhanced CRAC channel activity, it is important to note that neither 24 nor 72hours post stimulation exhibited the same phenotype. Based on the current paradigm of the role of DRAK2 regulation by calcium following TCR-mediated activation, we were surprised to observe that increased calcium mobilization was not universally preserved following activation.

Interestingly, when it came to the examination of calcium mobilization in naïve cells, we report no difference following thapsigargin treatment however TCR-crosslinking induced by selective streptavidin concentrations did support enhanced mobilization. Thapsigargin is a pharmacological agent that releases ER stored calcium, which in turn promotes CRAC channel activity [32]. Whereas TCR-ligation activates PLCγ1 which promotes the binding of IP_3 to IP_3R's located on the ER, thus triggering Ca2+ release. We determined that very specific concentrations of stimulation were required to demonstrate enhanced mobilization in CD4+ T cells. Furthermore, CD8+ T cells do not appear to bear a defect in the calcium mobilization following TCR-driven CRAC activation.

As a caveat to this examination, one problem with using two different Ca2+ indicators, with different Kd's for Ca2+, is that they may reflect different subcellular loading patterns, thus no pixel-by-pixel ratioing should be preformed [33]. Of relevance, Floto and colleagues reported trouble with determining subcellular Ca2+ signals because of strong intercellular loading variations of Fluo-3 and Fura Red [33]. Usage of Fluo-4 instead of Fuo-3 paired with Fura Red would be advantageous given their similar intensity distribution.

Despite this caveat, these data suggest that the difference in Ca2+ mobilization is not intrinsic to the ER and support the hypothesis that DRAK2 may be associated with TCR proximal signaling. As an alternative hypothesis, DRAK2-deficiency may alter mitochondrial calcium buffer, which would in turn potentiate metabolic dysfunction. Given that previous research has examined TCR proximal signaling including NFAT and NFkB signaling and found no difference, we centered our efforts towards metabolic profiling. As a future experiment, specific inhibitors could be used to block calcium entry into the mitochondria. In the experiments presented here, we measured the total amount of

calcium released into the cytosol. However the calcium concentration at various cellular compartments may be altered in the absence of DRAK2, which may be undetectable by measuring total calcium. Therefore to better understand if the enhanced Ca2+ is promoting mitochondrial metabolism research should pair monitoring of mitochondrial calcium with mitochondrial calcium uptake inhibition. These studies would allow for the examination of the effect of loss of DRAK2 on mitochondrial calcium stores. Perhaps loss of DRAK2 causes increased uptake of mitochondrial calcium, thus promoting metabolic deregulation and apoptosis initiation. However future experimentation will be needed to validate this hypothesis.

To investigate whether DRAK2 deficiency altered metabolism, we paired acute stimulations with examinations of GLUT1 expression, glycolytic rate and glucose uptake. Based on the reports of defective survival in activated DRAK2-/- T cells, we expected to observe reduced metabolic activity. Instead, we detected an overall increase in the DRAK2-/- T cell's metabolic rate. However it is worthwhile to address that these studies had been previously reported in Jeniffer Hernandez's thesis, yet DRAK2-/- T cells did not exhibit elevated metabolism. These disparate data indicate that further stimulations modalities are required to determine not only the optimal activation for DRAK2-/- T cells but also to confirm whether acute signaling alters metabolic machinery following activation.

Previous reports have demonstrated that GLUT1 dependence differs depending on the T cell subset [17]. In addition to cMyc [34], the estrogen related receptor α (ERRα)[35], mTOR and mTORC1 have all been shown to promote Glut1 trafficking, glycolysis and lipid synthesis. Developmentally, T cell restricted deletion of mTOR kinase resulted in the functional generation of Tregs but inhibited the generation of effector T cells [36].

However, alternative reports have shown that mTORC1 deletion is sufficient to hinder regulatory T cell suppressive function [37, 38]. Given that DRAK2-/- T cells exhibited elevated GLUT1 expression, future research could be geared to determine the reliance of this transporter for proliferation by genetic deletion.

Given the enhanced calcium mobilization, GLUT1 surface expression, glycolytic rate and glucose uptake we hypothesized that DRAK2-/- T cells may exhibit a hypermetabolic profile. Therefore we examined protein expression of key metabolic genes and, to our surprise, found no difference. Mitochondrial membrane potential is an important parameter for modeling channel current activity because it affects the ability of charged particles to pass across a membrane. Despite multiple experiments supporting heightened metabolic activity in DRAK2-/- T cells, our data does not indicate a hyperpolarization within the mitochondrial membrane but it does indicate that T cells require a functional ETC in order to proliferate.

Lonidamine remains an attractive therapeutic agent given its capacity to promote energy metabolism in normal cells but inhibit metabolism in cancer cells [39]. Moreover, lonidamine has been demonstrated to suppress proliferation and metastasis in various cancers *in vitro*, *in vivo* and in clinical trials [40]. Yet the exact mechanism of lonidamine remains controversial. Reports have suggested that it is able to induce permeability transition by inhibiting either ANT or HK but can also be inhibited by Bcl-2 [41-43]. More recently, lonidamine has been proposed to inhibit complex II by interfering with the reduction of ubiquinone thus limiting oxidative metabolism [44]. Additionally, Halestrap and group have reported the lonidamine prevents the efflux of glycolytically derived L-

lactic acid and subsequent oxidation and the corresponding drop in intracellular pH inhibits glycolysis [45].

Despite the mechanism of action being unclear, lonidamine treatment of activated T cells resulted in a dose dependent reduction in recovery. Interestingly, treatment of wildtype cells with lonidamine caused a greater inhibition of proliferation than in the DRAK2-/- T cells. These data support the hypothesis that the altered metabolic activity of DRAK2-/- T cells may compensate for the metabolic inhibition elicited by lonidamine. As an alternative hypothesis, DRAK2-/- T cells may have a different distribution of mitochondrial versus cytosolic hexokinase. Therefore, while the mitochondrial hexokinase may be inhibited by lonidamine the cytosolic hexokinase may compensate. Future studies could include measurement of intracellular pH to provide evidence of the inhibition of lactic acid export from cells subsequent to lonidamine treatment. Hexokinase inhibition by lonidamine would result in a decrease of lactic acid production and would therefore not cause a decline intracellular pH.

Using pharmacological agents can have off target effects, which may contribute to T cell function, thus caution must be taken when interpreting the data. For example, rotenone has the ability to increase ROS resulting from the inhibition of Complex I [46, 47]. Alternatively, a genetic manipulation approach would allow for a more direct examination, in which specific T cell intrinsic alterations could be examined.

Although excluded from this dissertation, our initial experiments using anti-CD3 stimulation did in fact display a survival deficit in DRAK2-/- T cells. However, following the acquisition of a new lot of serum, we were unable to recapitulate those results. Although both serums were suitable for tissue culture work, the presence of hormones or nutrient

composition may have skewed T cell development. For example, the hormone leptin has been demonstrated to reduce the proliferation of human Treg cells and *in vitro* leptin neutralization during anti-CD3/CD28 stimulation enhances Treg proliferation [48]. These observations led us to appreciate the sensitivity toward micro-environmental conditions and resulting metabolically aberrant T cell profile. These findings may also provide further insight into why Hernandez and I report differential glycolytic activity following 24hour anti-CD3/28 stimulation. Despite no difference in recovery, it is possible that the altered metabolic efficiency may be differentially impacting subset differentiation.

Biological systems have feedback control, robustness and sensitivity built into network complexity. Sensitivity to apoptotic stimuli is a concern to maintain the proper immune balance and ability to respond to infections. Interestingly, DRAK2-/- mice have demonstrated robust resistance to EAE, which has been attributed to enhanced T cell effector death. Yet the mechanism behind the enhanced apoptosis has yet to be determined. The impaired T cell function in DRAK2 deficient T cells was not due to enhanced calcium mobilization, as we observed comparable recovery between DRAK2-/- and wildtype splenocytes. Yet we sought to determine whether the altered calcium was modulating T cell metabolism. Here, our approach to directly alter metabolic efficiency with pharmaceutical reagents allowed us to selectively promote oxidative metabolism and inhibit glycolytic differentiation. From these studies we determined that DRAK2-/- T cells did display altered metabolic activity however the basis for this perturbation has yet to be determined. Given that the phenotypic survival defect was not reproducible in this system under sub-optimal conditions we next sought to profile DRAK2-/- T cells under a more physiologically relevant signal.

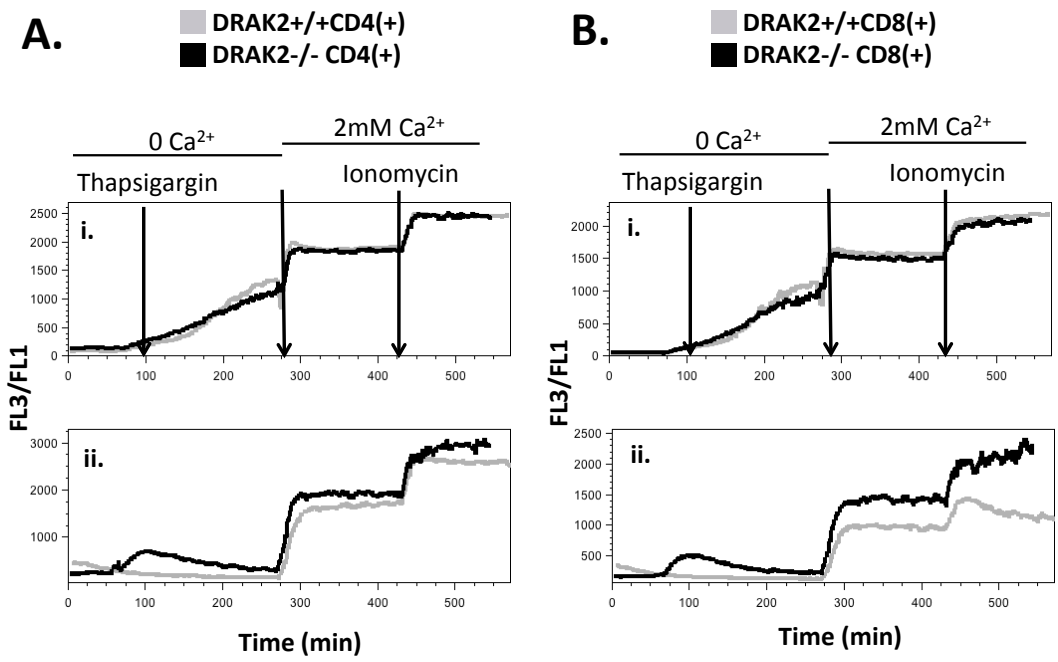


Figure 3.1: Enhanced calcium mobilization in activated T cells. Calcium flux examination through ratiometric analysis of Fluo-3 and Fura Red over time. Plots depict calcium mobilization in response to thapsigargin (1μM) administered to cells in HBSS without calcium followed by the addition of 2mM Ca2+. (A) Flow cytometry analysis of CD4(+) T cells. (B) Flow cytometry analysis of CD8(+) T cells. Cells were either left untreated (i) or stimulated for 48hrs with anti-CD3 and anti-CD28 (ii).

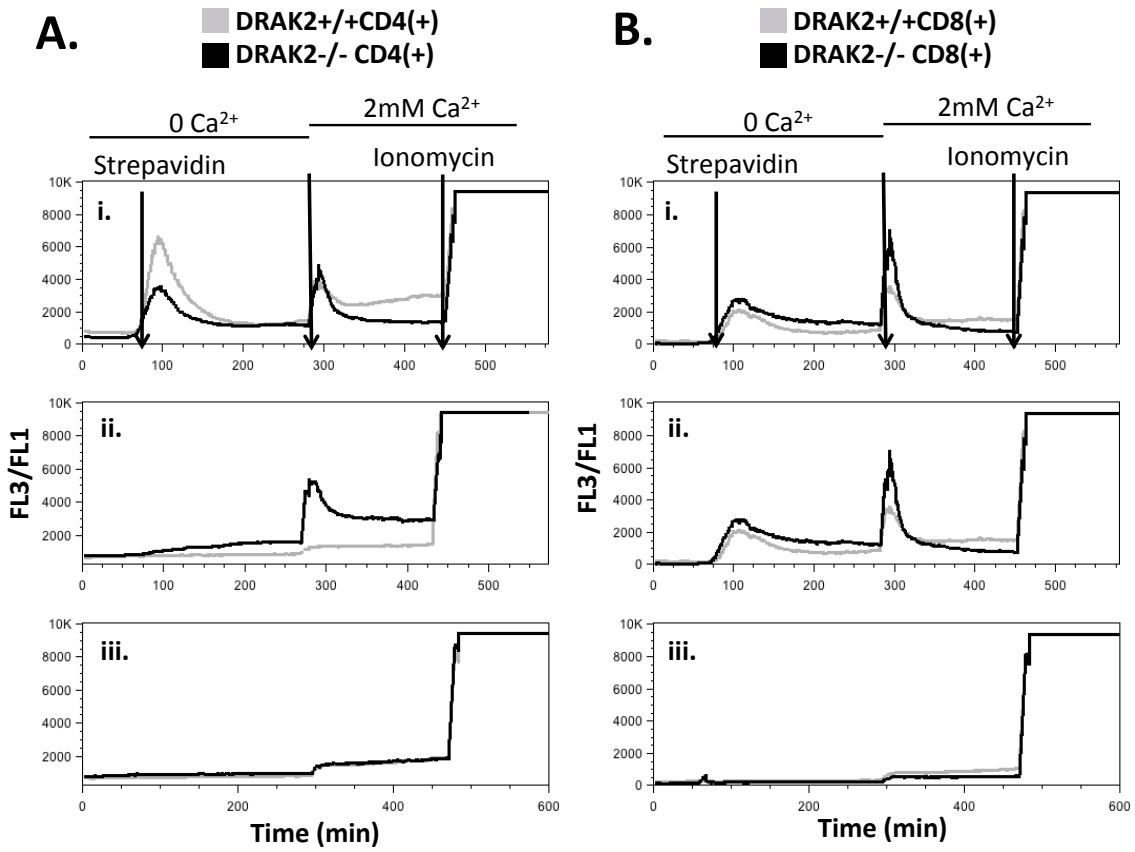


Figure 3.2: Loss of DRAK2 in naïve T cells does not lead to a universal increase in CRAC activation. T cells from DRAK2+/+ (grey lines) and DRAK2-/- (black lines) mice were purified and labeled with Fluo-3, Fura red, CD4 (A), and CD8 (B). Cells were incubated with various concentrations of biotin anti-CD3: 10ug/ml (i), 3µg/ml (ii), and 0.3µg/ml (iii). SOCE was monitored by flow cytometry and after establishing a baseline reading, streptavidin (40µg/ml) was added as a stimulus. Calcium flux was reported as a ratio of the change in fluorescence of the two dyes.

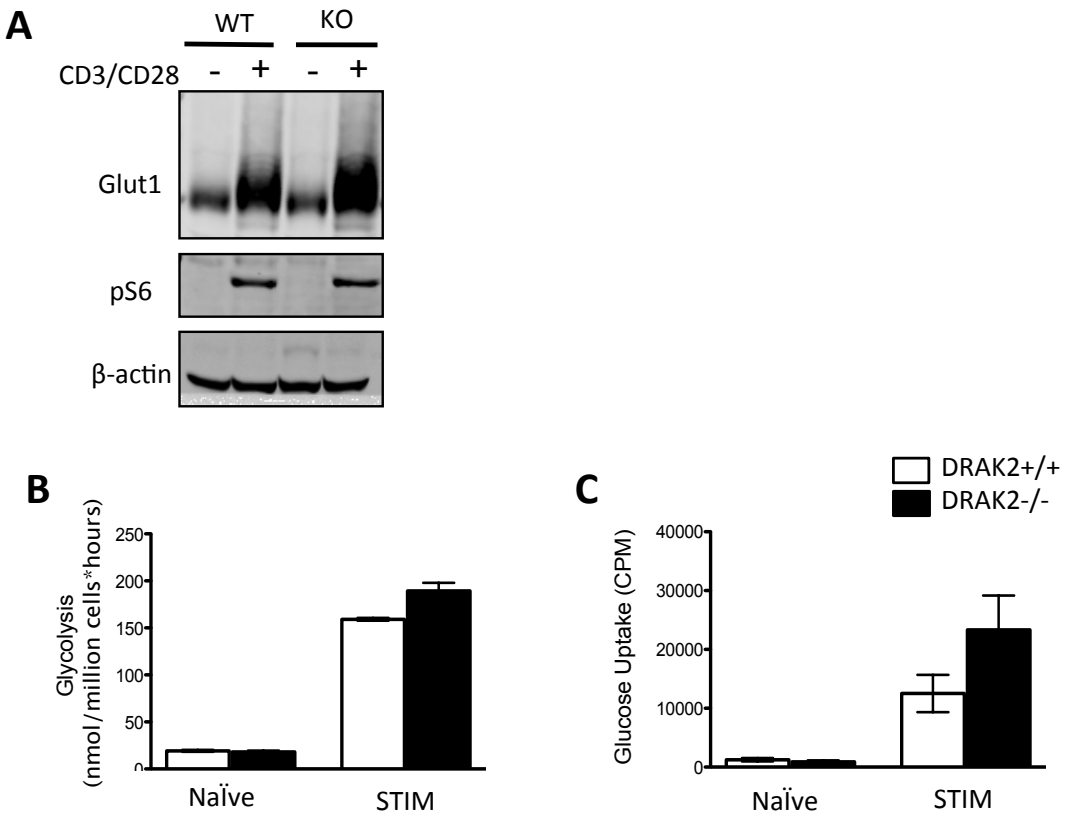


Figure 3.3: Loss of DRAK2 leads to altered metabolism in activated T cells. Naïve T cells were isolated from DRAK2+/+ (WT) and DRAK2-/- (KO) mice and were either left untreated or stimulated with anti-CD3 and anti-CD28 for 24hrs. (A) Immunoblot of GLUT1. Actin was used as a loading control. (B) The ability of T cells to utilize glucose was measured by the conversion of [5-3H]-glucose to 3H20 (*C*) T cells were harvested and their ability to uptake the nonhydrolyzable glucose analog [3H]2-deoxyglucose ([3H]2-DOG) was measured; units are in counts per minute (CPM). (B-C) Represent 2 independent experiments and graphs are displayed as an average.

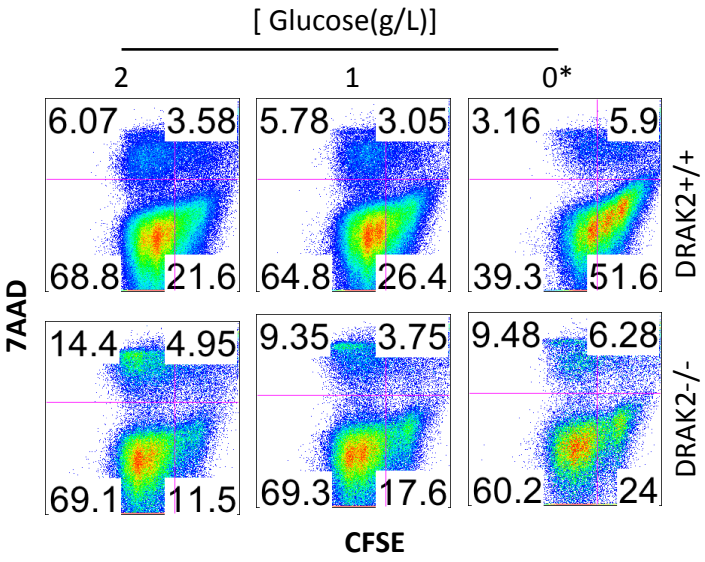


Figure 3.4: DRAK2-/- T cells are resistant to glucose titration. CFSE labeled-DRAK2+/+ and DRAK2-/- T cells were stimulated for 3 days with anti-CD3/28 in the presence of titrating glucose. Following stimulation, cells were labeled with CD4, CD8 and 7AAD. Representative plot shows the proliferation of total live T cells. *0 glucose conditioned media did not contain dialyzed serum.

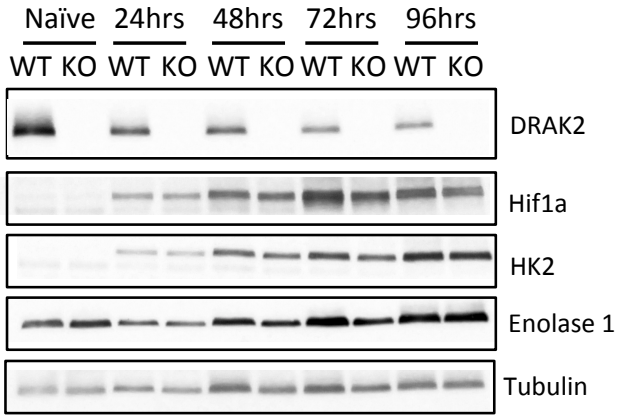


Figure 3.5: Expression of genes involved in metabolic reprogramming.
Immunoblot analysis of lysates from DRAK2+/+ (WT) and DRAK2-/- (KO) T cells stimulated with anti-CD3 and anti-CD28. 1 hour prior to the indicated time points, MG132 (10μM) was added to the culture to prevent proteasome-dependent degradation of HIF-1a.

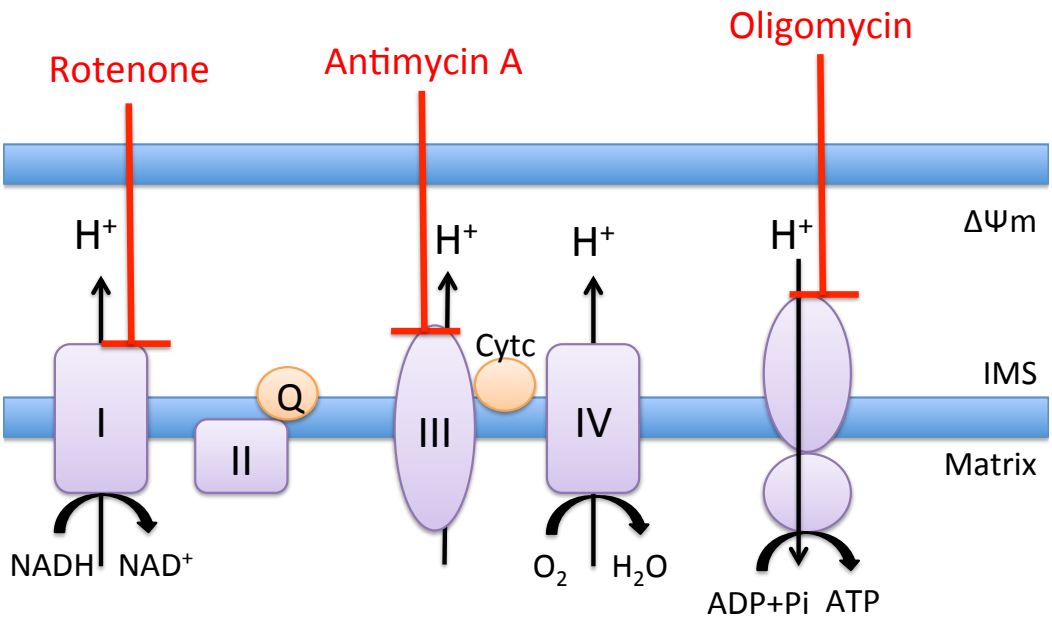


Figure 3.6: Proton gradient across the inter mitochondrial membrane. Diagram indicating the electron transport chain complexes along with their substrates and the inhibitors that target them (red text: rotenone, antimycin a, and oligomycin).

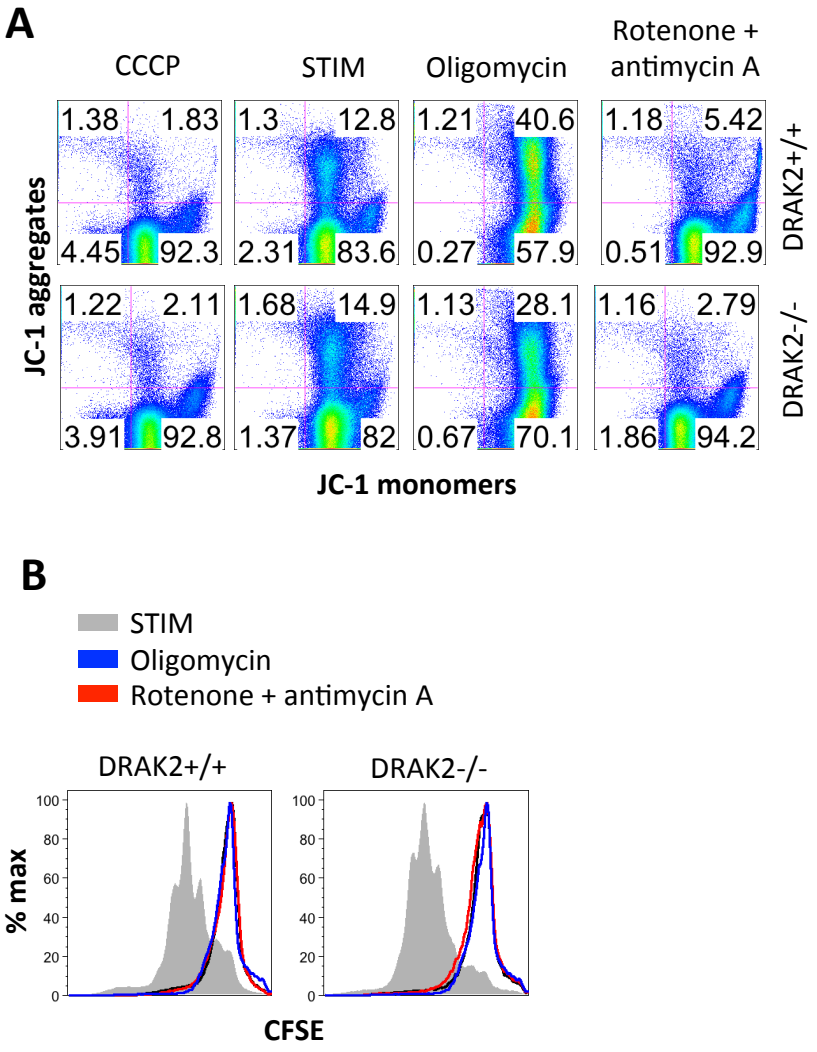


Figure 3.7: Functional ETC is necessary for T cell proliferation. DRAK2+/+ and DRAK2-/- T cells were stimulated for 3 days with anti-CD3/28. (A/B) 1 hour prior to collection cells were left untreated or treated with either CCCP, oligomycin (2μM) or rotenone (1μM) and antimycin A (1μM). (A) Cells were stained with the T cell marker CD3 and the mitochondrial membrane potential dye JC-1 then analyzed by flow cytometry. (B) CFSE-labeled T cells were stimulated in the presence of oligomycin (2μM) or rotenone (1μM) and antimycin A (1μM).

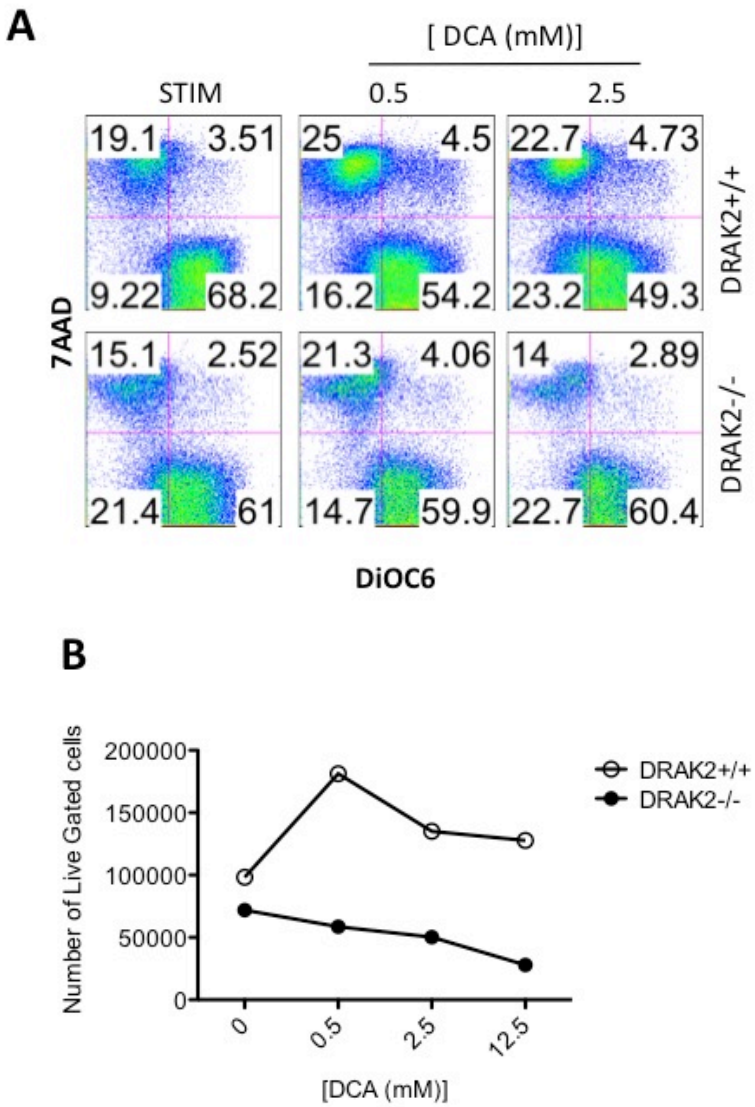


Figure 3.8: DCA treatment differentially polarizes T cells. Examination of mitochondrial membrane potential following stimulation with DCA. (A) Representative FACS plot stained with DiOC6 and 7AAD following 3 day anti-CD3/28 stimulation. (B) Recovery of live gated cells following treatment with DCA.

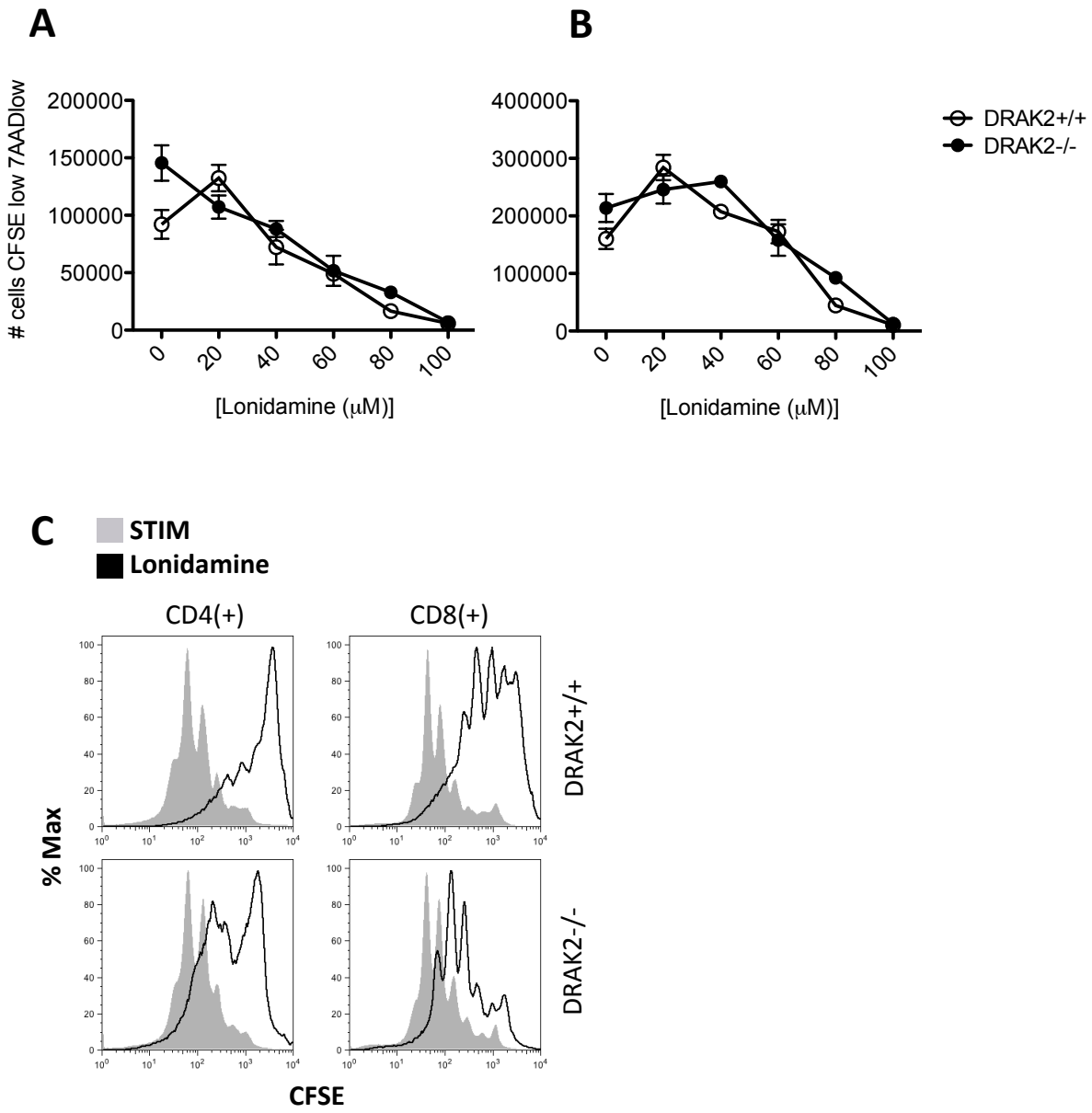


Figure 3.9: Inhibition of aerobic glycolysis differentially affects proliferative capacity of activated T cells. (A) T cells were labeled with CFSE and stimulated for 3 days with anti-CD3/28 in addition to titrating concentrations of lonidamine. (A) Number of live, proliferating CD4+ T cells. (B) Number of live, proliferating CD8+ T cells. (C) Representative CFSE overlay histograms from T cells were stimulated with lonidamine (80μM) for 3 days.

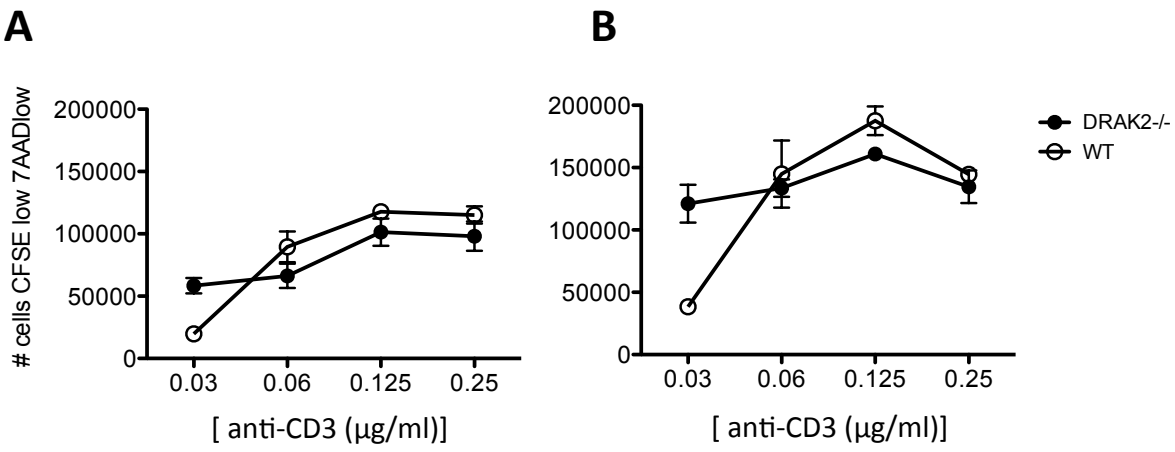


Figure 3.10: Phenotypic survival deficiency of DRAK2-/- T cells not found under titrating CD3 stimulation. Isolated T cells were activated for 3 days in the absence of CD28 under titrating concentrations of anti-CD3. (A) Recovery of live (7AAD-), proliferating (CFSE-) CD4+ T cells. (B) Recovery of live (7AAD-), proliferating (CFSE-) CD8+ T cells.

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CHAPTER 4

DRAK2 promotes T cell glycolytic differentiation upon SEB activation

Abstract

The lymphoid-restricted serine/threonine kinase DRAK2 is a negative regulator of TCR signaling and loss of DRAK2 in mice enhances allogeneic transplant survival and limits EAE progression. We report that following superantigen stimulation, Drak2-/- T cells exhibit defective proliferation, increased ROS and mitochondrial membrane hyperpolarization. Our work demonstrates that DRAK2 orchestrates changes in mitochondrial permeability that are essential for high rate aerobic glycolysis, blastogenesis and clonal expansion. Collectively, this study shows that T cells proliferate in response to stimulation through a positive feedback pathway involving mitochondrial ROS (mROS) that functions to enable rapid proliferation in a manner dependent on DRAK2. Thus, these studies define a function for DRAK2 as a mediator participating in the feedback to regulate mitochondrial permeability, metabolic state and initiation of cell division in activated T cells.

Introduction

In Chapter 3, the metabolic alterations associated with loss of DRAK2 were examined in anti-CD3/28 activated T cells. Although informative, these studies did not address the mechanism of cell death associated with loss of DRAK2. Evaluating DRAK2 function through a more physiologically relevant activation signal could therefore highlight pathways that were otherwise masked by artificial T cell activation. In this chapter, we explore how loss of DRAK2 alters mitochondrial remodeling and metabolic reprogramming during stimulation with Staphylococcal enterotoxin B (SEB). SEB activates a polyclonal T cell response by crosslinking the class II MHC of an antigen-presenting cell to the TCR of a T cell [1, 2]. Furthermore SEB has already been shown to induce abortive proliferation in activated DRAK2-/- T cells [3]. However, a potential mechanism to account for this survival defect has yet to be proposed.

The mitochondrion has been widely considered an essential hub of metabolic activity and cell death [4]. Activation induced by TCR ligation and costimulation metabolically reprograms T cells for rapid anabolic growth and biomass accumulation. A key point of metabolism regulation in lymphocytes is the control of nutrient uptake promoted by extrinsic signaling [5, 6]. Likewise, the phosphatidyl-inositol 3-kinase (PI3K)/Akt pathway, initiated upon T cell stimulation, has been shown to promote GLUT1 surface expression and activity [7, 8]. Upon insufficient stimulation, nutrient uptake decreases, leading to the inhibition of proliferation [5, 9]. Likewise, insufficient glucose can promote apoptosis in activated T cells through the activation of pro-apoptotic Bcl-2 family proteins [10].

Mitochondria are double-membrane organelles, consisting of inner [4] and outer mitochondrial membranes (OMM), separated by an intermembrane space (IMS). Spanning

both the outer and inner mitochondrial membrane, the mitochondrial permeability transition pore (mPTP) regulates the flow of solutes between the mitochondrial matrix and cytosol [5]. The structure of the mPTP has been widely debated, though the consensus molecular constituents that have been proposed include the voltage dependent anion channel (VDAC), the adenine nucleoside translocase (ANT) and Cyclophilin D (CypD) [11-13]. VDAC gating controls the passage of ATP and metabolites through the outer membrane [14]. This in turn enables, ANT to exchange ADP and ATP in a 1:1 stoichiometry between matrix and the cytosol. Lastly, CypD belongs to the family of cyclophilin proteins that induce conformational changes by catalyzing the cis-trans isomerization of proline amino acid peptide bonds [15].

The regulation of the mPTP is closely related to metabolic reprogramming and has been shown to be redox, Ca2+, voltage, adenine nucleotide, and pH sensitive [16, 17]. Mitochondrial permeability transition (MPT) refers to the persistent opening of the pore characterized by the uncoupling of the respiratory chain, overproduction of free radicals and mitochondrial swelling. Eventual rupture or mitochondrial outer membrane permeabilization (MOMP) triggers the release of apoptogenic factors thus promoting downstream apoptotic signaling. While MOMP typically promotes caspase-dependent apoptosis, reports document caspase-independent cell death probably driven by ROS production and mitochondrial dysfunction [18-21]. Although long-lasting mPTP opening can be deleterious, reversible opening of the mPTP during respiration, serves to periodically depolarize the inner membrane and allows the mitochondria to avoid matrix Ca2+ overload [22, 23]. Thus, these transitory openings allow intra- and extramitochondrial solutes to equilibrate.

Work described in this chapter addresses the *in vitro* role of DRAK2 in the regulation of mitochondrial membrane potential upon SEB stimulation. We show that upon stimulation DRAK2-/- T cells have an enhanced lag period and as a result a reduced fraction of cells enter blastogenesis. Gene expression data suggested that DRAK2-/- T cells had a defect in their ability to utilize aerobic metabolism. Indeed, DRAK2-deficiency led to an enhanced accumulation of small, hyperpolarized cells in addition to greater ROS, suggesting greater oxidative metabolism. Following the identification of ANT2 as a binding partner of DRAK2, studies confirmed that DRAK2-/- splenocytes had lower mPTP activity. Together, these data have identified the phosphorylation of ANT2 by DRAK2 as a key regulatory signaling step mediating the transition from oxidative metabolism to aerobic glycolysis.

Materials and Methods

Cell culture and inhibitor assays. Total splenocytes were isolated, single cell suspensions generated and cells were cultured in 96-well U-bottomed plates with 2ug/ml of SEB (Toxin Technology Inc.). Cells were maintained in RPMI 1640 supplemented with sodium pyruvate, nonessential amino acids, Penn strep-glutamine, 2-ME and FCS. After the various time points, cells were harvested and analyzed. Where indicated, 100μM MnTBAP (Sigma) or 12.5μM Q-VD-OPH (Sigma) was added to the culture.

Proliferation studies. Proliferation assays were based on eFluor670 (eF670; eBioscience) dilution. Cells were thoroughly washed with phosphate buffered saline (PBS), labeled with 5μM eF670 in PBS for 15 min at 37°C. After washing with PBS, cells were plated for stimulation.

Analysis of Mitochondrial Membrane Potential. In order to analyze mitochondrial membrane potentials (ΔΨm) cells were labeled with either 2μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes) or 50nM tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) in PBS for 15min at 37°C. After washing with PBS, cells were further processed for analysis using flow cytometry.

Determination of reactive oxygen species by flow cytometry. The intracellular level of superoxide generation by T cells was measured by loading cells with 20μM Dihydroethidium (HE; Molecular Probes) or with 5 μM MitoSOX™ Red (Molecular Probes)

for 15min at 37°C. After washing with PBS, cells were further processed for analysis using flow cytometry.

Measurement of mPTP opening. Mitochondrial PTP opening was assessed by the quenching of calcein-AM fluorescence by cobalt chloride. Cells were loaded with 10nM calcein-AM (Molecular Probes) and 400μM $CoCl_2$ (Fisher) at 37°C in the dark for 15min. calcein-AM is a membrane permeable fluorophore that diffuses to all subcellular compartments. Whereas $CoCl_2$ quenches calcein fluorescence from all subcellular compartments except the mitochondrial matrix due to it's impermeability to Co^{2+} cations. mPTP opening was indicated by a reduction in mitochondrial calcein signal as monitored by flow cytometry. Two controls were set by either leaving samples unstained or incubating with calcein-AM alone.

Measurement of mitochondrial content. Mitochondria were stained with the ΔΨm – insensitive fluorescent dye MitoTracker Green FM (Molecular Probes) at 100nM for 15 min at 37°C.

Gene expression array. T cells were isolated and cultured with wildtype APCs pulsed with 2μg/ml of SEB. Following 4 days of stimulation, Vβ7/8+ T cells were isolated and biotin-labeled cRNA was hybridized to an Affymetrix GeneChip mouse (430 v. 2.0) array. Triplicate RNA samples from wildtype and DRAK2-/- cultures were prepared by polling the T cells from 2 independent mice. Following analysis, the raw data was processed using Affymetrix software, and subsequent enrichments were preformed using iPathwayGuide.

Upon clustering genes based on regulation profile, heat maps were generated using the Morpheus software.

Calcium phosphate transfection of 293T cells. The cells were counted and seeded in plates 24h prior to transfection. The number of cells was calculated such that the cells would be ~ 70% confluent on the day of the transfection. A total of 20 µg DNA to be transfected was mixed with 50 µl 2.5M CaCl2 and made up to a volume of 500 µl with HBSS. Chloroquine (25µM) was added to the center of the petri dish followed by the transfection mixture, which was added drop-wise into the wells. Then the plates were shaken slowly side-to-side or forward and backward to evenly distribute the transfection mixture. The cells were incubated at 37°C for 12 hours. Lysates were harvested by trypsin dissociation. In general, the efficiency of transfection was over 80%.

Immunoprecipitation. Following transfection, cells were resuspended in HBSS and stimulated for 10 minutes with 1mg/ml of PMA at 37°C. Cells were then washed with HBSS, pelleted, and resuspended in 1ml of 0.25% formaldehyde solution. Cells were incubated with crosslinking solution at room temperature for 10 minutes under gentle agitation. Next, cells were placed on ice for 1 minute followed by pelleting and supernatant aspiration. The crosslinking was quenched by adding 1ml of 0.125M glycine solution. Following IP lysate preparation, 20 µl of 50% bead slurry was added to the cell lysate at 1mg/ml. Cells were incubated with rotation at 4°C for 60 min. The supernatant was transferred to a new tube and 1 µg of the pull down antibody was added and allowed to incubate overnight at 4°C. Next, cells were incubated for 2hrs at 4°C with 20 µl of 50% bead slurry. Following incubation, pellet was washed 5X with 500 µl of 1X IP lysis buffer.

Immunoblotting. Whole cell lysates were prepared by lysis in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, with phosphatase and protease inhibitors (Sigma). Protein concentrations were calculated using a BCA Protein Assay (Thermo Scientific). 50µg of whole cell protein was loaded per condition, separated by SDS-PAGE, and transferred to PVDF membranes for overnight probing with various antibodies in 5% BSA at 4°C. Bands were detected by incubating blots with HRP conjugated secondary antibodies for one hour at room temperature followed by ECL detection.

2D Gel Electrophoresis. During the isoelectric focusing, IPG strips (BioRad) were loaded passively during rehydration. 500µg protein sample in 260µl 2D protein solubilization buffer were loaded in strip holder and the IPG strip was placed on top followed by mineral oil. Moistened Whatman paper was placed between the ends of the strip and the electrodes to promote conductivity. Isolelectric focusing was performed using the following voltage scheme: (1) 250V, linear, 20min (2) 8000V, linear, 2hrs 30min (3) 8000V, rapid, 12hrs. After focusing, strips were either processed directly or frozen at -80°C. Second dimension electrophoresis was standard SDS-PAGE followed by transfer to PVDF membranes for overnight probing with various antibodies in 5% BSA at 4°C. Bands were detected by incubating blots with HRP conjugated secondary antibodies for one hour at room temperature followed by ECL detection.

Amnis Image Analysis. A different single color sample was acquired for each fluorophore in the experiment for the compensation matrix. Test samples were acquired, saved, and analyzed by the spot count feature in the IDEAS program. Spot count is a feature that counts the number of designated small masks in an image.

Results

SEB stimulation results in enhanced death and reduced blastogenesis in DRAK2-/- T cells following SEB stimulation

To investigate whether DRAK2 may contribute to survival signaling, wildtype and DRAK2-/- splenocytes were stimulated *in vitro* with the superantigen, SEB. Consistent with previous reports [3], DRAK2-/- T cells displayed a survival defect in response to SEB, as evidenced by decreased recovery (Fig. 4.1A). In order for cells to proliferate and enter into cell cycle, they increase macromolecular biosynthesis to increase cell mass. Thus, activation of T cells can be monitored by an increase in their size and granularity [24]. Upon stimulation, we found that DRAK2-/- T cells are relatively smaller and non-granular, whereas activated DRAK2+/+ T cells are both larger and display a higher granularity (Fig. 4.1B). These data suggest that DRAK2 may regulate survival through the progression of cell cycle. To test that hypothesis, we next examined the functional capacity of the responding T cells to enter into division.

To visualize cell division, purified splenocytes were labeled with eF670, stimulated and harvested at the indicated time points (Fig 4.1C). Given that DRAK2-/- T cells underwent reduced blastogenesis, we sought to determine if there was an intrinsic defect in cell division. At the 72-hour time point, while both wildtype and DRAK2-/- cells had entered into division, wildtype cells had a smaller fraction of undivided cells, thus supporting faster entry into cycle. Indeed, the observation of three to four cell divisions between 48 and 72 hours after stimulation suggests that the initial doubling times are ~6 hours. We hypothesized that if DRAK2-/- T cells had a defect in their ability to divide, then this would result in a progressive reduction in entering division. Interestingly, once

DRAK2-/- T cells engaged cell division, they appeared to divide at the same rate as the wildtype control. Taken together these data suggest that DRAK2-/- T cells have a problem with the initial commitment to divide rather than an intrinsic cell division rate issue.

Blockade of ROS production by MnTBAP does not enhance the recovery of activated DRAK2-/- T cells

Mitochondria tightly balance the production and detoxification of ROS by regulating oxidative metabolism [25, 26]. Previous studies have demonstrated that DRAK2 participates in a negative feedback loop mediated by Ca2+ mobilization and regulation of ROS [27]. Interesting, SEB activated T cells have been shown to die by a Fas- and TNFα-independent apoptosis characterized by enhanced ROS and cell membrane permeability [28]. Consistent with enhanced oxidative metabolism, flow cytometry with the mitochondrial oxidant probe MitoSOX Red showed that production of mitochondrial oxidants was increased upon loss of DRAK2 (Fig. 4.2).

To determine whether the enhanced ROS was inducing cell death, activated cells were cultured with the cell permeable superoxide dismutase (SOD) mimetic, Mn(III)tetrakis (4-benzoic acid) porphyrin, MnTBAP, which acts as a catalytic antioxidant (Fig 4.3). As shown in Figure 4.3B, MnTBAP caused a reduction in the generation of mitochondrial ROS. However MnTBAP was not sufficient to overcome the survival deficit (Fig. 4.3A). These results indicate that the ROS associated with loss of DRAK2 are not causing the survival defect.

DRAK2 deficiency promotes hyperpolarization

To obtain further evidence supporting that loss of DRAK2 leads to mitochondrial dysfunction upon T cell activation, we assessed ΔΨm. ΔΨm is a critical parameter determining the viability of a mitochondrion because it regulates respiratory activities and ATP synthesis. ROS production is also known to be regulated by the ΔΨm [29]. We hypothesized that the mitochondria of activated DRAK2-deficient T cells may be functionally compromised due to the elevation in ROS. Stimulated cells were treated with the ratiometric fluorophore 5,5′,6,6′- tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and examined by flow cytometry (Fig. 4.4A,B). JC-1 can selectively enter the mitochondria as a monomer where it reversibly changes color as a result of membrane potential. Hyperpolarization of mitochondria increases the aggregate fluorescence (red, 590nm emission) and depolarization raises the monomer fluorescence (green, 530nm emission).

As shown in Figure 4.4A, DRAK2-/- T cells had a greater fraction of polarized cells compared to the wildtype control. It has been shown that there is a correlation between the ratio of JC-1 red to green fluorescence and the membrane potential of mitochondria [30, 31]. Thus, the JC-1 fluorescence ratio was evaluated and DRAK2-/- T cells exhibited a significantly higher ΔΨm (Fig. 4.4B). To further corroborate the increase in ΔΨm, we stained activated cells with the potentiometric lipophilic cationic dye, Tetramethylrhodamine Methyl Ester (TMRE). As expected, we found that DRAK2-/- T cells displayed enhanced levels of TMRE (Fig 4.4D). Enhanced membrane staining could be attributed to a greater number of mitochondria. Therefore, as a control, we evaluated mitochondrial content using MitoTracker Green, which localizes with mitochondria

irrespective of ΔΨm, and found no significant difference between DRAK2-deficent and wildtype T cells (Fig 4.4C).

To further characterize the polarized cell populations, forward scatter and membrane potential dyes were gated against one another (Fig 4.5A,C). Next, we quantified the number of cells recovered from each polarized gate (Fig 4.5B,D). In accord with the blasting phenotype, we found that DRAK2-/- splenocytes had a markedly reduced fraction of large, intermediate ΔΨm cells. Interestingly, we found that the reduced recovery phenotype was not simply a result of increased cell death but rather that the DRAK2-/- splenocytes appeared to accumulate as small, polarized cells. As previously stated, upon T cell stimulation, metabolic reprogramming favors aerobic metabolism over oxidative phosphorylation.

Given this, we next sought to determine if DRAK2-deficiency altered the ability of activated T cells to reprogram their metabolism.

DRAK2 promotes the expansion of glycolytic cells independent of mitochondrial remodeling

In order to explore whether the transition in ΔΨm corresponded with the Warburg effect, a gene array was performed using RNA from 4 day SEB stimulated T cells. We reasoned that if dysfunctional metabolism was related to the proliferative defect in activated DRAK2-deficient T cells, this should be detectable in gene expression profiles. After sorting the top 5000 genes based on most statistically significant fold change between DRAK2-/- and wildtype T cells, metabolism associated genes were enriched by pathway analysis (Fig 4.6). From this clustering we found 42 statistically significant genes, 24 of which were up-

regulated and 18 down-regulated. It is important to note that not only was the key glycolytic transcription factor, HIF-1α, down regulated, but also many of its downstream targets including Pdk1 and Slc2a1 were also down. These data are consistent with DRAK2-/- T cells exhibiting altered aerobic glycolysis. Additionally there were no cell division genes that appeared to be differentially regulated. Overall, these data support that DRAK2 deficient T cells exhibit altered metabolic signaling which may explain the delay in initiating cell division and reduction in ΔΨm.

To directly test whether metabolic reprogramming was promoting the transition of small, high ΔΨm to large intermediate ΔΨm cells, we treated the cells with the glycolytic inhibitor dichlotoacetate (DCA). DCA indirectly inhibits glycolysis by promoting the conversion of pyruvate into acetyl-CoA. Specifically, DCA inhibits PDK, which is an inhibitor of PDH, the enzyme responsible for converting pyruvate into acetyl-CoA. Consistent with a role in promoting oxidative metabolism, activated T cells displayed enhanced polarization within their mitochondrial membrane (Fig 4.7A). Interestingly, the addition of DCA reduced the fraction of large, intermediate potential cells and increased the fraction of small, hyperpolarized cells (Fig 4.7B). The failure of DCA treatment to differentially induce polarization between DRAK2-/- and wildtype cells suggests that PDK's are not a direct target of DRAK2. These data support the hypothesis that switching to a glycolytic metabolism allows for the accumulation of biomass and reduction in ΔΨm.

Interestingly, different T cell subsets have been associated with different mitochondrial phenotypes. Effector T cells have more punctate mitochondria whereas memory T cells have a greater fused network [32, 33]. Fusion of mitochondria not only promotes the formation of supercomplexes within the ETC thus stimulating oxidative

metabolism but it also enhances membrane associated ER contacts, which is important for Ca2+ flux [34, 35]. Given that DRAK2-deficiency leads to enhanced calcium mobilization, elevated ROS and mitochondrial hyperpolarization, we hypothesized that DRAK2-/- T cells would have a greater proportion of fused mitochondria, thus favoring oxidative phosphorylation. To investigate whether DRAK2-/- T cells exhibited altered mitochondrial density and distribution, we stimulated cells for 4 days with SEB and analyzed them by ImageStream (Fig 4.8). By using the spot count feature, within the IDEAS software, we were able to identify pixel staining that corresponded to multiple mitochondrion within a cell. A demonstrative example comparing DRAK2+/+ and DRAK2-/- T cells with high mitochondrial content is shown in Figure 4.8A. A software algorithm then calculated the number of spots per cell and created a histogram based on the distribution (Fig 4.8B). Interesting, both DRAK2+/+ and DRAK2-/- T cells had a similar distribution of mitochondrial content. These observations demonstrate the altered polarization and proliferative capacity of DRAK2-/- cells cannot be attributed to altered mitochondrial morphology.

Blockade of caspase signaling with QVD fails to overcome survival defect in DRAK2-/- T cells

The observed mitochondrial membrane potential disruption may be related to enhanced cell death. To test this hypothesis, we treated cells with the pan-caspase inhibitor, Quinoline-Val-Asp-Difluorophenoxymethyl Ketone (QVD) upon stimulation with SEB (Fig 4.9). Although QVD treatment had no effect on the JC-1 aggregate to monomer distribution in wildtype cells, it did appear to have a minor reduction in the JC-1 aggregate

accumulation in DRAK2-/- splenocytes (Fig 4.9A). Further examination of the polarized cell fractions based on cell size revealed that while QVD enhanced the overall recovery of activated T cells, it did not promote the generation of large, intermediate ΔΨm cells (Fig 4.9B,C). Similar results were obtained using the pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone, Z-VAD-FMK (data not shown). Collectively these data suggest that caspase activation does not inhibit the generation of large, intermediate ΔΨm cells.

Costimulation with CD28 is sufficient to restore the proliferation and survival of activated DRAK2-/- T cells

While both ROS attenuation and inhibition of caspases were not sufficient to overcome the survival deficit or normalize the mitochondrial membrane potential, we next sought to alter the signal strength. The rate of glucose uptake in activated T cells increases as the strength of antigenic stimulus increases [36]. Moreover, costimulation with CD28 has been documented to promote the glycolytic switch by inducing the PI3K-dependent activation of AKT [8, 37]. To determine the impact of signal strength on driving metabolic reprogramming, cells were labeled with the proliferation dye eF670 and following stimulation stained with TMRE (Fig 4.10A). Interestingly, examination of the differentially sized polarized subsets revealed that, small, hyperpolarized DRAK2-/- splenocytes were unable to proliferate (Fig 4.10B). However, promoting signal strength with CD28 enabled the proliferation of small, hyperpolarized DRAK2-/- splenocytes, thus restoring the accumulation of large, intermediate membrane potential cells (Fig4.10B,C). These data suggest that DRAK2-/- T cells are unable to generate large, intermediate ΔΨm cells as a

result of defective proliferation found within the small, hyperpolarized subset. Taken together, these data support that costimulation can promote the proliferation of polarized cells and allow glycolytic metabolic reprogramming that lowers the membrane potential.

DRAK2 phosphorylates ANT2

To better understand the role of DRAK2 in T cell signaling, we next sought to isolate and characterize the unknown binding partners of DRAK2. Initially, we performed yeast two-hybrid and co-immunoprecipitation studies but these were met with limited success. Kinase-catalyzed protein phosphorylation is an important post-translational modification that controls a variety of cellular functions. However, kinase-substrate interactions are often transient and must therefore be stabilized. For this purpose, formaldehyde was used to promote crosslinking and stabilization of transient interactions [38]. Following transfection, DRAK2-Myc-DDk 293T cells were immunoprecipitated with an anti-DRAK2 antibody and subjected to western blotting (Fig 4.11). Interestingly, a high molecular weight band (~100kDa) was detected upon crosslinking with formaldehyde. No complex was detected in the control, in which cells were not crosslinked. Given that the other components of this complex were unknown, we next sought to identify them.

To interrogate the signal transduction network and associated binding partners of DRAK2, we preformed mass spectrometry comparing DRAK2-Myc-DDk transfected 293 T cells against an untransfected control. Overall, our complex background consisted of 205 proteins from non-transfected cells and included 246 proteins from transfected samples. Out of this background we identified 44 unique proteins from the transfected 293T cells using a threshold enrichment where the false discovery rate was set at $p<0.05$ (Table 4.1).

The false discovery rate is calculated by dividing the number of false positives by the total number of hits [39]. While DRAK2 had the most identifications, there were several mitochondria associated hits including ANT2, PGAM5, and AIF. Of these, ANT2 was the second most abundant protein detected by mass spectrometry. Given that ANT2 is a functional component of the mPTP and we had established a hyperpolarization phenotype within DRAK2-/- T cells, we sought to further characterize the interaction between DRAK2 and ANT2.

As a measure of validation, we preformed immunoprecipitations on both thymocytes and 293T cells that had either been left untreated or transfected with DRAK2-Myc-DDk and blotted for ANT2 (Fig 4.12). In the 293T cells transfected with DRAK2-Myc-DDk and immunoprecipitated with anti-myc, lane 3, we found expression of ANT2 (Fig 4.12A). Similarly, thymoycte immunoprecipitation revealed a modest expression of ANT2 (Fig 4.12B). However it is noteworthy that DRAK2 is developmentally regulated and as a result, we would expect lower DRAK2 activity in thymocytes then in cells that have been ectopically expressed. Collectively these data support that upon immunoprecipitation of DRAK2, the resulting lysate is enriched with ANT2.

To identify whether DRAK2 phosphorylated ANT2, we decided to isolate thymocytes from either WT or DRAK2-/- mice and subject their proteins to 2D gel electrophoresis. Given that 2D gels separate proteins according to two properties, molecular weight and pH, they result in better separation than 1D gels. As shown in Fig 4.13, the protein spot corresponding to ANT2 is indicated with an arrow. Consistent with our hypothesis, the DRAK2-/- thymocytes exhibit a more alkaline protein spot when compared to wildtype sample. Thus, we identified a charge variant suggestive of

phosphorylation of ANT2 by DRAK2 as evidenced by the acidic isoelectric point. Collectively these data support the hypothesis that upon activation, DRAK2 phosphorylates ANT2, which in turn promotes aerobic glycolysis and diversion of pyruvate into biomass synthesis.

DRAK2 promotes mPTP activity

By not only acting as a translocase, but also as a functional component of the mPTP, ANT is intricately involved in the control of cell fate. mPTP activity was detected in stimulated splenocytes by monitoring the movement of the membrane-impermeant fluorescent dye, calcein-AM out of the mitochondria upon mPTP opening. Representative histograms demonstrate that DRAK2-/- cells exhibit an enhanced retention of calcein-AM compared to the wildtype cells suggesting a lower pore conductance (Fig 4.14A). While the relative differences may not appear striking, this was found under all stimulations. Furthermore, analysis of the differentially polarized subsets demonstrated that between 72 and 96 hours, small, hyperpolarized wildtype splenocytes underwent opening of the mPTP whereas DRAK2-/- splenocytes maintained their enhanced retention (Fig 4.14C). Taken together with previous findings, these data suggested that DRAK2 promotes aerobic glycolysis by promoting mPTP activity through the phosphorylation of ANT2.

To evaluate the kinase activity of DRAK2 in a heterologous system, DRAK2- or empty-pMIT plasmids were transiently transfected in 293T cells by the calcium phosphate method (Fig 4.15). While we detected successful transfection as evidenced by Thy1.1 expression (Fig 4.15A), overexpression of DRAK2 did not alter the mitochondrial membrane potential (Fig 4.15B). Interestingly, examination of the mPTP revealed that

DRAK2-overexpressing 293T cells had a much lower retention of calcein-AM (Fig 4.15C), suggesting that DRAK2 promotes the opening of the mPTP. Furthermore, we demonstrated that calcein-AM worked as an accurate reporter of mPTP activity because upon pharmacological inhibition using cyclosporine A (CsA), we were able to increase the MFI of calcein-AM (Fig 4.15E). Collectively, these data support that DRAK2 promotes pore opening.

Pharmacological opening of the mPTP by ionomycin promotes expansion whereas treatment with mPTP inhibitor drives polarization

Given that the DRAK2-/- splenocytes appeared to have a defect in opening their mPTP, we next asked how pharmacological preconditioning would alter cell survival. To that end, stimulated cells were cultured in the presence of atractyloside, mPTP opener, or bongkretic acid, mPTP inhibitor [40, 41]. Given that neither drug was capable of inducing a shift in either ΔΨm or calcein-AM fluorescence we next sought to determine if the drugs were directly modulating pore activity (data not shown). However, when either activated cells or naïve cells were acutely treated with atractyloside or bongkretic acid neither drug resulted in a reproducible shift in calcein-AM fluorescence (data not shown). Given these inconclusive results, we next sought to utilize endogenous mPTP openers.

Calcium has long been known to act as a mediator promoting mPTP opening [42, 43]. To that end, we evaluated the ability of the calcium ionophore, ionomycin, to promote mPTP opening in thymocytes (Fig 4.16). Consistent with calcium, ionomycin addition resulted in a dose dependent reduction in the MFI of calcein-AM, thus supporting mPTP opening (Fig 4.16A). Having established a positive role for ionomycin in opening mPTP, we

next questioned how T cell activation would be altered in the presence of ionomycin. We hypothesized that if ionomycin was sufficient to induce mPTP opening then it would promote the generation of large, low ΔΨm cells. In accord with this hypothesis, we found that ionomycin promoted the expansion of both low and high ΔΨm large cells (Fig 4.16C).

Given that the mPTP appeared to promote the accumulation of biomass, we turned our attention to short term mPTP inhibition. Splenocytes were activated for 72 hours with CsA addition to the culture following 48 hours (Fig 4.17). In accord with our hypothesis, CsA addition resulted in an increased fraction of small, hyperpolarized cells (Fig 4.17B). Furthermore, evaluation of calcein-AM retention revealed that CsA increased the MFI of calcein-AM in both DRAK2-/- and to a greater degree DRAK2+/+ splenocytes (Fig 4.17C). Taken together, these data support that the mPTP opening promotes generation of biomass for proliferation.

These studies provided evidence, although indirect, that during T cell activation the mPTP was responsible for the reduction in ΔΨm. Thus, upon T cell activation the increase in mitochondrial matrix Ca2+ promotes the Krebs cycle, which in turn generates ROS (Fig 4.18). Mitochondrial generation of ROS has been shown to be sufficient for DRAK2 activation, which has now been shown to phosphorylate ANT2 and promote transient opening of the mPTP. The opening of the mPTP complements metabolic reprogramming and the diversion of energy towards biomass production.

Discussion

Although activated T cells primarily rely on glycolysis for metabolism, the signals that drive the induction of aerobic glycolysis remain unclear. In this study, we investigated how loss of DRAK2 alters mitochondrial events regulating metabolic reprogramming and leads to enhanced cell death upon stimulation with SEB. While the exact mechanism remains unclear, we determined that DRAK2 signaling supports the expansion of activated T cells by promoting glycolytic metabolism. We identified ANT2 as a novel binding partner of DRAK2, which facilitated mPTP activity and a favorable reduction in $\Delta\Psi m$ that allowed biomass synthesis. Taken together, this work demonstrates the importance of mPTP signaling for metabolic homeostasis.

Naïve T cells rely on metabolism for basic housekeeping functions whereas activated T cells prioritize diversion of energy into synthesis of biomass. Therefore, the changes associated with T cell activation include a complex rewiring of catabolic signaling. The reprogramming of cellular metabolism towards macromolecule synthesis is critical to supplying enough intermediates for a cell to divide. During the period of cell growth prior to the first cell division, lag phase, T cells upregulate synthesis of proteins and lipids [44]. The lag phase may represent a period wherein the innate immune response has the opportunity to mitigate an infection [45]. If successful, this would limit the circulating antigen thus promoting a less inflammatory environment. As we, and others have previously reported, after an initial lag period activated T cells rapidly divide [45-47].

The metabolic transcriptome undergoes global changes in response to metabolic reprogramming. For example, activation of HIF-1α leads to the inhibition of oxidative metabolism by promoting the expression of PDK1 which inhibits PDH activity [48]. As a

result of PDH inhibition, pyruvate diverts toward the production of lactate and synthesis of intermediates. Although dispensable for the initial metabolic reprogramming following stimulation, HIF-1α remains necessary for Th17 differentiation [49, 50]. Previous studies have found that the reduction in differentiation of Th17 CD4+ T cells in HIF-1α -deficient CD4+ T cells was concurrent with an increase in differentiating regulatory T cells [50]. Based on the gene expression profiles and accumulation of small, hyperpolarized cells, we hypothesize that DRAK2-/- T cells are unable to properly differentiate into glycolytic effector T cell subsets. In contrast to this hypothesis, we have generated data showing that the *in vitro* Th17 skewing of DRAK2-/- T cells remains unaffected compared to wildtype control [3]. However these studies were done with anti-CD3 stimulation, which as shown in Chapter 3, is sufficient to overcome the survival defect. Whether or not DRAK2-deficiency alters T cell subset effector differentiation upon SEB stimulation has yet to be determined but preliminary data suggests enhanced T cell death in DRAK2-/- T cells is mediating EAE resistance.

One of the hallmarks of the adaptive immune system is the ability of T cells to respond to foreign antigen by growing, proliferating and differentiating into effector lineages. Balance between regulatory and effector T cells is crucial to elicit immune protection without promoting autoimmunity. Interestingly, effector T cells have been shown to strongly induce glycolysis whereas memory and regulatory T cells have an oxidative metabolic profile [51-53]. Given that these subsets have their respective metabolic program, there has been speculation into whether modulation may provide a way to prevent or suppress autoimmunity.

Upon T cell activation, mitochondria undergo biogenesis and remodel their structure through fission and fusion events [54]. Interestingly, effector T cells have more punctate mitochondria whereas memory T cells have a greater fused network [32, 33]. Fusion of mitochondria not only promotes the formation of supercomplexes within the ETC thus stimulating oxidative metabolism but also enhances membrane associated ER contacts, which is important for Ca2+ flux [34, 35]. Likewise, memory T cells rely on FAO for survival whereas effector T cells utilize aerobic glycolysis. Failure to engage specific metabolic programs impairs the differentiation of T cells [55]. While we did not observe a defect in either mitochondrial fusion or fission, we did generate data suggesting that DRAK2-/- T cells have a defect in their aerobic glycolytic reprogramming. Available evidence points to a transient activation of the mPTP, by DRAK2 through ANT2, as a prime mediator of metabolic transitioning. Thus, it is likely that while mitochondrial morphology can correlate with metabolic preference, these two are not mutually exclusive.

By not only acting as a translocase but also as a functional component of the mPTP, ANT is intricately involved in the control of cell fate. The physiological role of ANT is to catalyze the exchange of ATP/ADP across the mitochondrial membrane [56]. By coupling cytosolic delivery of ADP to mitochondrial ATP synthesis, ANT plays a central role in energetic metabolism. The question as to whether ANT can function independently or requires the association of additional binding partners has yet to be defined. When incorporated into liposomes, ANT acts as a Ca2+ responsive non-selective high conductance channel [57]. Overexpression studies found ANT1 and ANT3 facilitate mitochondrial permeability transition driven apoptosis through caspase activation [58, 59]. Examination of proteoliposomes containing ANT demonstrated that pore function was

favored by Bax and inhibited by Bcl-2, whereas, in contrast, the translocase activity was favored by Bcl-2 and inhibited by Bax [60, 61]. Additional studies revealed that the Bcl-2 family of proteins bind to the VDAC in order to regulate the mitochondrial membrane potential and the release of cytochrome c during apoptosis [62]. Interestingly, mouse liver mitochondria lacking ANT1 and ANT2 still exhibit a CsA-sensitive permeability transition, thus ANT's appear to have a dispensable role in mPTP formation [63]. Moreover, mitochondria isolated from ANT-knockout mice respond to both MPT activation and inhibition with Ca2+ and CsA respectively [63]. The studies described in this thesis are among the first efforts to comprehensively examine the mechanism whereby the mPTP is involved in the metabolic reprogramming associated with T cell activation.

Previous work has established that DRAK2 localizes to the mitochondria and becomes activated as a result of mitochondrial ROS [27]. Here, we have demonstrated that DRAK2 also serves to regulate the mPTP through its phosphorylation of ANT2. Interestingly, mPTP opening has been associated with both increased Ca2+ and ROS yet the exact mechanism remains elusive [64-66]. Reports have also implicated complexes I and III of the ETC as regulators of the mPTP [67, 68]. Therefore, we propose a new mechanism whereby T cell activation promotes Ca2+ flux, which in turn promotes oxidative metabolism, thus generating ROS. These ROS trigger the activation of DRAK2, which phosphorylates ANT2 thus promoting mPTP activity. As a consequence, DRAK2 serves as a candidate drug target to specifically alter autoimmune and alloimmune responses by preventing the expansion of glycolytic effector T cells.

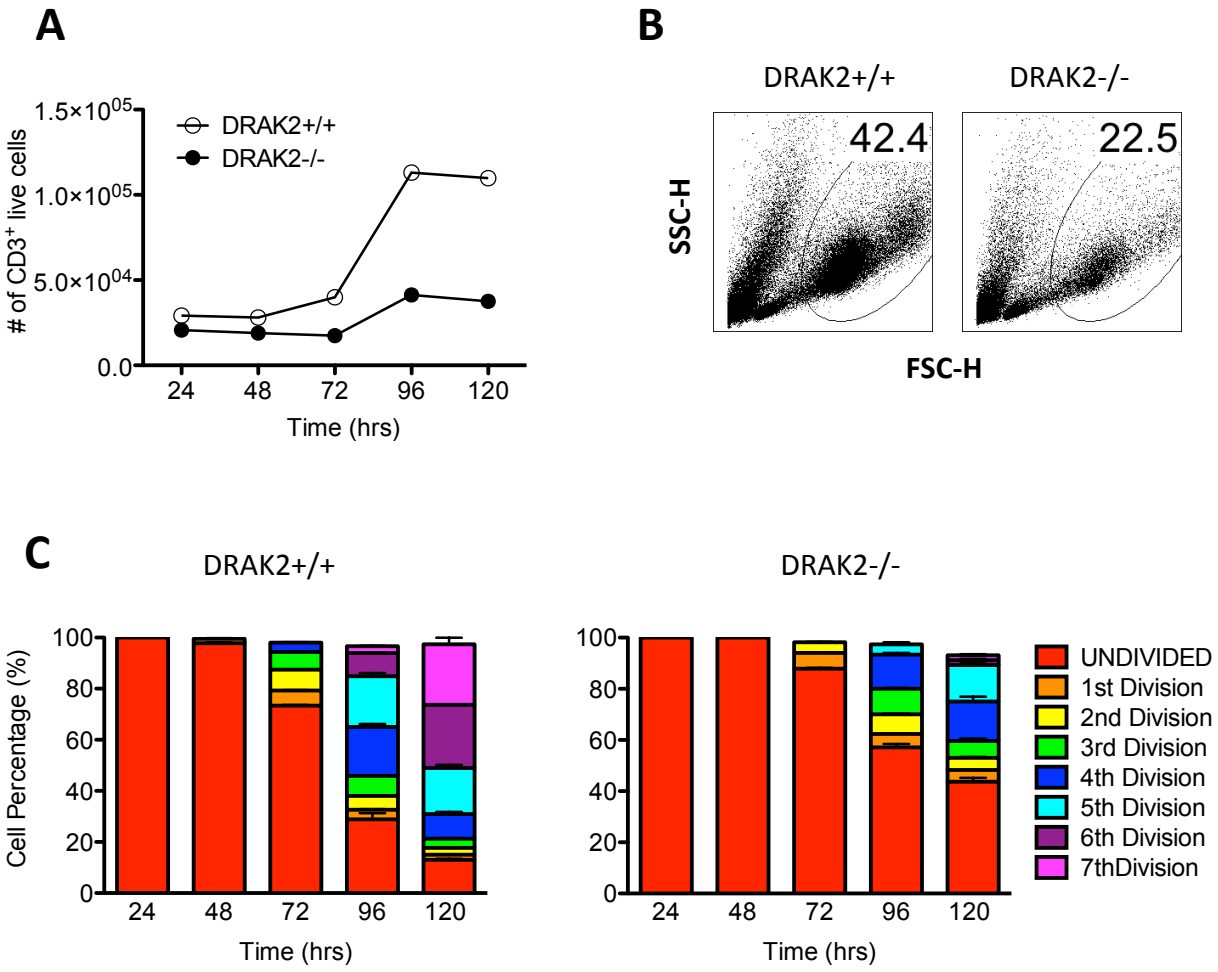


Figure 4.1: Survival deficit within DRAK2-deficent T cells upon SEB stimulation. At the indicated times cells were harvested, labeled with anti-CD3, and live cells (as judged by forward-scatter and side-scatter criteria) were analyzed by flow cytometry.(A) Time course of CD3 recovery following stimulation expressed as mean +/-SD of triplicate cultures. (B) Representative dot plot example of blasting lymphocytes (CD3+) identified by forward (FSC) and side scatter (SSC) following 72 hour stimulation. Numbers in the FACS-gated regions show percentage of lymphocytes undergoing blasting. (C) At the indicated time points, percentage of cells in each stage of division was calculated based on the elution of eF670. Data are representative of one independent experiment expressed as mean ± SEM.

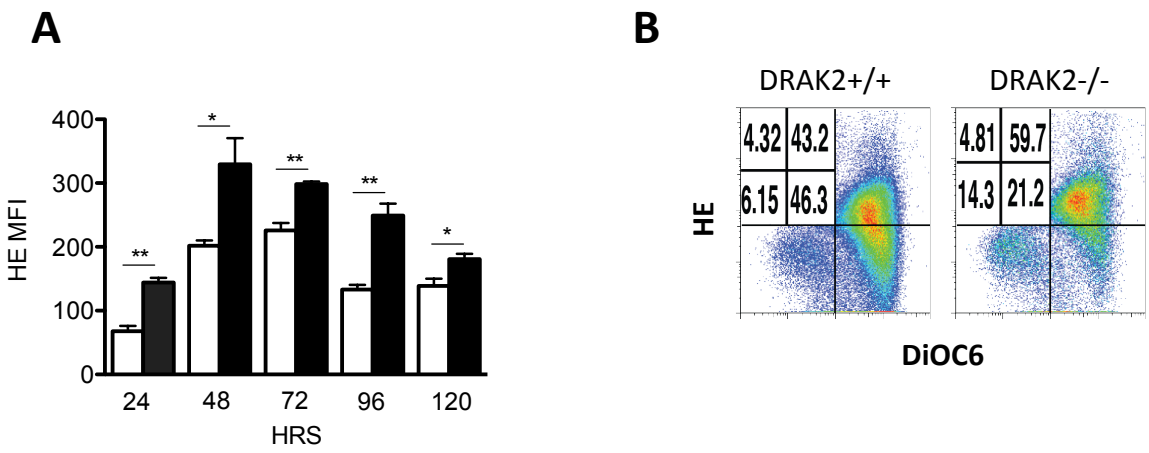


Figure 4.2: Elevated ROS production within DRAK2-/- T cells.
Following stimulation with SEB, activated T cells were collected and stained for flow cytometry. (A) Measurement of ROS levels by dihydroethidium (HE) as mean fluorescent intensity (MFI). (B) Following 96 hours, cells were stained with DiOC6(3) and HE to determine $\Delta\Psi m$ and ROS production respectively. The numbers represent the percentage of cells in each quadrant.

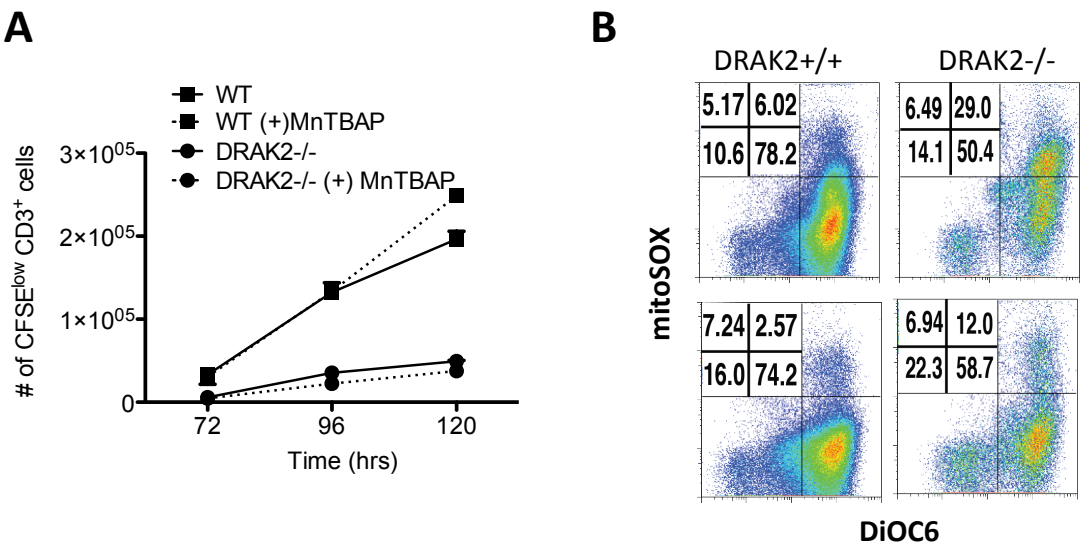


Figure 4.3: ROS attenuation does not enhance recovery of DRAK2-/- T cells. T cells were activated in the presence of MnTBAP (100uM) and at the indicated time points cells were collected for flow cytometry. (A) Number of recovered CD3+ T cells recovered at 72, 96 and 120hrs post stimulation with MnTBAP treatment. (B) Representative FACS plot showing cells cultured for 96hrs with and without MnTBAP and then stained with DiOC6(3) and mitoSOX. The numbers represent the percentage of cells in each quadrant.

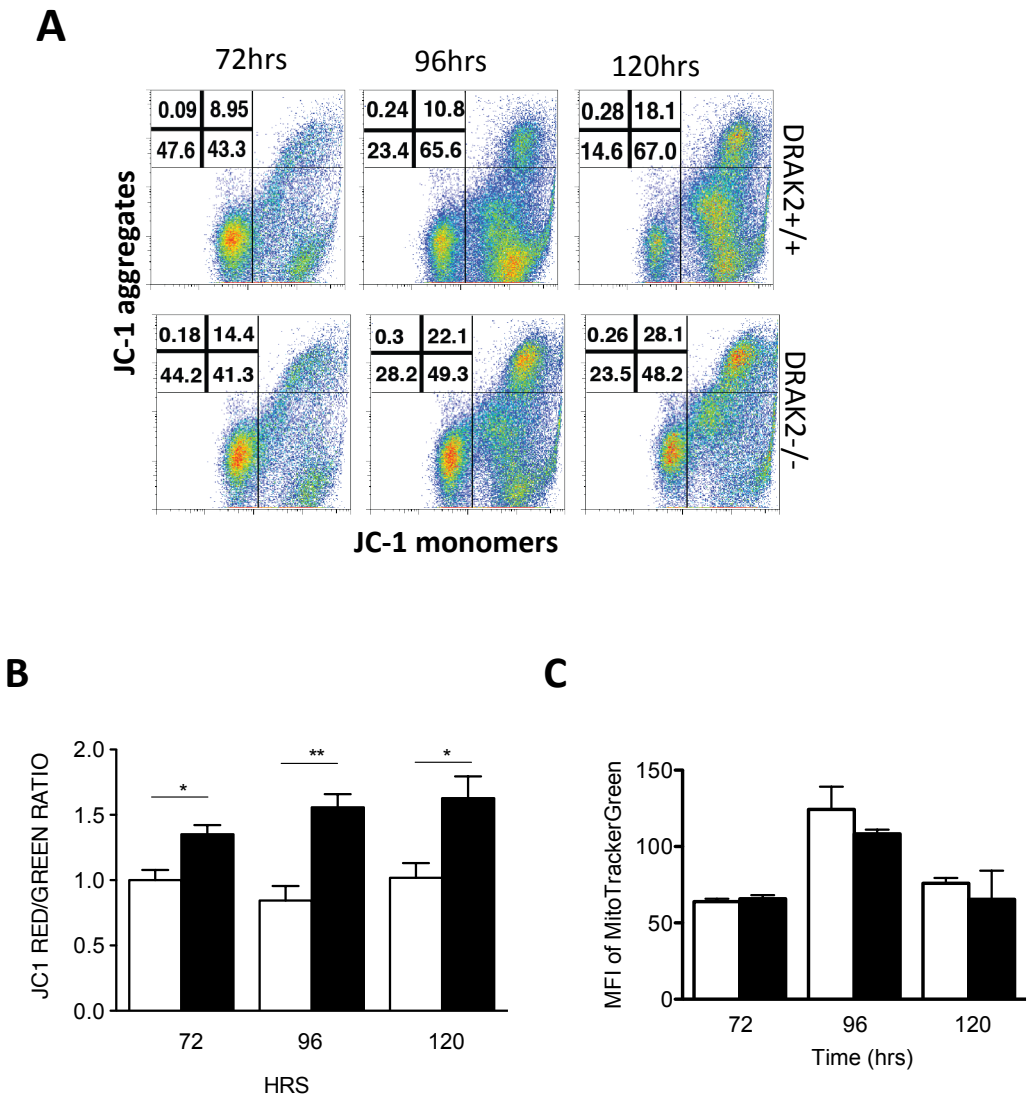


Figure 4.4: DRAK2-deficiency induces mitochondrial hyperpolarization independent of mitochondrial content. (A) Representative dot plot analysis of the JC-1 red (aggregates) and JC-1 green (monomers) profiles by flow cytometry following SEB stimulation. The numbers represent the percentage of cells in each quadrant. (B) Quantification of ΔΨm expressed as a ratio (aggregates/monomers) at different times. Average and SD of three samples are shown. (C) Mitochondrial density measured by relative MitoTracker Green MFI.

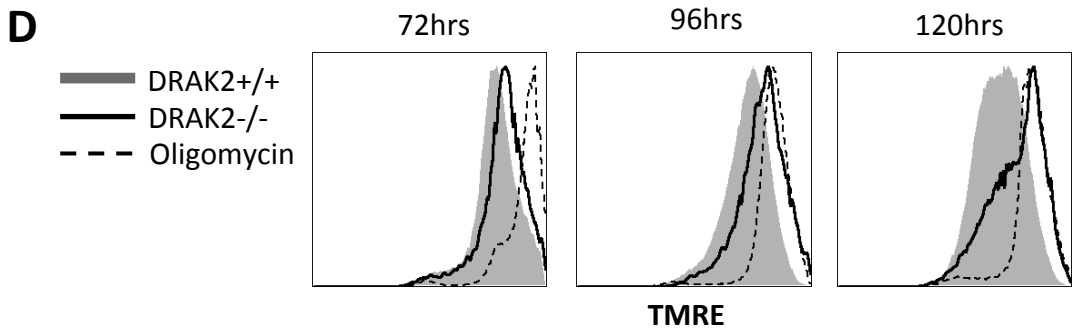


Figure 4.4: DRAK2-deficiency induces mitochondrial hyperpolarization independent of mitochondrial content. (D) Representative histogram overlays of TMRE fluorescence of stimulated cells. As a positive control, oligomycin (dashed black line) was added to the cells 1 hour prior to staining.

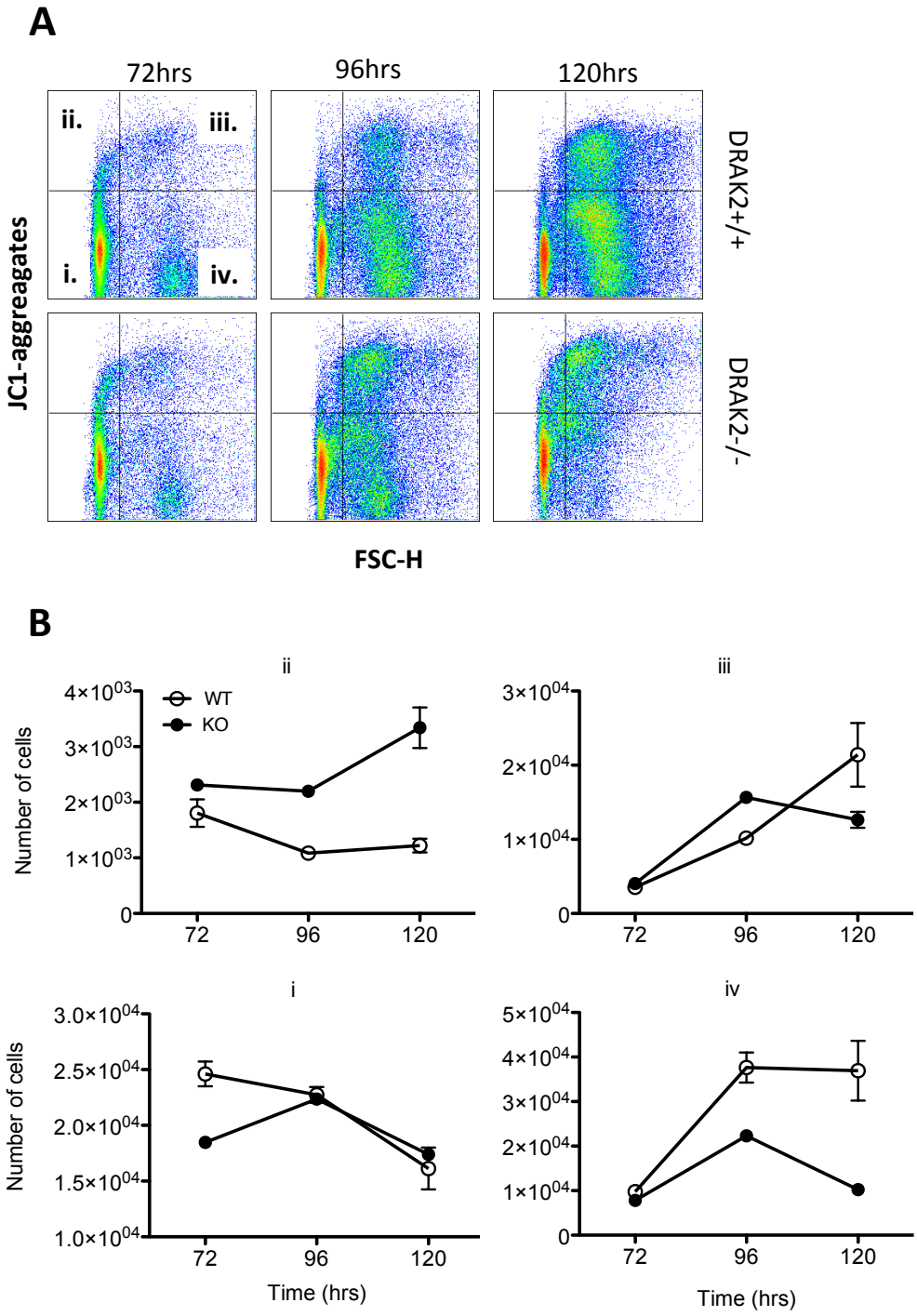


Figure 4.5: Loss of DRAK2 inhibits the generation of expanding polarized cells. Examination of mitochondrial membrane potential following stimulation with SEB. (A) Representative dot plot shows the expression of JC-1 and forward scatter on CD3+ cells at the indicated time points. (i) Small low ΔΨm, (ii) small high ΔΨm (iii) large high ΔΨm and (iv) large low ΔΨm. Cells within each gate were subsequently analyzed. (B) Number of live CD3+ cells recovered at 72, 96 and 120hrs post stimulation for each polarized quadrant as indicated in (A).

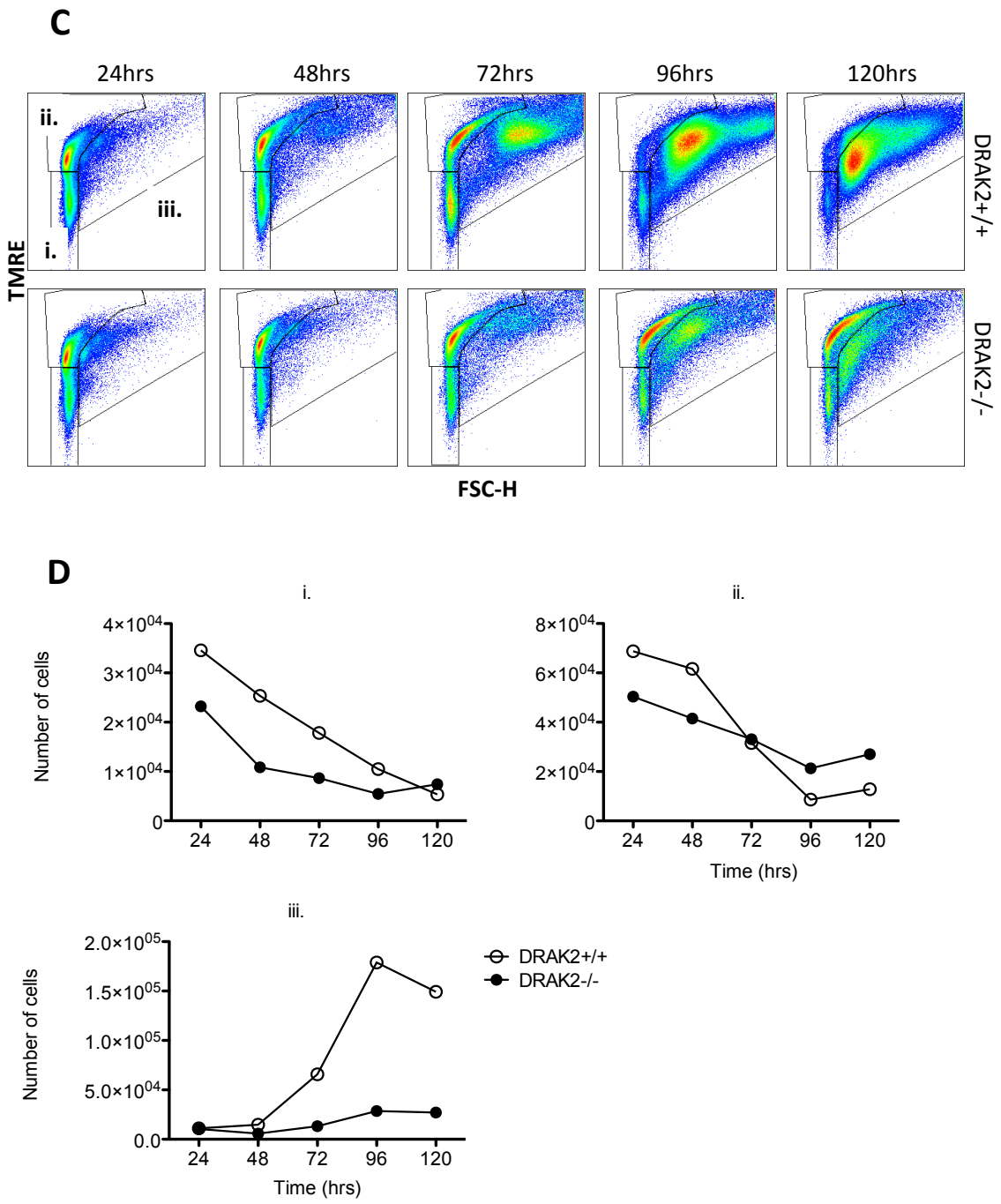


Figure 4.5: Loss of DRAK2 inhibits the generation of expanding polarized cells. (C) Representative FACS plot with the expression of TMRE and forward scatter on activated splenocytes. (i) Small low ΔΨm, (ii) small high ΔΨm and (iii) expanding polarized. Cells within each gate were subsequently analyzed. (D) Number of live splenocytes recovered for each polarized quadrant as indicated in (C).

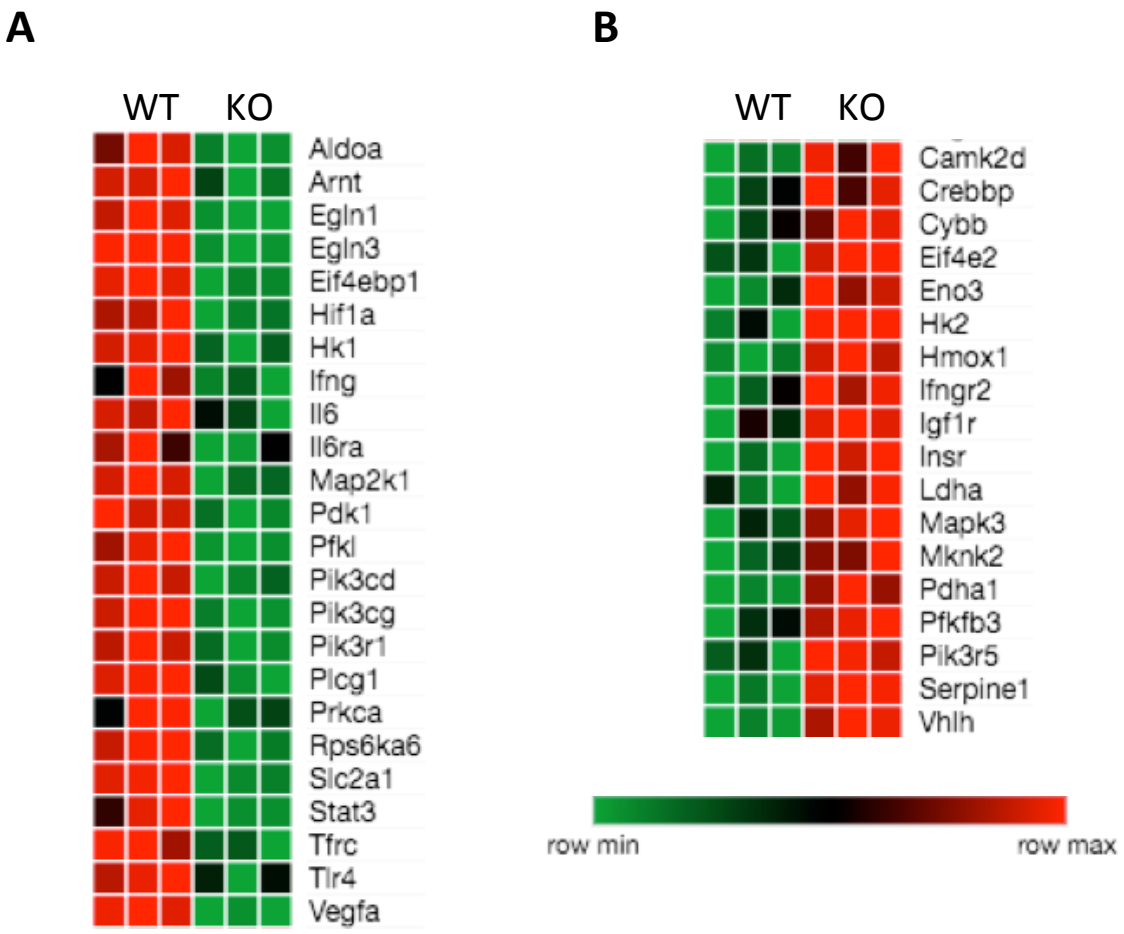


Figure 4.6: Loss of DRAK2 alters expression of genes involved in metabolic signaling. Splenocytes were stimulated for 4 days with SEB. (A) Heat map of down-regulated (green) genes based on log2 raw expression values. (A) Heat map of up-regulated (red) genes based on log2 raw expression values.

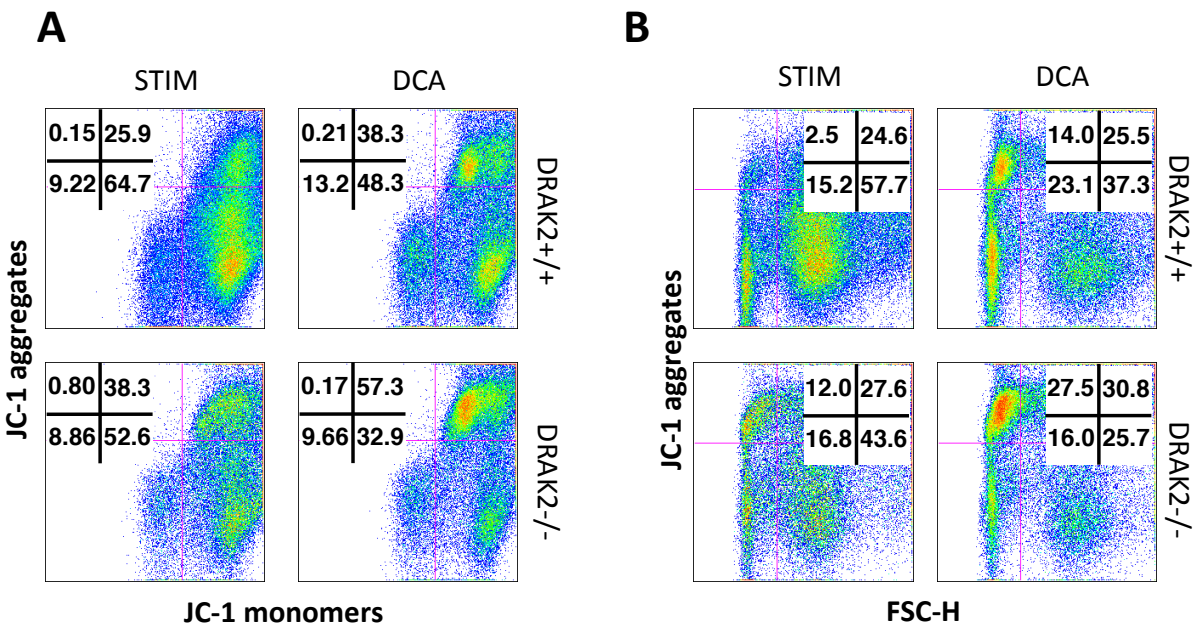


Figure 4.7: DCA treatment promotes the accumulation of small hyperpolarized cells. Total splenocytes were cultured for 4 days with SEB in the presence of absence of 10mM DCA. (A) Representative dot plots of JC-1 96hrs post stimulation. The numbers represent the percentage of cells in each quadrant. (B) Representative dot plots of JC-1 vs. forward scatter following stimulation. The numbers represent the percentage of cells in each quadrant.

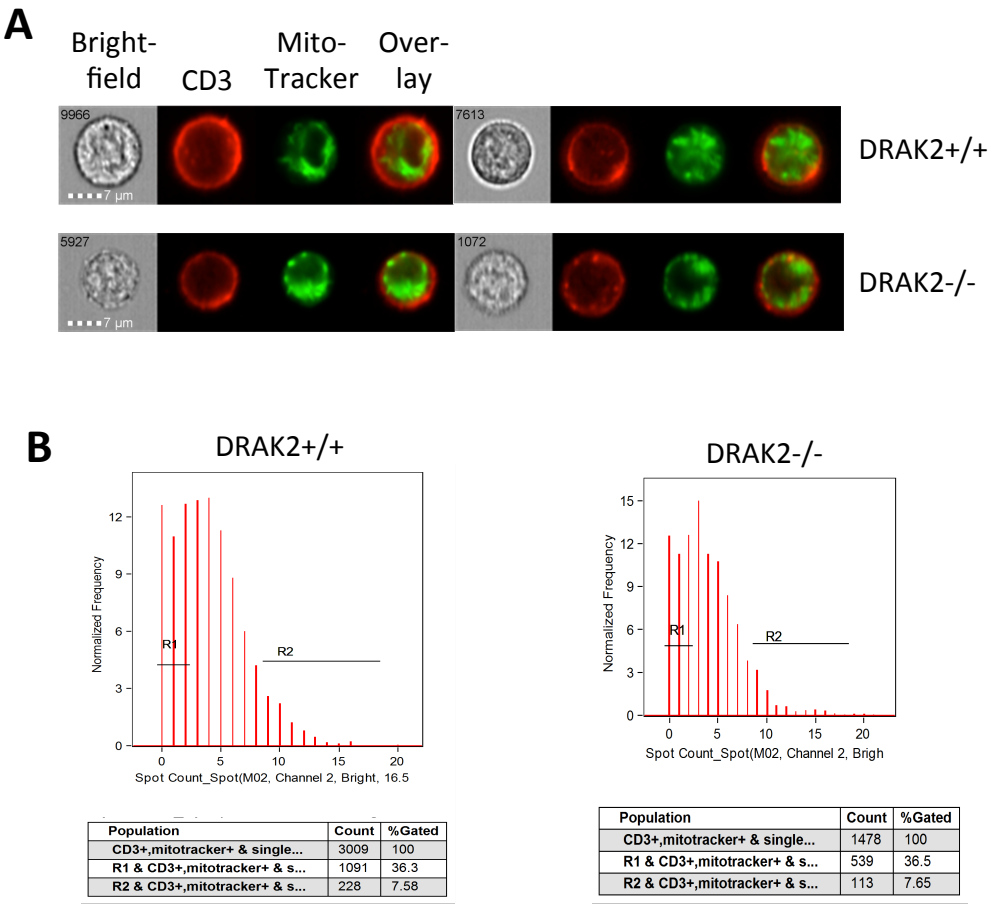


Figure 4.8: Mitochondrial content and distribution does not differ between activated wildtype and DRAK2-/- T cells.. Total splenocytes were cultured for 4 days with SEB and analyzed by Amnis brand ImageStream imaging flow cytometer. (A) Representative brightfield, anti-CD3, and mitotracker green staining from high spot containing cells. (B) Representative spot count histograms for activated wildtype and DRAK2 T cells.

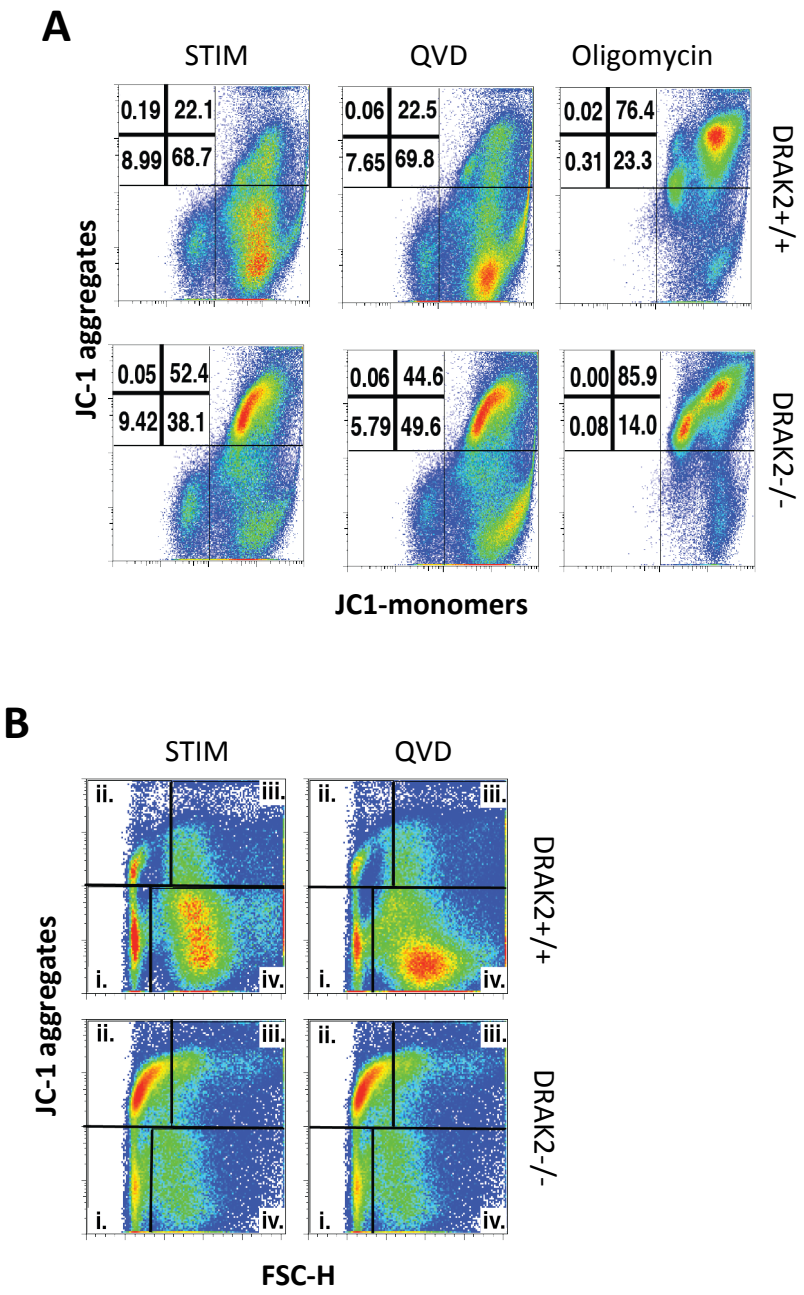


Figure 4.9: Caspase inhibition does not restore the polarization of DRAK2-deficient splenocytes. (A) Representative dot plots of JC-1 96hrs post stimulation. Upon stimulation cells were treated with 12.5μM QVD. Oligomycin was added for 1hr prior to staining as a control (B) Representative dot plots of JC-1 aggregates by forward scatter. (i) Small low ΔΨm, (ii) small high ΔΨm (iii) large high ΔΨm and (iv) large low ΔΨm. Cells within each gate were subsequently analyzed.

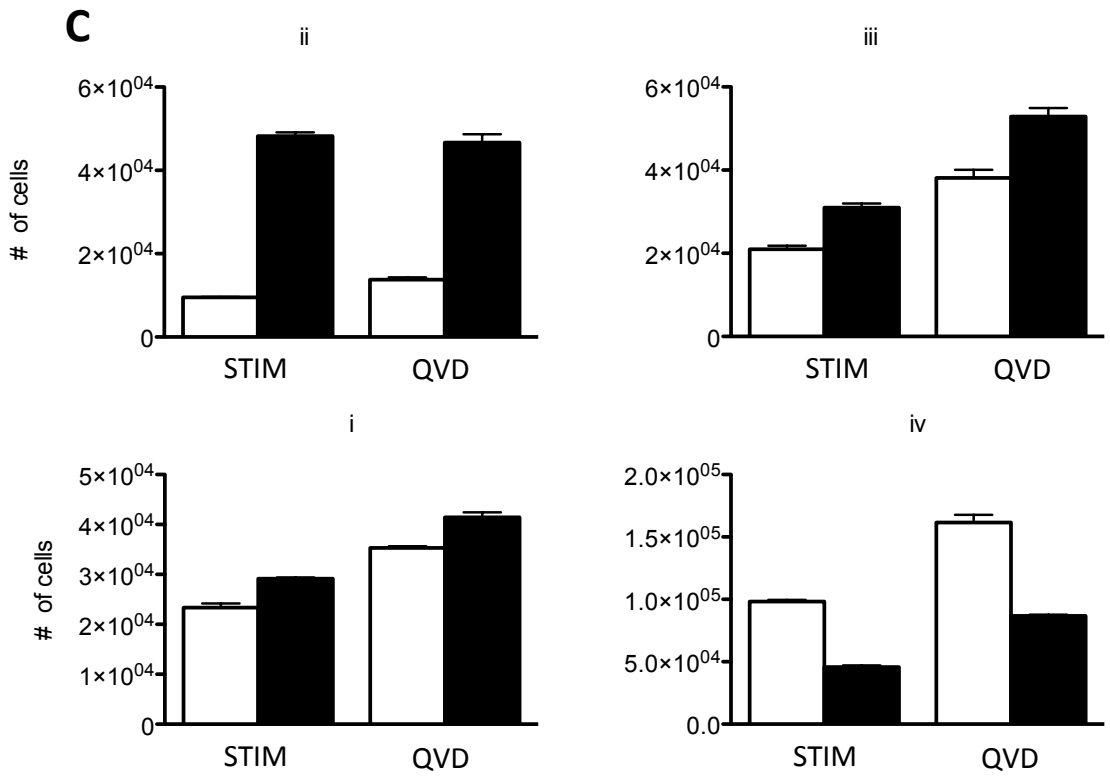


Figure 4.9: Caspase inhibition does not restore the polarization of DRAK2-deficient splenocytes. (C) Number of live splenocytes recovered 96 hours post stimulation for each polarized quadrant as indicated in (B). Two independent experiments were done with similar outcome.

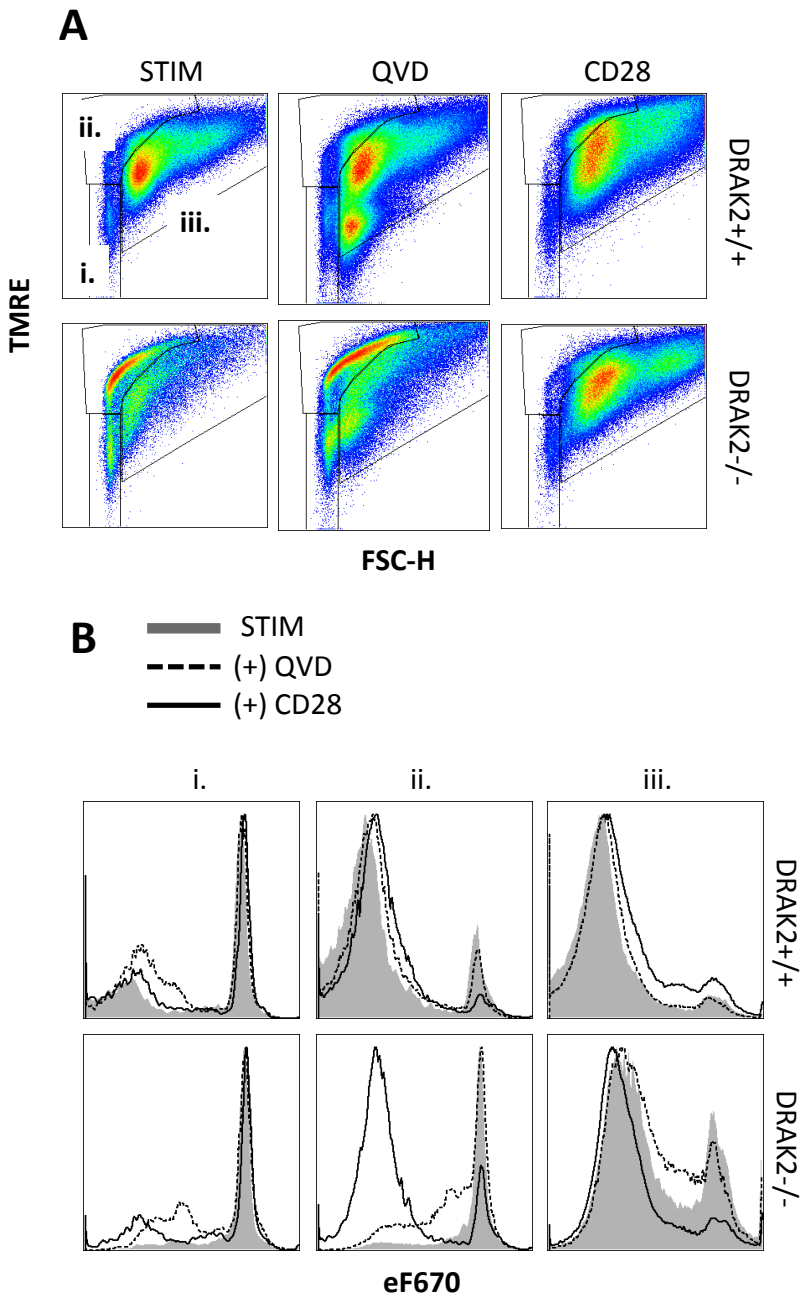


Figure 4.10: CD28 costimulation protects DRAK2-/- splenocytes. Following 5 day SEB stimulation, ΔΨm was measured by TMRE fluorescence and proliferation rates were assessed by ef670. (A) Splenocyte TMRE profile, indicating the specific populations analyzed (i) Small low ΔΨm, (ii) small high ΔΨm and (iii) large intermediate ΔΨm. (B) Following 120hrs post stimulation, stimulated (shaded histogram), CD28 (black line) and QVD (dashed line) splenocytes were analyzed by eF670 histogram overlays for each population.

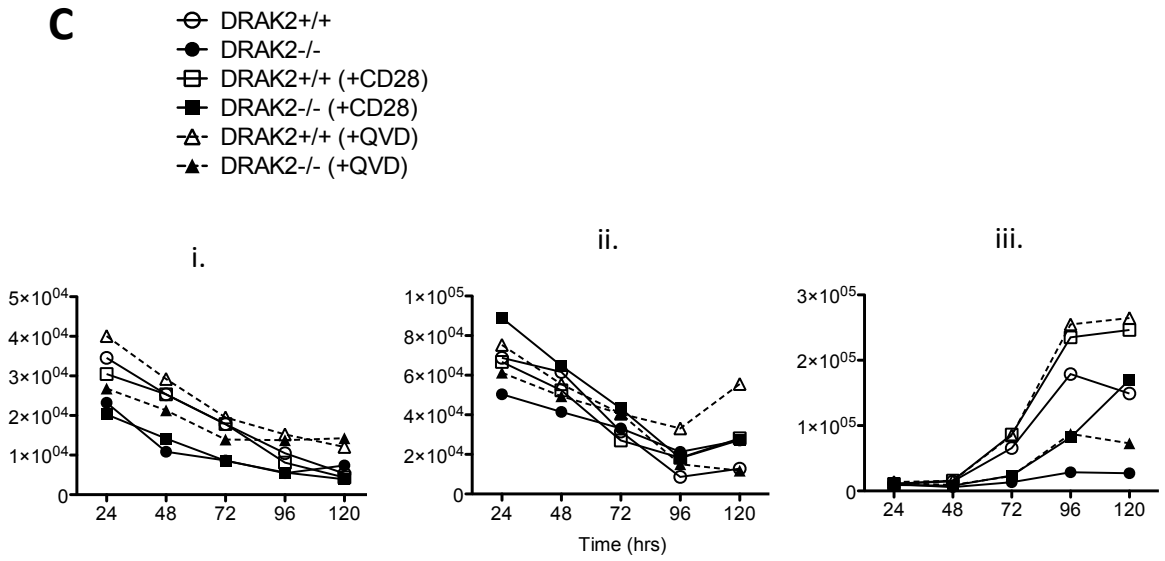


Figure 4.10: CD28 costimulation protects DRAK2-/- splenocytes. (C) Number of live splenocytes recovered at 24, 48, 72, 96 and 120hrs post stimulation.

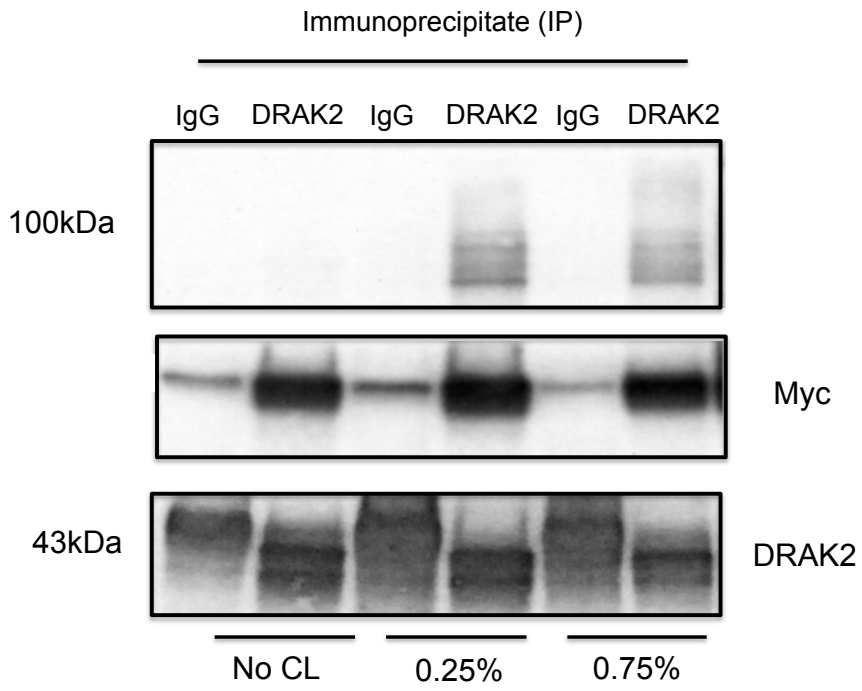


Figure 4.11: High molecular weight binding partners of DRAK2 appear following crosslinking in 293T cells. 293T cells were transfected with myc-ddk. Transfected cell lysates were immunoprecipitated by adding anti-DRAK2 or IgG (negative control). Lysates were either left untreated (lanes 1,2) or crosslinked with 0.25% (lanes 3,4) or 0.75% (lanes 5,6) formaldehyde.

Table 4.1: Protein binding partners of DRAK2. LC-MS performed on 293T cells transfected with DRAK2-Myc-DDK plasmid.

Accession	Description	Abundance
3::Q8BG48	Serine/threonine-protein kinase 17B OS=Mus musculus	203
2::ADT2_HUMAN	ADP/ATP translocase 2 OS=Homo sapiens	130
2::TCPD_HUMAN	T-complex protein 1 subunit delta OS=Homo sapiens	101
2::TBA1A_HUMAN	Tubulin alpha-1A chain OS=Homo sapiens	79
2::IF4A1_HUMAN	Eukaryotic initiation factor 4A-I OS=Homo sapiens	66
2::SERA_HUMAN	D-3-phosphoglycerate dehydrogenase OS=Homo sapiens	62
2::AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1 OS=Homo sapiens	55
2::RS8_HUMAN	40S ribosomal protein S8 OS=Homo sapiens	50
2::TCPH_HUMAN	T-complex protein 1 subunit eta OS=Homo sapiens	49
2::RS4X_HUMAN	40S ribosomal protein S4, X isoform OS=Homo sapiens	48
2::HS71L_HUMAN	Heat shock 70 kDa protein 1-like OS=Homo sapiens	46
2::RS18_HUMAN	40S ribosomal protein S18 OS=Homo sapiens	41
2::TCPG_HUMAN	T-complex protein 1 subunit gamma OS=Homo sapiens	39
2::RL7A_HUMAN	60S ribosomal protein L7a OS=Homo sapiens	39
2::RS3_HUMAN	40S ribosomal protein S3 OS=Homo sapiens	39
2::RUVB1_HUMAN	RuvB-like 1 OS=Homo sapiens	38
2::HNRPD_HUMAN	Heterogeneous nuclear ribonucleoprotein D0	37
2::HNRPM_HUMAN	Heterogeneous nuclear ribonucleoprotein M	34
2::CCD14_HUMAN	Coiled-coil domain-containing protein 14	34
2::RS25_HUMAN	40S ribosomal protein S25 OS=Homo sapiens	34
2::PGAM5_HUMAN	Serine/threonine-protein phosphatase PGAM5	33
2::TRY1_HUMAN	Trypsin-1 OS=Homo sapiens	31
2::CH60_HUMAN	60 kDa heat shock protein,	31
2::HNRPC_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2	30
2::RL4_HUMAN	60S ribosomal protein L4 OS=Homo sapiens	29
2::AT2A1_HUMAN	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	29
1::P00735	SWISS-PROT:P00735 (Bos taurus) Prothrombin precursor	28
2::DJC10_HUMAN	DnaJ homolog subfamily C member 10 OS=Homo sapiens	28
2::H90B2_HUMAN	Putative heat shock protein HSP 90-beta 2 OS=Homo sapiens	28

Table 4.1: Continued

2::M2OM_HUMAN	Mitochondrial 2-oxoglutarate/malate carrier protein	27
2::RL11_HUMAN	60S ribosomal protein L11 OS=Homo sapiens	27
2::C1TC_HUMAN	C-1-tetrahydrofolate synthase, cytoplasmic OS=HS	26
2::HNRPU_HUMAN	Heterogeneous nuclear ribonucleoprotein U OS=HS	24
2::RAB1A_HUMAN	Ras-related protein Rab-1A OS=Homo sapiens	24
2::PSDE_HUMAN	26S proteasome non-ATPase regulatory subunit 14	24
2::AIFM1_HUMAN	Apoptosis-inducing factor 1, mitochondrial OS=Homo sapiens	23
2::RUVB2_HUMAN	RuvB-like 2 OS=Homo sapiens	23
2::EF1A1_HUMAN	Elongation factor 1-alpha 1 OS=Homo sapiens	22
2::RAN_HUMAN	GTP-binding nuclear protein Ran OS=Homo sapiens	22
2::CMC1_HUMAN	Calcium-binding mitochondrial carrier protein Aralar1 OS=Homo sapiens	22
2::ARMC9_HUMAN	LisH domain-containing protein ARMC9 OS=Homo sapiens	22
2::CG013_HUMAN	Uncharacterized protein C7orf13 OS=Homo sapiens	22
2::ACTA_HUMAN	Actin, aortic smooth muscle OS=Homo sapiens	21
2::EF2_HUMAN	Elongation factor 2 OS=Homo sapiens	18

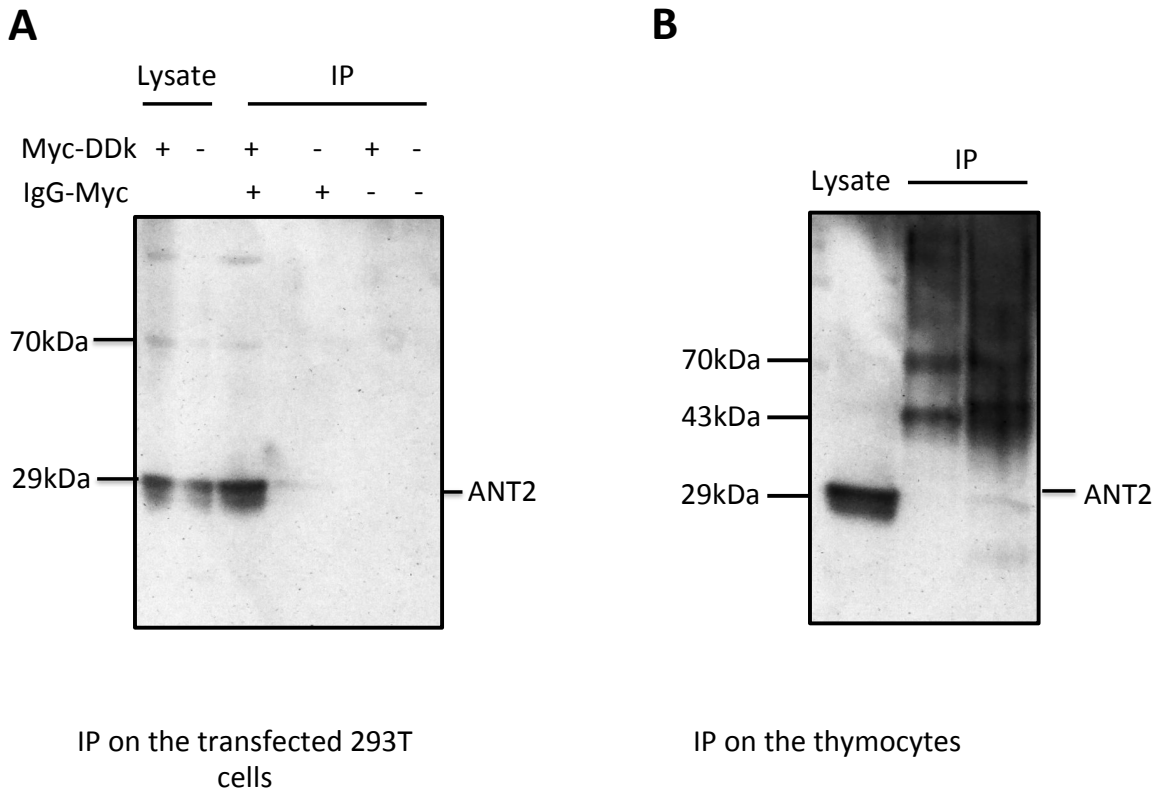


Figure 4.12: Immunoprecipitation of DRAK2 confirms ANT2 expression. (A) Following transfection, Myc-DDk 293 T cells were subjected to immunoprecipitation followed by immunoblotting for ANT2. Immunoprecipitations were done using an IgG-Myc antibody or a IgG-mouse control. (B) Immunoblot of ANT2 comparing thymocyte whole cell lysate against immunoprecipitated samples.

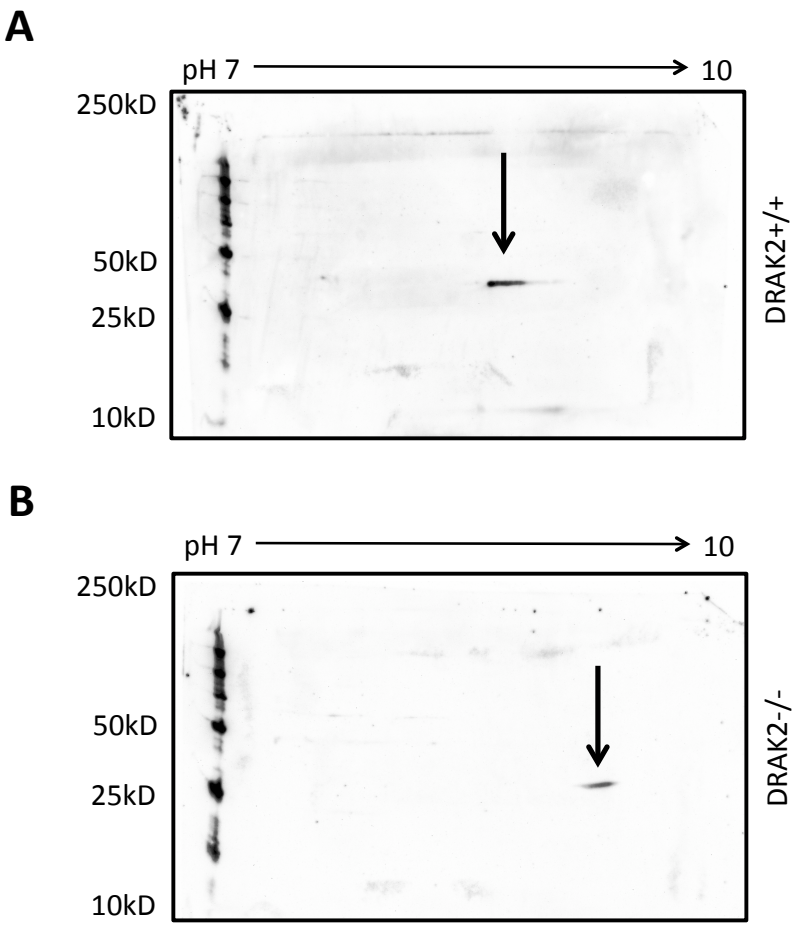


Figure 4.13: DRAK2 promotes phosphorylation of ANT2. Two-dimensional gel analysis of phosphorylated ANT2. Thymocytes from either DRAK2+/+ (A) or DRAK2-/- (B) were harvested and separated by 2d gel electrophoresis followed by SDS-PAGE. The gels were electrophoretically transferred on to PVDF membrane and incubated with the ANT2 antibody. The phosphorylated proteins were indicated with arrows.

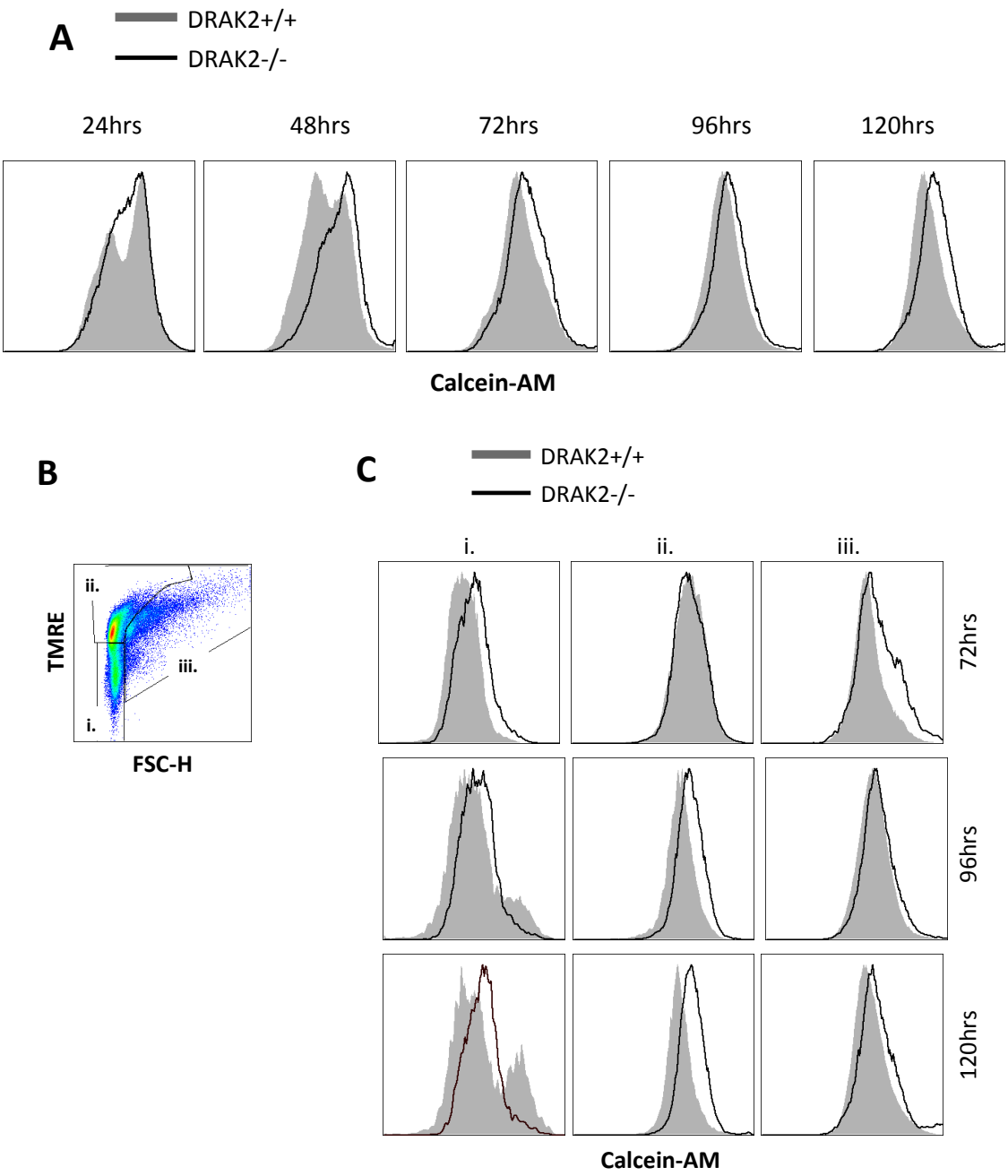


Figure 4.14: DRAK2-deficent splenocytes display lower mitochondrial permeability transition pore activity. Splenocytes were activated with SEB and at the indicated timepoints cells were harvested and analyzed for Calcein-AM retention. (A) Timecourse examination of the live gated fraction of activated cells. (B) Gating scheme based on TMRE and forward scatter. (i) Small low ΔΨm, (ii) small high ΔΨm and (iii) expanding polarized. Cells within each gate were subsequently analyzed. (C) Histogram representation of Calcein-AM retention from the indicated populations found in (B).

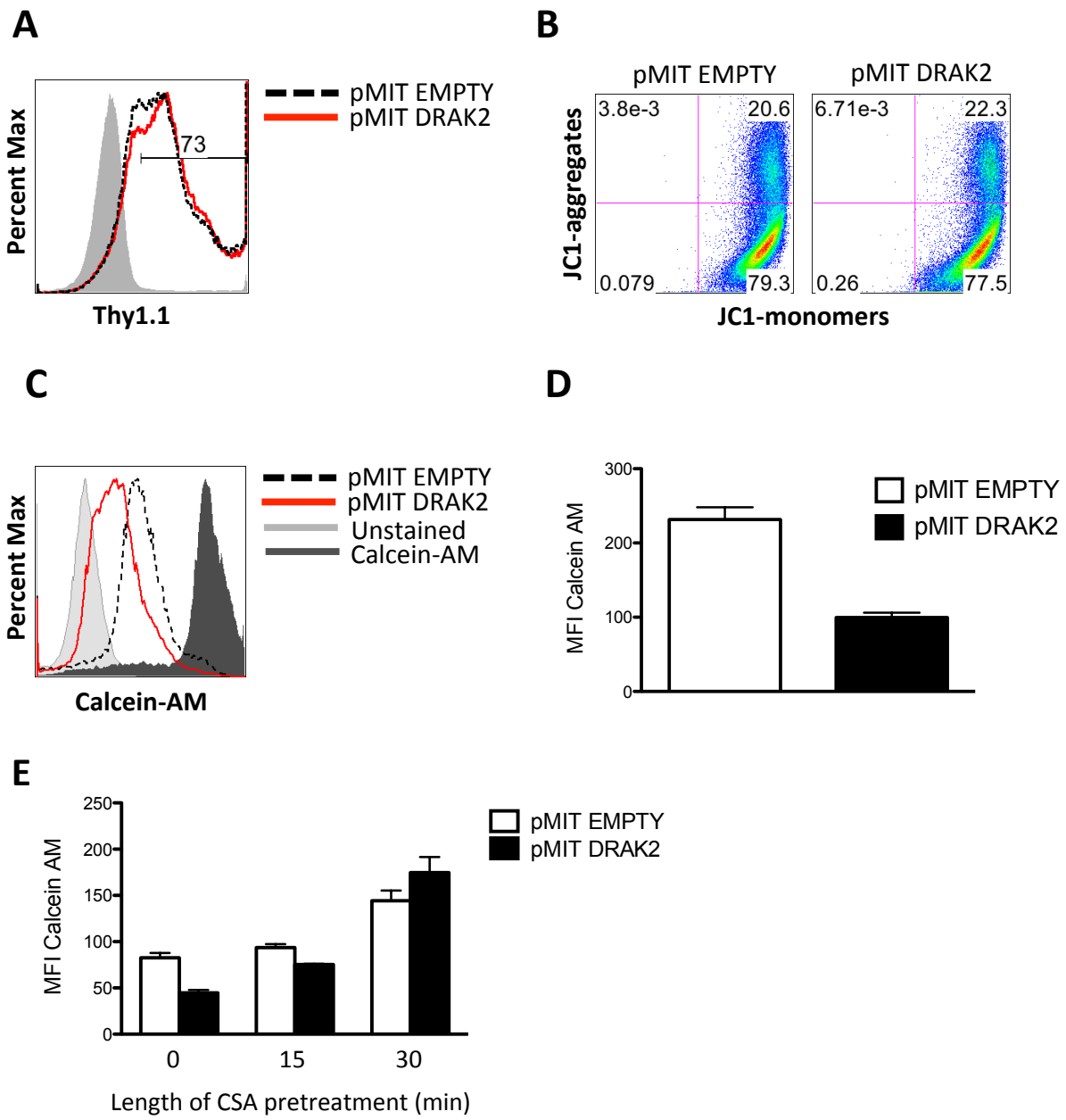
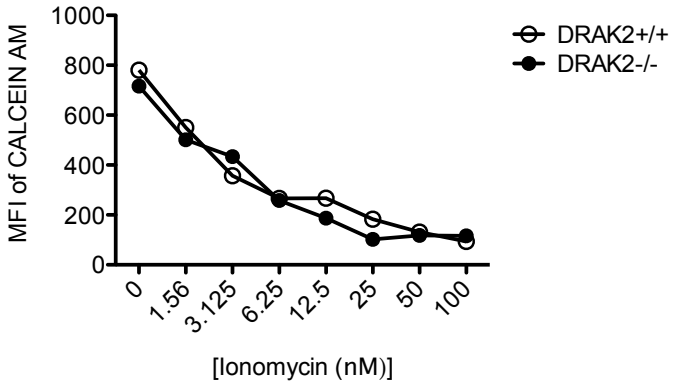
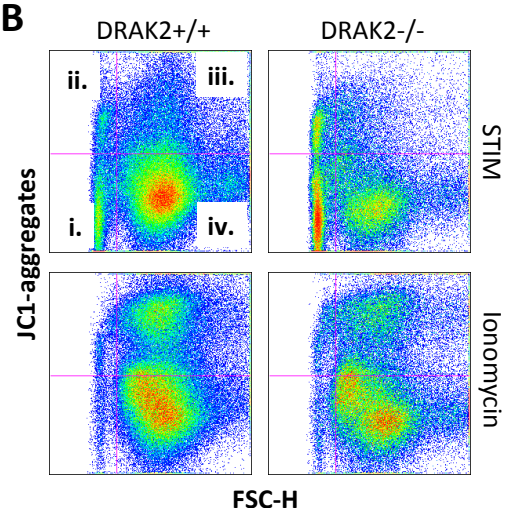


Figure 4.15: Overexpression of DRAK2 alters mitochondrial permeability. 293T cells were transfected overnight with either pMIT empty or pMIT DRAK2 plasmids. (A) Transfection efficiency as determined by Thy1.1 expression. (B) Representative dot plot of JC-1 distribution. (C) Following 15min PMA stimulation, Calcein-AM retention for either pMIT empty (black dashed) or pMIT DRAK2 (red line) transfected cells was determined by flow cytometry. Unstained and calcein-am only stains were included as controls. (D) MFI of Calcein-AM was determined from triplicate samples in one independent experiment. Three independent experiments were done with a similar outcome.

A



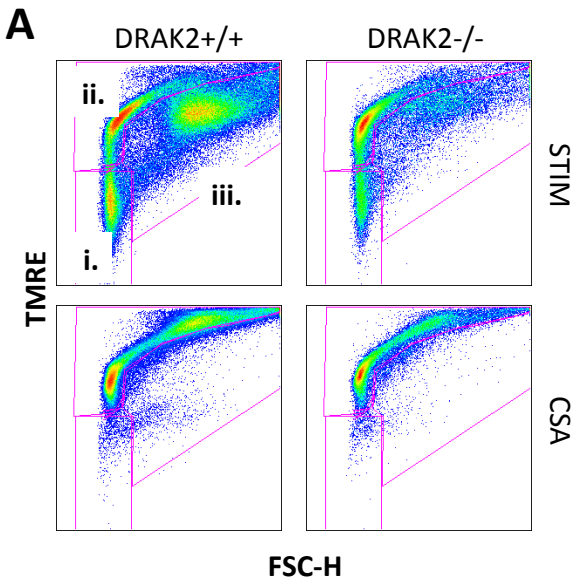
B



C

	DRAK2+/+	DRAK2+/+ (+) Ionomycin	DRAK2-/-	DRAK2-/- (+) Ionomycin
i.	10.1%	1.84%	26.9%	4.07%
ii.	3.37%	1.78%	9.24	3.31%
iii.	18.6%	38.1%	13.1%	32.6%
iv.	67.9%	58.2%	50.8%	60.1%

Figure 4.16: Ionomycin promotes mPTP opening and promotes the generation of biomass. (A) Thymocytes were treated with increasing concentrations of ionomycin and their mPTP activity was monitored by calcein-AM. (B) Splenocytes were stimulated for 4 days with or without ionomycin and representative FACS dot plot is shown. Gating scheme is indicated by i, ii, iii, and iv. (C) Fraction of cells belonging to the quadrant gates outlined in (B).



B

	DRAK2+/+	DRAK2+/+ (+) CSA	DRAK2-/-	DRAK2-/- (+) CSA
i.	14.7%	0.94%	14.1%	1.35%
ii.	30.9%	87.6%	62.5%	91.7%
iii.	53.2%	10.2%	21.6%	5.97%

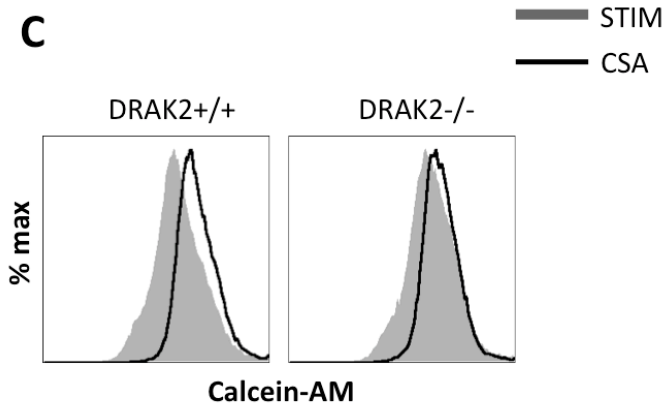
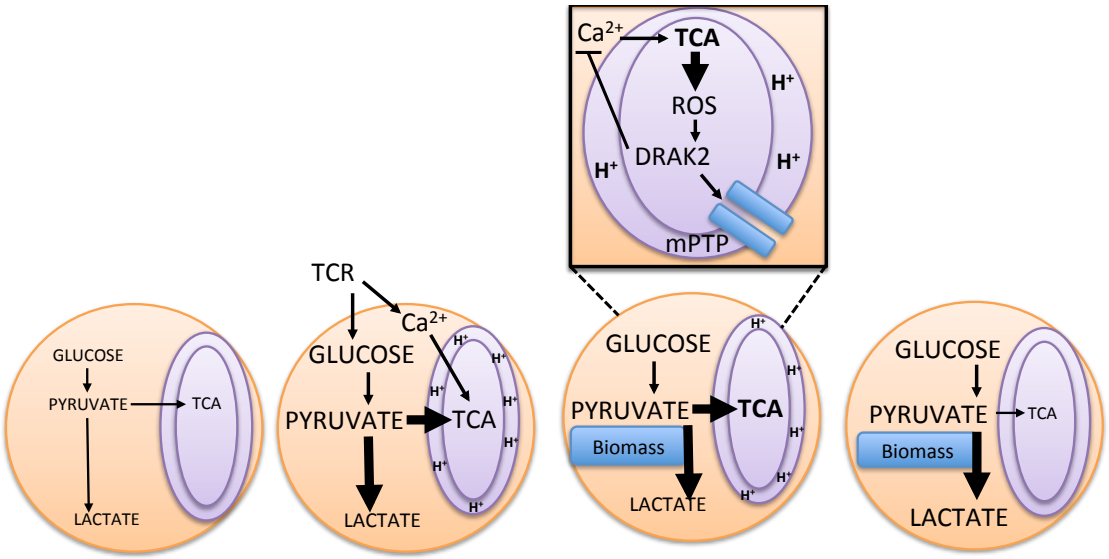


Figure 4.17: CsA promotes mPTP closing and accumulation of hyperpolarized cells. (A) Representative FACS plot following 72 hours of stimulation with CsA addition occurring at 48 hours. Gates marked with i, ii and iii for subsequent analysis. (B) Fraction of cells for each polarized gate fractions, as indicated in A, are shown. (C) Histogram analysis of calcein-AM retention analyzed as an indicator of mPTP activity.



	Stage 1	Stage 2	Stage 3	Stage 4
OXPHOS	LOW	HIGH	HIGH	LOW
GLYCOLYSIS	LOW	HIGH	HIGH	HIGH
ΔΨm	LOW	HIGH	HIGH	MED
Cell Size	SMALL	SMALL	SMALL ➔LARGE	LARGE

Figure 4.18: Model of DRAK2s activity on metabolic activity and intermediate synthesis

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CHAPTER 5

Conclusions

There are currently 12 disease modifying therapies that have been approved by the European Medicines Agency [1] and U.S. Food and Drug Administration (FDA) for MS treatment [2]. Among the drugs currently available, Interferon-β-1a (Avonex) and Glatiramer acetate have both been used in practice for over two decades and are often first-line treatment options for the relapsing-remitting form of MS [3]. However these therapies involve broad immunosuppression and act by non-selectively inhibiting immune cells. Therefore, there is demand to develop selective therapies that can inhibit specific T cell effector differentiation and promote regulatory T cell accumulation.

The negative regulation of T cell activation by DRAK2 is crucial for the survival of T cells following stimulation. Enhanced T cell receptor sensitivity traditionally lowers central tolerance; for example loss of Cbl-b, leads to enhanced EAE progression [4]. However, DRAK2-deficiency limits the induction of T1D, allograph rejection and EAE by inhibiting the accumulation of T cells following stimulation [5]. Despite exhibiting T cell hypersensitivity, DRAK2-/- mice showed no instances of spontaneous autoimmunity. Given that DRAK2 does not function as a tumor suppressor [6], research has focused on targeting it for the treatment of T cell mediated autoimmune diseases. The investigations performed in this dissertation aimed to examine and clarify more precise mechanisms by which DRAK2 limits the expansion of activated T cells.

T cell accumulation is a result of efficient survival and proliferation. We found that defective proliferation, in addition to decreased survival, contributed to the survival defect in SEB-activated DRAK2-deficient T cells. However, we did not observe a defect in DRAK2-deficient T cells following anti-CD3 stimulation, but this does not exclude the possibility that loss of DRAK2 may alter survival and/or subset differentiation. Our preliminary data-assessing metabolism in DRAK2-/- T cells indicates that loss of DRAK2 increased glucose transport, glycolytic rate, and resistance to nutrient deprivation following acute activation (Chapter 3). These data led us to hypothesize that the dysregulated metabolism may be driving cell death. Given that anti-CD3 activations are considered an artificial stimulus, due to the lack of APC's, we next sought to utilize a more physiologically relevant source. *In vitro*, SEB stimulations, provided an optimal activation signal in which recovery of DRAK2-/- T cells was markedly decreased relative to wildtype. Following activation, DRAK2-deficiency reduced the recovery of T cells and this survival defect could be overcome with appropriate costimulation.

As afore mentioned, in T cells, the costimulatory receptor CD28 participates in an intracellular signaling pathway that activates the kinase AKT [1, 7]. By activating NFkB, activated AKT enhances transcription of various genes including Bcl-xL. [8]. Increased AKT signaling has been attributed to increased glycolysis due to its ability to increase key glycolytic enzymes [9-11] in addition to increasing surface localization of GLUT1 [1, 7]. However in other cell types, including adipocytes and fibroblasts, AKT signaling promotes oxidative metabolism and fatty acid synthesis [12, 13]. Therefore, CD28 signaling promotes the appropriate balance of nutrient uptake and energy production in a context-specific

manner. Although multiple signals also strongly influence T cell effector differentiation and proliferation, metabolic regulation is a key contributor..

The cumulative study has proposed that DRAK2 is involved in the regulation of the mPTP gating and that loss of DRAK2 disrupts mitochondrial homeostasis and therefore the development of glycolytic effector cells, suggesting loss of DRAK2 and mitochondrial remodeling are concomitant events. However, the precise mechanism underlying the glycolytic switch elicited through transient mPTP opening is still elusive. In this study our observation indicates that mPTP opening, mediated by DRAK2, results in the development of glycolytic effector cells. Therefore, we propose a new mechanism whereby T cell activation promotes Ca2+ flux, which in turn drives oxidative metabolism, thus generating ROS. These ROS trigger the activation of DRAK2, which phosphorylates ANT2 thus promoting mPTP activity. As a consequence, DRAK2 serves as a candidate drug target to specifically alter autoimmune and alloimmune responses by preventing the expansion of glycolytic effector T cells.

While the metabolic reprogramming that occurs upon T cell activation is well described, less is understood about the associated mitochondrial changes. Here, a timecourse, cell intrinsic approach was taken to determine the effect of activation on T cell differentiation and mitochondrial homeostasis (Chapter 4). Our data suggest that manipulating mPTP activity can have profound consequences that impact metabolic fate and ultimately T cell differentiation and recovery. We found that DRAK2 regulated mPTP in activated T cells, which facilitated efficient metabolism, as evidenced by $\Delta\Psi m$ and a favorable redox balance. Based on preliminary studies using anti-CD3 stimulation, we originally hypothesized that loss of DRAK2 would result in hyper-metabolism which in turn

promoted cell death. However we found that following SEB stimulation, DRAK2-/- T cells appeared to accumulate as small, hyperpolarized cells unable to not only dissipate their ΔΨm but also divide.

In the context of autoimmunity, understanding the activation of transcriptional programs underlying T cell metabolic fate is crucial in understanding the mechanisms of modulating subset differentiation. Interestingly, effector T cells have been shown to strongly induce glycolysis whereas memory and regulatory T cells favor an oxidative metabolic profile [14-16]. The question of what signals drive the metabolic differences between T cell subsets has yet to be determined. The work outlined here was performed on CD3+ T cells, which could be further classified into effector subsets, allowing the relative importance of DRAK2 signaling in these effector cells to be examined. It also remains to be seen whether large, intermediate ΔΨm cells correspond with glycolytic metabolism, or alternatively are associated with a specific effector T cell subset.

To determine if large intermediate ΔΨm correlate with a glycolytic phenotype, cells could be FACS sorted and investigated using fluorescence-lifetime imaging microscopy (FLIM). FLIM could be used to determine the relative pools of NADH and NADPH [17]. NADH is the principal electron acceptor in glycolysis and electron donor in oxidative phosphorylation [18]. Therefore, the shorter lifetime of NADH corresponds with glycolysis whereas the longer fluorescing, bound form indicates oxidative phosphorylation. Alternatively, following FACS separation based on the polarized quadrants, T cells could be plated on the Seahorse metabolic flux analyzer, which can distinguish between glycolytic and oxidative levels by measuring the media acidification and consumption of oxygen, respectively. If large intermediate ΔΨm cells correlate with glycolytic metabolism, it could

be further investigated through quantitative polymerase chain reaction (qPCR). I hypothesize that many of the transcription factors, such as HIF-1α, that are known to regulate glycolytic metabolism would be upregulated in the large intermediate ΔΨm population. It is possible that multiple signals may regulate glycolytic metabolic fate, including CD28.

Here, we showed that CD28 was sufficient to overcome the survival defect in activated DRAK2-deficent T cells. However, it is noteworthy to comment on evidence not presented in this thesis wherein activated DRAK2-deficient T cells did not display a survival phenotype following SEB stimulation. Although not normative, periodically DRAK2-deficient T cells were sufficiently stimulated with SEB to overcome the survival defect and phenotypic distribution of polarized cells. Superantigens, including SEB, have been reported to facilitate not only the interaction of MHC-II with TCR but also B7-2 with CD28 [19], thus providing costimulation. To overcome this periodic occurrence, CTLA4-Ig, an antagonist of CD28, was added to culture. Upon attenuating excessive B7 signaling, using CTLA4-Ig, DRAK2-/- T cells exhibited the normal distribution of polarized subsets in addition to the survival defect. Although tremendous effort has been made at elucidating the signaling pathway(s) initiated by CD28 stimulation, the detailed mechanism by which CD28 operates has yet to be defined, in part due to the complexity of distinguishing the TCR- versus CD28-driven signals in primary T cells. Taken together with the anti-CD3 stimulation experiments, we believe that signal strength, if sufficient, can overcome the characteristic survival defect in activated DRAK2-deficient T cells perhaps by supporting glycolytic differentiation.

In the absence of DRAK2, CD28 stimulation efficiently enhances T cell responses induced by TCR ligation. In fact, adequate costimualtion circumvents the need for DRAK2 following activation. Since DRAK2 promotes mPTP gating, we reasoned that CD28 would likely do the same. However examination of mPTP activity in CD28 cultured cells did not indicate differential modulation of mPTP activity by CD28 (data not shown). Because of the difficulty in dissecting the CD28 signaling cascade driving glycolytic differentiation, it is still possible that other T cell family kinases activated through CD28 may compensate for the loss of DRAK2, although further experimentation would need to be performed. Interestingly, calcium mobilization has also been implicated as a target of CD28 costimulation [20, 21]. Downstream of TCR activation, Ca2+ flux activation promotes the phosphatase activity of calcineurin, which dephosphorylates Drp1 at Ser637, thus promoting fission [22]. In previous studies, phosphorylation of Drp1 at its activating site Ser616 was found following 1 day of stimulation [23]. Consistently, CD28 has been reported to regulate mitochondrial sphericity and cristae morphology in activated T cells [24]. Furthermore, reports have suggested that specific mitochondrial morphology correlates with metabolic phenotype [23].

Given these findings and the observation that DRAK2 is a target of calcium signaling, we profiled mitochondrial morphology and found no difference in mitochondrial distribution following activation. However, it is worthwhile to comment on data not presented in this dissertation wherein we stimulated PGAM5-/- T cells as a control for altered mitochondrial dynamics. PGAM5 serves to dephosphorylate and activate mitochondrial fission protein Drp1, thus promoting fission. However confocal microscopy examination of PGAM5-/- splenocytes did not indicate elongated mitochondria (data not

shown). The problem of discrepant findings in the various studies, suggests that further examination of mitochondrial distribution and morphology should be undertaken, perhaps with electron microscopy.

CypD has also been proposed as a calcium sensitive regulator of mPTP by decreasing the threshold of intra-mitochondrial calcium necessary for pore opening. Cylophilin D knockout mice showed markedly reduced clinical disease severity in EAE [25]. The data demonstrated that by inactivating CyPD, mitochondria had enhanced ability to sequester Ca2+, thereby protecting mitochondria against high cytoplasmic Ca2+ in neurons and axons, resulting from disease. These data suggested a direct link between mitochondrial function, axonal destruction and Ca2+ overload during EAE. CsA binds matrix CypD, resulting in inhibition of mPTP by increasing relative calcium insensitivity [26]. Indeed, CsA has been shown to reduce tissue injury in EAE [27]. However, long-term treatment with CsA is not possible given its associated toxicity [28]. Upon opening of mPTP, released calcium could trigger the activation of calcium dependent proteases, including calpain, which may contribute to neuronal cell death [29]. Taken together, these data support the feasibility behind targeting DRAK2's activity on mPTP as a potential therapeutic target.

Increased calcium mobilization in DRAK2-/- T cells may drive intrinsic apoptotic pathways that are able to be overcome through selective upregulation of survival pathways driven by costimulation. Possibly due to enhanced expression of anti-apoptotic genes such as Bcl-xL that can occur following CD28 costimulation [8]. DRAK2-/- T cells were shown to undergo abortive proliferation, as evidenced through experiments utilizing DRAK2-/- mice bred onto a transgenic background that specifically over-expresses Bcl-xL in T cells [30]. In

DRAK2-/- mice, susceptibility to EAE was restored following Bcl-xL over-expression. Although Bcl-xL expression and effect on apoptosis have been well documented, the effects of Bcl-xL on mitochondrial dynamics in activated T cells are largely unexplored. By specifically overexpressing the anti-apoptotic protein in DRAK2-/- T cells, we could more closely clarify the role of Bcl-xL on mitochondrial function by monitoring the development of large intermediate $\Delta\Psi m$ in addition to calcein-AM retention.

One caveat to these studies is that, in addition to T cells, DRAK2 is also expressed in B cells. Possible defects in these cells may have extrinsic effects on T cells isolated from a DRAK2-/- mouse. For example, APC's expressing indoleamine 2,3 dioxygenase (IDO) play a key role in regulating responses orchestrated by T cells through tryptophan catabolism. IDO is the enzyme responsible for metabolizing tryptophan into kyneurnine. By deleting tryptophan sources and producing kynurenine, IDO can promote the stress-response kinase GCN2 which can inhibit T cell proliferation and promote regulatory T cell accumulation [31]. As a result, it is possible to hypothesize that the oxidative metabolic profile of DRAK2-/- T cells may be a result of kynurenine promoting regulatory T cell accumulation. Therefore, when stimulated cells are compared, it is difficult to distinguish an intrinsic effect of DRAK2 in T cells versus the extrinsic effect from the environment. As previously mentioned, further experimentation would need to be preformed to determine whether the enhanced oxidative cells from DRAK2-/- T cells correlate with the regulatory T cell subset.

In contrast to this hypothesis, our initial SEB stimulation model presented by Ramos did in fact isolate DRAK2-/- T cells and culture them with purified wildtype APCs. Given that her model did indicate a survival defect, these data led the investigators to conclude

that the survival defect was T cell autonomous. However as cautioned earlier, the immunological context and microenvironmental cues can drastically alter T cell responses. As discussed in Chapter 3, McGargill reported a survival defect following anti-CD3 stimulation in the absence of costimulation yet more recent stimulations have been unable to reproduce these results, perhaps as a result of serum composition. Given these caveats, future research should utilize a stimulation model in which DRAK2-/- T cells are cultured with wildtype APCs.

In this project, the attempt to define the relationship between DRAK2 and mPTP was limited to utilizing SEB stimulation. While the results of SEB stimulation suggest that DRAK2 activation is upstream of the mPTP, further experiments using different stimulations are likely necessary to establish the relationship given the complexity of signaling cascades. Such studies should aim to elicit a survival deficit in activated DRAK2-/- T cells and may include altered peptide ligands or the antigen-independent mitogen, Concanavalin A (ConA).

Extending this work to human T cells is another important area of study. Although activated human T cells resemble the metabolic profile of activated mouse T cells [32], it is critical to determine whether the regulation of the mPTP we have described in mouse T cells are similar to human T cells. Given that humans are more genetically diverse than mice, there may be vast differences in the regulation of metabolism. Therefore, future studies will need to recruit a large number of donors to analyze T cell metabolic profiles. By extending these studies to human T cells, researchers will be better suited for designing therapeutic strategies targeted at metabolic manipulations.

While OXPHOS to aerobic glycolysis is now becoming established as a mechanism utilized by activated T cells, the data presented in this study argue that mPTP control is also a feature of glycolytic differentiation. In fact, our studies suggest the feasibility behind pharmacologically modulating T cell metabolism and subset differentiation through mPTP activity. While the structural components of the mPTP have yet to be defined, the ongoing search for contributing mPTP proteins will undoubtedly help define other regulators that may be important in DRAK2-dependent activation of the mPTP. Here we identified critical roles for DRAK2-mediated induction of glycolytic metabolism in the activation of T cells through the control of mPTP activity.

Since DRAK2 activity is dispensable for T cell activation yet indispensible for effector function, elution of the mechanism by which DRAK2 regulates effector differentiation is crucial. Overall, this study uncovers how T cells differentiate into glycolytic effector cells under SEB stimulation and how DRAK2 influences T cell proliferation by altering mPTP activity in these cells. In conclusion, we have described a DRAK2 regulatory mechanism that acts as a rheostat: in the differentiation of glycolytic effector T cells and by modulating the bioenergetics requirements between homeostasis and antigen-driven activation and differentiation. We believe our study opens a new avenue for the future use of DRAK2 inhibitors to modulate T cell metabolism that can be applicable to multiple sclerosis, as well as to other T cell mediated inflammatory diseases.

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