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# Metabolic regulation of CD8 T cell functions in the tumor microenvironment

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# Metabolic Regulation of CD8 T cell Functions in the Tumor Microenvironment

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# ABSTRACT OF DISSERTATION

Metabolic Regulation of CD8 T cell Function in the Tumor Microenvironment

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Washington University in St. Louis, 2017

Professor Erika L. Pearce, Professor Paul M. Allen, Co-Chairperson

Failure of T cells to protect against cancer is thought to result from lack of antigen recognition, chronic activation, and/or suppression by other cells. Using a mouse sarcoma model, we show that glucose consumption by tumors metabolically restricts T cells in the tumor microenvironment, leading to their dampened mTOR activity, glycolytic capacity, and IFN-γ production, and thereby allowing tumor progression. We demonstrate that enhancing glycolysis in an antigenic 'regressor' tumor is sufficient to override the ability of T cells to respond to a major tumor rejection antigen, allowing progression of tumors that are normally rejected. Checkpoint blockade therapy is used clinically to promote immune rejection of progressing tumors. We show that checkpoint blockade (anti-CTLA-4, anti-PD-1, and anti-PD-L1) monoclonal antibodies (mAbs) restore glucose in the microenvironment of progressing tumors, permitting T cell glycolysis and IFN-γ production. Furthermore, we unexpectedly found that blocking PD-L1 directly on tumors dampens glycolysis by inhibiting mTOR activity and decreasing expression of glycolysis enzymes. These data reflect a novel role for PD-L1 in tumor glucose utilization. Together our results establish that tumor-imposed metabolic restrictions can mediate T cell

hyporesponsiveness during cancer and suggest that checkpoint blockade therapy may be targeting this mechanism.

Glucose has been viewed as a major carbon source for adenosine triphosphate (ATP) generation, which is not only essential for the maintenance of cellular bioenergetics, but also critical for the regulation of effector T cell function. Consistent with the model that glucose consumption by antigenic tumors can metabolically restrict infiltrating T cells (TILs) and directly dampen their cytokine production, leading to tumor progression, it is conceivable that supplementation of glucose may rescue T cell hyporesponsiveness as a result of nutrient depletion. However, resupplying TILs with glucose *ex vivo* does not fully restore cytokine production, indicating that mechanisms beyond substrate limitation contribute to loss of effector functions in TILs. Exactly how T cells reprogram their metabolism in a nutrient restricted microenvironment and how this influences their effector functions remains unclear. In the second part of this study, we focus on understanding the metabolic alteration of T cells experiencing long-term glucose restriction. We found that T cells deprived of glucose lose responsiveness to exogenous glucose over time. Their inability to acquire glucose and thus engage glycolysis contributes to a hyporesponsive state characterized by impaired cytokine production. However, glucose restricted T cells remain responsive to acetate, which is the substrate for acetyl coenzyme A (acetyl-CoA) synthesis and thus is positioned at the intersection of metabolism and genetic regulation. The rescue of effector cytokine production of T cells by acetate supplementation was dependent on the nuclear-cytoplasmic acetyl-CoA synthetase enzyme (ACSS2). Our results demonstrate an alternative metabolic pathway engaged by T cells in a dearth of glucose. Combining therapies that blunt

tumor metabolism with those that promote glycolysis in T cells or distinct metabolic pathway triggered by alternative metabolites that directly regulate cytokine gene expression may generate more effective T cell based immunotherapies in the context of human disease including cancer.

# CHAPTER 1

# T CELL METABOLISM AND IMMUNITY

## § 1.1 Introduction

An effective adaptive immune response is initiated when naïve T cells encounter foreign antigens in the form of peptides presented by MHC molecules on professional APCs and become activated. In the context of appropriate costimulation signals, T cells will transition to a period of rapid clonal expansion, producing a variety of effector molecules that lead to the elimination of the pathogen. To support their activation, clonal expansion and exertion of effector functions, T cells must undergo metabolic reprogramming to shift from a quiescent phenotype generating energy by oxidative metabolism to a predominantly anabolic and intermediary metabolism that requires a high nutrient flux into the cells (Vander Heiden et al., 2009, van der Windt and Pearce, 2012, Wang and Green, 2012, MacIver et al., 2013, Pollizzi and Powell, 2014). Upon pathogen clearance, most effector T cells undergo apotosis, leaving behind a small proportion of long-lived antigen specific memory T cells that can respond to antigens quicker and stronger in the future. Consistent with their distinct characteristics, memory T cells must also reprogram cellular metabolic pathway to support their differentiation, longevity and “rapid recall” ability (van der Windt et al., 2012). As a result, engaging in a proper metabolic pathway is required for a protective T cell immune response.

Metabolism fundamentally underpins T cell functions. The processes of cellular activation, differentiation and massive proliferation are unique aspects in T cell biology compared to most terminal differentiated healthy cell types, thus there is great interests in understanding how metabolic pathway support vigorous changes in cellular activities, influences immune response and ultimately dictate disease

progression. Combination of modern tools for analyzing and tracing these cells both *in vitro* and *in vivo* make it suitable for exploring this complex network of biochemistry reaction involved in energy production and macromolecular biosynthesis, especially the molecular mechanisms that govern metabolic reprogramming in the immune system (Wang and Green, 2012, Pearce and Pearce, 2013). In addition, each step of these metabolic modifications that happen during the normal T cell development is intimately linked to the cell fate and function, which represents potential points for clinical intervention. The ability of eliciting or enhancing desired T cell response may assist in controlling or at least mitigating multiple diseases including infections, cancers and autoimmune disorders. As a result, targeting these cells by manipulating their metabolism hold promise for developing novel therapies to improve human health (Finlay and Cantrell, 2011, Wang and Green, 2012, Pearce and Pearce, 2013, Pearce et al., 2013).

## § 1.2 The dynamics of T cell metabolism

Glucose is a critical substrate for adenosine triphosphate (ATP) production (Greiner et al., 1994), which supports cellular bioenergetics in all living cells, including T lymphocytes. During the course of glycolysis, glucose is broken down into two molecules of pyruvate, generating two reduced nicotinamide adenine dinucleotide (NADH) molecules and two net ATP molecules per molecule of glucose. This process dose not require the participation of oxygen. In case of terminally differentiated, nonproliferating cells, pyruvate can be completely oxidized in the tricarboxylic acid (TCA) cycle, yielding NADH and reduced flavin adenine dinucleotide ($FADH_2$) to fuel oxidative phosphorylation (OXPHOS), which is a oxygen dependent process generating up to 36 molecules of ATP per glucose molecule. On the other hand, pyruvate can be fermented into lactate in an oxygen independent process termed aerobic glycolysis, regenerating $NAD^+$ for subsequent use. Notably, only two molecules of ATP are gained per molecule of glucose via aerobic glycolysis. From a bioenergetics perspective, engaging OXPHOS maximizes the amount of ATP that can be derived from glucose (Vander Heiden et al., 2009).

Bioenergetic profiling of T cells has revealed that T cell metabolism changed dynamically with the activation state. Resting naïve T cells have a metabolically quiescent phenotype, which metabolize glucose, fatty acid and amino acids to generate reducing equivalents that fuel oxidative phosphorylation (OXPHOS) in the mitochondria (Yusuf and Fruman, 2003, Fox et al., 2005, van der Windt et al., 2012). Upon activation, highly proliferative effector T cells augment aerobic glycolysis, a mitochondrion-independent metabolism of glucose in the cytoplasm although

sufficient oxygen is present to utilize OXPHOS. Although aerobic glycolysis is less efficient when ATP is generated in this pathway, it may afford a metabolic advantage not only allows for rapid ATP production in effector T cells in favor of glucose replete environment but also supplies various metabolites for the synthesis of lipids, proteins and nucleic acids, as well as provides a means for maintaining redox balance (Anastasiou et al., 2011, Kidani et al., 2013). In addition, it has also been shown that T cells can use either OXPHOS or aerobic glycolysis to survive and proliferate. Instead, they need to engage aerobic glycolysis for the acquisition of full effector function via a posttranscriptional mechanism (Cham and Gajewski, 2005, Cham et al., 2008, Chang et al., 2013, Pearce et al., 2013). In the absence of this glycolytic flux, the glycolysis enzyme GAPDH acts as an RNA-binding protein, binding to and inhibiting the translation of effector cytokine mRNA (Chang et al., 2013). Glutamine also presents as another essential carbon source for TCA cycle in form of α-ketoglutarate through the process of glutaminolysis, or can contribute to the citrate pool via reductive carboxylation. Deletion of glutamine or glucose transporters impairs T cell activation and function (Carr et al., 2010, Metallo et al., 2011, Sinclair et al., 2013, Macintyre et al., 2014).

Several signaling pathways and transcriptional factors tightly control the metabolic transition T cells experience after activation. The recognition of peptide-MHC complex by the T cell receptor (TCR) with CD28 costimulation triggers the activation of Phosphatidylinositol 3 Kinase (PI3K), AKT and Mechanistic Target of Rapamycin (mTOR) signaling pathways, and increased surface trafficking of the glucose transporter Glut1 (Rathmell et al., 2003, Jacobs et al., 2008, Powell et al., 2012, Macintyre et al., 2014). mTOR is a serine/threonine kinase comprised of two protein

complexes—mTORC1 and mTORC2—has a fundamental role in coordinating anabolic and catabolic processes associated with nutrient levels, energy status, cell stress response, as well as growth factor signaling (Duvel et al., 2010, Laplante and Sabatini, 2012). Opposing mTOR is the 5’ AMP-activated protein kinase (AMPK), which acts as a metabolic stress sensor and promotes oxidative metabolism (Tamas et al., 2006, Blagih et al., 2015). Transcriptionally, metabolic reprogramming in activated T cells is driven by the expression of the transcription factors Myc and hypoxia inducible factor 1α (HIF-1α) (Wang et al., 2011, Finlay et al., 2012, Doedens et al., 2013, Sullivan et al., 2013, Tannahill et al., 2013). Myc regulates cell cycle progression by controlling the expression of several essential cell cycle regulators both directly and indirectly. It has been shown that maximum expression of Myc is required for the induction of enhanced glycolysis activity following T cell activation (Wang et al., 2011). HIF-1α is another transcriptional factor that actively shunts glucose metabolism from OXPHOS in the mitochondria to aerobic glycolysis to maintain ATP production and prevent toxic ROS generation in oxygen depleted environments, protecting cells from hypoxia-induced apoptosis (Doedens et al., 2013, Sullivan et al., 2013, Tannahill et al., 2013). Collectively, these factors that promote glycolytic gene expression and posttranscriptional regulation are essential to enforce effector T cell metabolism and function.

In contrast to effector T cells, memory T cells adopt a metabolic profile similar to that of naïve T cells, which is using a catabolic metabolism characterized by increased reliance on OXPHOS and lower rates of nutrient uptake (O'Sullivan et al., 2014). However, memory T cells maintain substantial spare respiratory capacity (SRC) and have increased mitochondrial mass, both of which confer a metabolic advantage for

survival and recall when re-encountering pathogens (van der Windt et al., 2012, van der Windt et al., 2013). SRC is the maximal respiratory capacity reserved for cells to generate more energy and promote cell viability under increased stress or nutrient limitation (Yadava and Nicholls, 2007, Nicholls, 2009). From this vantage, memory T cells maybe viewed as being metabolically primed, with mitochondrial metabolism fueling the rapid recall response to reinfection. The memory T cell promoting cytokine interleukin (IL)-15 plays a key role in this catabolic switch by promoting mitochondrial biogenesis (van der Windt et al., 2013).

The machinery controlling the differentiation of T cells from effector to memory states are still not clear, but recent studies suggest that transitions in metabolism may play a role in this process. For example, mitochondrial FAO has been demonstrated to stimulate downstream of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which is required for memory $CD8^+$ T cells development (Pearce et al., 2009). Attenuation of aerobic glycolysis or the enhancement of OXPHOS can alter T cell phenotype dramatically. For example, rapamycin or AMPK activator metformin induced augmentation in catabolic pathway can eliminate the differentiation of $CD8^+$ T cells but enhance development of $CD8^+$ memory T cells (Pearce et al., 2009). Similarly, the inhibition of mTOR by rapamycin or blockage of glycolysis by hexokinase inhibitor 2-deoxyglucose (2-DG) can block the differentiation of T helper 17 (Th17) cells while promoting regulatory T (Treg) cell development (Kopf et al., 2007, Michalek et al., 2011, Shi et al., 2011). Moreover, enhancement of fatty acid oxidation (FAO) and energy conservation by activating AMPK pathway also tips the balance of $CD4^+$ T cell differentiation in favor of Treg (Hardie et al., 2012). Given these observations, memory T cell formation maybe influenced by a number of

enzymes and transporters involved in fatty acid synthesis, desaturation and oxidation, as well as availability of free fatty acids (FFA) to memory precursor cells. For instance, acetyl-CoA carboxylase (ACC2), the mitochondrial lipid transporter CTP1A, and the metabolites such as acetyl-CoA, malonyl-CoA has been considered as important players in this process (Abu-Elheiga et al., 2001, Deberardinis et al., 2006, van der Windt et al., 2012). AMPK activation and mTOR inhibition are also both potent activators of autophagy, a catabolic process induced during starvation that has been shown to be important for the maintenance of cellular bioenergetics and sustained T cell viability after activation (Kundu and Thompson, 2008, Hubbard et al., 2010).

## § 1.3 Metabolic regulation and T cell function

T cell metabolism changes over the course of an immune response. Naïve T cells are metabolically quiescent, which adopt a basal level of nutrient uptake and use OXPHOS as their primary pathway for ATP generation. Upon immune challenge, effector T cells switch to a statue of metabolic activation featured by increased nutrient uptake, elevated glucose and glutamine metabolism, biomass accumulation and reduced mitochondrial SRC. Effector T cells preferentially engage in glycolysis for ATP production. Transition from effector to memory T cells is characterized by a quiescent metabolism dependent on FAO to fuel OXPHOS. However mitochondrial mass and SRC are reserved in memory T cells, suggesting that these cells are metabolically primed to respond upon reinfection.

As described before, the ability to engage in glycolysis plays an essential role during T cells activation. Recent evidence indicates that mitochondrial OXPHOS is also important for T cells transiting from naïve to effector state. Blocking mitochondrial ATP synthase by oligomycin can block the expression of early activation markers after TCR ligation and blunts subsequent T cell proliferation (Chang et al., 2013). Mitochondrial derived reactive oxygen species (ROS) may function as a bioenergetics secondary messenger in shaping T cell response (Chaudhri et al., 1986, Devadas et al., 2002, Jackson et al., 2004). T cell deficient for ubiquinol-cytochrome c reductase (Uqcrfsl), a component of complex III of electron transport chain (ETC), displays impaired TCR-dependent ROS production and defects in antigen-specific proliferation (Sena et al., 2013). Intracellular calcium ($Ca^{+}$) flux, which happens early in TCR signal transduction, may also indicates a intimate link

between TCR ligation, mitochondrial OXPHOS, and cell proliferation because of the evidence that uptake of $Ca^+$ by mitochondrial could stimulate $Ca^+$ dependent dehydrogenases of TCA cycle, driving NADH as well as ATP production by OXPHOS during early T cell activation (Krauss et al., 2001). T cells without Bax and Bak, the apoptosis regulators important for $Ca^+$ homeostasis exhibit reduced $Ca^+$ dependent mitochondrial ROS production thus impair T cell proliferation after TCR stimulation. Although toxic in many tissues, OXPHOS derived ROS may prime and license full T cell activation.

Scientific advances in intermediate metabolism observed in cancer highlight a wealth of evidences that are likely to be relevant to T cell biology. Many metabolic pathways abnormally activated in cancer have been shown to play similar roles in normal lymphocyte physiology. Take aerobic glycolysis for example, it has been described as early as 1950s that tumor cells consume glucose at a high rate and produced lactate in a process termed the "Warburg effect" (Warburg, 1956). Proliferating T cells engaged in the same metabolic pathway which allow them to not only generate ATP quickly but also metabolic intermediates important for supporting cell growth and proliferation. One of the key enzymes governing glycolytic flux and cell proliferation is pyruvate kinase (PK). It catalyzes the terminal reaction of glycolysis by promoting the conversion of phophsenolpyruvate (PEP) to pyruvate and is one of two ATP-generating steps of glycolysis. There are two isoforms of PK and naïve T cells express both of them (PKM1 and PKM2) in the resting state but preferentially accumulate PKM2 when transiting to effector T cells (Marjanovic et al., 1990). Interestingly, PKM2 is actually less efficient at converting PEP to pyruvate than PKM1 but its expression is promoted in proliferating cells, including T lymphocytes. Multiple lines of evidence point to a role of Warburg metabolism in supporting

anabolic growth, in which enhanced PKM2 activity promotes the accumulation of glycolytic intermediates towards upstream biosynthetic pathway to support amino acid, triglyceride and nucleotide biosynthesis (Anastasiou et al., 2012, Kung et al., 2012). Moreover, PKM2 also exert some of its effect on cell proliferation through transcriptional and epigenetic regulation (Luo et al., 2011, Yang et al., 2012). PKM2 has been proved to phosphorylate signal transducer and activator of transcription 3 (STAT3) at $Tyr^{705}$, promoting STAT3-dependent transcription (Gao et al., 2012). The protein kinase activity of PKM2 can be viewed as a sensor to metabolic flux, flavoring high glycolysis PEP conditions and antagonizing low-energy high ADP concentration.

Another derivative from glycolysis as an anabolic precursor is 3-phosphoglycerate (3GP), which is a key intermediate in both amino acid and nucleotide biosynthesis. 3GP is the starting point for the glucose-dependent biosynthesis pathway of serine and glycine via serine biosynthesis pathway (SBP). Key enzymes involved in SBP are phosphoglycerate dehydrogenase (PHGDH) and serine hydroxymethyltrasferase (SHMT), generating glycine and methylene-THF to fuel downstream nucleotide biosynthesis and methylation reaction. Serine is also an allosteric activator of PKM2 (Chaneton et al., 2012) and thus provides feedback to the glycolytic pathway to regulate 3PG levels and serine biosynthesis. The amplification of PHGDH that can enhance flux through SBP may confer a growth advantage to proliferation cells by generating NADH to fuel mitochondrial OXPHOS for making ATP. The conversion of 3-phosphohydroxypyruvate to 3-phosphoserine by phosphoserine aminotransferase (PSAT) requires glutamate and α-KG, thus SBP may indirectly promote alternative pathway of α-KG as well as its dependent enzymes activity (Possemato et al., 2011).

Furthermore, recent study indicates production of reduced glutathione (GSH) derived from serine and glycine via SBP can be used as a buffer against oxidative damage (Maddocks et al., 2013). Together, it makes more sense that proliferating effector T cells actively engage in SBP upon activation regardless of abundant exogenous serine.

Similar to glycolysis, TCA cycle in proliferating cells functions as a source of biosynthetic precursors in addition to its role in ATP production (DeBerardinis et al., 2008). Glucose derived pyruvate that is not converted to lactate will enter mitochondrial and be converted into acetyl-CoA by pyruvate dehydrogenase (PDH) complex. Together with rate-limiting substrate oxaloacetate (OAA), glucose-derived acetyl-CoA generate citrate in the TCA cycle, which is essential in lipid biosynthesis. $CD8^+$ T cells unable to engage this acetyl-CoA-dependent lipid biosynthetic pathway display defect in antigen-driven blastogenesis and clonal expansion in response to pathogens (Kidani et al., 2013). Another key metabolite generated through TCA cycle is α-KG from glutamine via glutaminolysis. Groundbreaking work indicates that α-KG can be converted to citrate through reductive carboxylation under conditions of stress such as hypoxia or mitochondrial dysfunction, which bypasses the conventional TCA cycle by using glutamine to generate acetyl-CoA for fatty acid synthesis (Metallo et al., 2011, Mullen et al., 2011, Wise et al., 2011). This alternative pathway for lipogenesis implicates the possibility that T cells are able to use reductive glutamine for fatty acid biosynthesis under hypoxic condition (Metallo et al., 2011). Such metabolic plasticity prepares T cells to response to environmental cues and in order to maintain proliferation and /or effector function.

In mammalian cells, fluctuations in cellular energy are monitored by heterotrimetric AMPK complex, which contains nucleotide-binding sites of the γ regulatory subunit that binds ATP, ADP or AMP. Elevation of cellular AMP:ATP ration leads to increased phosphorylation of AMPK at Thr172 of its activation loop through the kinase LKB1, which make AMPK functions as a sensor for cellular energy charge (Oakhill et al., 2011, Xiao et al., 2011, Hardie et al., 2012). Together with LKB1, AMPK promote catabolic pathway for ATP generation and antagonized mRNA translation through negative regulation of mTORC1 (Shackelford and Shaw, 2009). Recent work suggests that LKB1-AMPK signaling can influence T cell metabolism and function. Lymphocytes exclusively express the α1 subunit of AMPK indicating its role in promoting catalysis. TCR engagement promote LKB1 dependent AMPK activation while in turn lost of LKB1-AMPK signaling enhance glycolysis as well as mTORC1 activity with elevated $T_H1$ cytokine interferon-γ production by effector T cells (Tamas et al., 2010, MacIver et al., 2011, Faubert et al., 2013). Deletion of either LKB1 or AMPK α1 disrupts normal lymphocytes homeostasis, leading to an accumulation of activated $CD8^+$ T cell population in animals (MacIver et al., 2011). Furthermore, it was recently shown that AMPK-deficient T cells are defective in generating $CD8^+$ memory T cells during infection (Rolf et al., 2013). In summary, AMPK may serve as a metabolic checkpoint in T cells to regulate its metabolic fitness and immune functions coordinating metabolic change in response to nutrient fluctuation in the microenvironment.

All in all, the implication of these findings for immunologists is that we may gain control over cell growth and survival by directly influencing metabolic currency of

cells, energetic intermediates and metabolites involved in bioenergetics and biosynthetic reactions through metabolic reprogramming.

## § 1.4 T cell dysfunction in tissue microenvironment

T cells are influenced by nutrients and other supportive signals, such as those provided by growth factor cytokines, which are available in their environment. In settings such as chronic infection and cancer, T cells can become anergic, or exhausted, losing the capacity to exert their effector function thus leading to disease progression. Take cancer for example, one of the primary mechanisms of T cell dysfunction is thought to be long-lasting stimulation from immunogenic antigens expressed by tumor cells. Chronic TCR stimulation, lack of proper costimulation, and active suppression by other cell types in the tumor microenvironment has all been implicated in T cell dysfunction (Wherry, 2011, Crespo et al., 2013, Pauken and Wherry, 2015). Evidences in gene expression analysis of exhausted T cells indicates that several genes involved in energy metabolism transcriptionally downregulated and inhibiting leucine or glucose metabolism during T cell activation can lead to an anergic phenotype (Zheng et al., 2009, Wherry, 2011). Moreover, upregulated expression of inhibitory receptors on exhausted T cells, such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein-1 (PD-1) has been shown to inhibit elevated glucose and glutamine metabolism induced by TCR ligation and co-stimulation during T cell activation (Parry et al., 2005, Boussiotis et al., 2014). Especially, ligation of PD-1 through its ligands PDL-1 can alter T cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation (Patsoukis et al., 2015). This metabolic alteration regulated by PD-1 contributes to bioenergetics insufficiencies through mTOR signaling thus presents as an early driver of CD8 T cell exhaustion (Bengsch et al., 2016). All these findings implicate that T cell hyporesonsiveness may be in

large part due to an inability of the cells to optimal utilize appropriate metabolic pathway.

Nutrient competition between cells can influence cell growth, survival, and function – and a fierce competition likely exists between cells in the tumor microenvironment, as the demand for resources is high (MacIver et al., 2013, Siska and Rathmell, 2015). Tumor cells promote Warburg metabolism over oxidative metabolism for quick ATP generation to support their rapid cell growth, so do proliferation T cells. We speculate that lymphoid organs where lymphocytes resides and activated are nutrient-replete and normoxia but high level of glycolytic activity in cancer cells coupled with poor angiogenesis can lead to glucose depletion and accumulation of lactate in the local tumor sites infiltrated by T cells. Nutrient availability in the tumor microenvironment can post particular metabolic challenges for antitumor T cell responses. For example, tumor cells can express indoleamine 2,3-dioxygenase (IDO), an enzyme that depletes tryptophan from the microenvironment and thus inhibits T cell proliferation and effector function (Munn et al., 1999, Munn and Mellor, 2013). Tumor-derived lactate can also suppress T cell function directly by suppressing lactate export from T cells, thus disrupting their ability to maintain glycolysis (Fischer et al., 2007). Metabolic interaction between tumor cells and immune cells in metabolic restrictive environment may present a potential mechanism of T cell hyporesponsiveness during cancer. Because glucose and glutamine are crucial for T cell differentiation and function, and depletion of glucose impairs cytolytic activity as well as IFN-γ production (Chang et al., 2013). Culture of T cells in glucose-depleted conditions has been shown to inhibit signaling through mTOR activation and selectively down-regulate gene to impair cell cycle

progression, cytokines production and cytotoxic ability of effector T cells (Cham and Gajewski, 2005, Cham et al., 2008). It was also found to be true that mutation BRAF V600E, which is commonly expressed in melanoma with constitutive activation of the MEK-MAPK pathway, generate a tumor that has a strongly immunosuppressive microenvironment. T cells isolated from BRAF V600E tumors had decreased CD40L expression and IFN-γ production, which support the idea that tumor metabolism may directly alter anti-tumor T cell immunity (Ho et al., 2014). All of these pieces of evidences suggest that metabolic reprogramming is intimately tied with T cell function and is therefore is central to effective immunity against tumors. The outcome of an antitumor response may be swayed one way or the other by the ability of T cells to cope with modulation in nutrient availability in the microenvironment.

While it is relatively easy to envisage how T cells in a solid tumor could be at a competitive disadvantage for nutrients, and that this would negatively affect their function, this paradigm can additionally be extended to other setting that perhaps less obvious. Gut environment is a special hub where groups of bacteria species, termed “microbiota” reside and produce multiple metabolites that can interact with host tissues and immune system thus can have profound effects on T cell development and function (Nicholson et al., 2012, Mockler et al., 2014). For example, the bacteria production of short-chain fatty acids such as butyrate within the gut has been shown to alter the balance of Th17 and Treg cells as well as altering T cell mTOR signaling (Berod et al., 2014). Acetate, another metabolites fermented by intestinal microbiota has been determined to regulate the size and function of colonic Treg pool thus promotes colonic homeostasis and health (Smith

et al., 2013). Notably, when acetyl-CoA carboxylase, an important enzyme in fatty acid synthesis is blocked either genetically or by the bacterial metabolites Soraphen A, naïve T cells favor to polarize to Treg cell fate instead of Th17 cells. The alternation in metabolic pathway that bias T cell differentiation away from Th17 cell development has been implicated to contribute to Th17 mediated diseases including multiple sclerosis or Crohn's disease (Wilke et al., 2011). Given evidences above, it is possible that other metabolites generated by commensal bacteria that modulate cell metabolites and influence immune responses, possibly regulating autoimmune susceptibility in human. All together, understanding how diverse cell populations or organisms influence the tissue microenvironment, and how this environment dictates metabolic pathway engagement by T cells, and thus differentiation and function is essential for developing effective T cell-based immunotherapies.

## § 1.5 Metabolites as signaling molecules connecting metabolism and gene regulation

As described before, signaling transduction pathway, such as T cell activation signal through its TCR can drive cellular metabolism above the capacity normally maintained in the quiescent. This proliferation signal mediated metabolic reprogramming required to match the biological need can not only influence downstream signals of cell surface receptors through these metabolic pathways but also serve as a feedback system to regulate metabolic flux through local nodes in the network. Energetic intermediates and metabolites generated in this process involved in bioenergetics and biosynthetic reaction that influence cell growth and survival. Metabolites within cells can also act as signaling molecules that participate in T cell signaling to shape T cell function and fate, and thus the availability of particular metabolites can dramatically affect both cellular metabolism and cell signaling.

One of the key metabolic intermediates acetyl-CoA is not only oxidized in TCA cycle for energy generation and fatty acid synthesis, but also involved in the regulation of gene transcription, transcription factor signaling, enzyme activity through protein acetylation (Wellen and Thompson, 2012, Choudhary et al., 2014). In the nutrient rich and normoxic conditions, the primary source of acetyl-CoA is glucose, which is converted to pyruvate through glycolysis via PDH enzyme complex. As one of the major carbon source, acetyl-CoA enters TCA cycle to generate reducing equivalents (NADH, FADH) and to drive electron transport chain. Mitochondrial acetyl-CoA can only pass across the mitochondrial membrane entering cytoplasm in form of citrate.

In the cytoplasm, the enzyme ATP-citrate lyase converts citrate back into acetyl-CoA to be used in fatty acid synthesis or protein acetylation. In contrast, under hypoxic condition, reduced PDH activity limits OXPHAS in the mitochondrial, thus synthesize less acetyl-CoA but more lactate through TCA cycle. Similarly, other substrates such as glutamine, which is important in cells to converted to glutamate by glutaminase, only make partial pass through TCA cycle and are exported from mitochondria as malate. As a result, the level of acetyl-CoA derived from glucose and glutamine drops, which may correlate to the levels of histone acetylation in an ACL-dependent process (Wellen et al., 2009).

Other metabolic intermediates also act as signal transducers drive diverse downstream biological processes. Take succinate for example, it is derived from TCA cycle, which can induce IL-1β inflammatory signal in macrophages through HIF-1α stabilization (Tannahill et al., 2013). The accumulation of fumarate, due to fumarate hydratase deficiency, leads to several changes within cancer cells, including to hypermethylation and HIF-1α stabilization (Sullivan et al., 2013). Transport of amino acid is also required for T cell metabolic reprogramming. Intracellular leucine level can influence mTOR activity via leucyl-rRNA synthetase thus low concentration of leucine will impair mTOR function. Accordingly, it was reported that expression of cytosolic branched-chain aminotransferase (BCATc) that transaminates leucine, negatively regulation mTOR activity during T cell activation to prevent overactivation (Ananieva et al., 2014).

One innovative way we consider the ability of metabolism within cells to influence their biological function is to govern gene expression through posttranscriptional

regulation network involving RNA, metabolites and metabolic enzymes. Many enzymes involved in metabolic pathway, including glycolysis, PPP and fatty acid metabolism, have been shown to bind RNA thus specifically regulate protein translation *in vitro* and in culture cells (Hentze and Preiss, 2010). In addition, RNA binding function of enzymes can be influenced by interaction with other metabolites or cofactors, demonstrating how metabolic state of cells can link to gene regulation through the control of RNA binding function of the enzyme. The well characterized metabolic enzyme as an RNA binding protein is cytosolic aconitase, which is the key regulator of cellular iron metabolism (Hentze and Argos, 1991, Rouault et al., 1991). It was reported that cytosolic aconitase share the same polypeptide with RNA binding protein IRP-1 which is available to insert or remove an iron sulfur cluster, thus switching the protein's function between RNA binding activity in low iron condition as IRP-1 and metabolic enzyme activity in high iron environment (Constable et al., 1992). This dual function of aconitase is essential in regulation iron homeostasis.

Following the idea that bifunctional enzymes with the ability of RNA binding may present a general mechanism of how metabolism and gene expression are coordinated, our lab identify another glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) function as a RNA binding protein, which can regulate posttranscriptional production of IFN-γ in T cells by engaging or disengaging the glycolysis pathway (Chang et al., 2013). Active T cells can dynamically switch between OXPHOS or glycolysis for ATP generation. When T cells do not engage in glycolysis pathway but using OXPHOS, GAPDH binds the 3'UTR of cytokine mRNAs, including IFN-γ and IL-2 to prevent translation of these mRNAs. Thus

although OXPHOS can support T cell survival or proliferation, only aerobic glycolysis can facilitate their full effector function. This control mechanism allows T cell uncouple its survival and proliferation from effector function, creating a checkpoint for T cells to differentially govern cytokine production when is not desirable during hemostasis. The activity of GAPDH is not only controlled by substrate availability but is also heavily influenced by redox balance within the cells. For example, $NAD^+$ is required for GAPDH enzymatic function but it is also interferes with mRNA binding in vitro (Nagy et al., 2000). NAD+/NADH redox balance, which may influence by other metabolic enzymes including LDHA and PHGDH, could also affect posttranslational modification of GPADH, altering its binding to mRNA, metabolites as well as localization in the cell (Colell et al., 2007, Tristan et al., 2011). All in all, these observations clearly demonstrate how cofactors and substrates generally considered for their direct effects on metabolism may also coordinate metabolism with gene regulation.

## § 1.6 Targeting T cell metabolism as a effective adoptive immunotherapy

Researches in the last decade has accumulated a wealth of evidence demonstrating how metabolic reprogramming intrinsically link to T cell biology, as well as how metabolic alternation impacts T cell activation, differentiation and ultimately, immunological consequence during infections and cancer. Emerging concepts about targeting metabolic pathway for more effective adoptive immunotherapies to recue T cell dysfunction in tumor microenvironment, as well as to prevent undesirable pathogenic T cell response in settings of autoimmunity will be highlighted in this chapter.

Substrate availability in the microenvironment has been shown as a major impact on T cell function *in vivo*, and clearly the environmental impact on T cells also present *in vitro* culture system. There has been a huge effort put into the development of adoptive cellular immunotherapy for cancer and viral infection, whereby naturally occurring or engineered T cell are stimulated and expanded *in vitro* before transferring back to the patients (Maus et al., 2014). The first use of chimeric antigen receptor (CAR)-modified T cells was in HIV infection, which engineered with receptors for HIV envelope protein fused to TCR downstream signaling molecule to induce antigen-specific T cell response. In setting of solid tumors, immune-based clinical interventions were proposed to convert TILs into effective cells, which includes systemic administration of cytokines such as IL-2 and interferon, antibody treatments aimed at modifying T cell activation and therapies targeting inhibitory pathway by relieving checkpoint blockade. Specially, checkpoint blockade antibodies against CTLA-4, PD-1/PDL-1, 4-1BB as well as CD40 has had remarkable result not

only in advanced melanoma, but also lung caner that has been preciously been described as “immunologically silent” with poor response (Hodi et al., 2010, Topalian et al., 2012). The combination of two checkpoints in melanoma significantly improved the response rate and time to tat over either therapy alone (Wolchok et al., 2013). Direct isolation and ex vivo activation of TILs have also been tested in multiple early-phase studies and result in durable responses in advanced melanoma (Rosenberg et al., 2011). Although phenomenal success has been achieved using adoptive cellular immunotherapy, many patients still failed to response following with poor clinic outcomes. Given the substantial amount of evidences showing the intrinsic link between T cell activation and metabolism, research directed at manipulating metabolic pathway which can positively affect T cell function and longevity may potentially enhance therapeutic efficacy and lead to better patient outcomes (Sukumar et al., 2013).

Altering culture conditions for adoptive cellular immunotherapy in order to change the metabolism is one way in which T cells could be restrained from terminal differentiation while being optimized for persistence *in vivo*. For example, many commonly culture media contains glucose concentration ranging from 10mM to 25mM, which is substantially higher than physiological glucose level in the normal blood stream. High glucose in the culturing media may potentially induce hyperglycemia in proliferating T cells that become overly dependent on glycolysis through metabolic reprogramming (Glick et al., 2014). It may risk the effect of T cells that are transferred back into patients when exposing to low physiological glucose level again because the augmentation of glycolysis in $CD8^+$ T cell limits its long-term survival (Sukumar et al., 2013). It has also ben shown that increased glycolysis

positively correlates to cell size, which is negatively impact mitochondrial gene expression as well as cell survival in vivo (Manjunath et al., 2001). Likewise, constitutive activation of Akt can enhance cellular glycolysis and increase surface expression of glucose transporters, which result in enlarged cell size. Conversely, limiting T cell glycolysis using low dose 2-deoxyglucose (2-DG) by inhibiting hexokinase in vitro can reduce cell size and increase longevity without impairing proliferation capacity. All together, these data indicate that appropriate culture conditions can improve adoptive T cell transfer therapy through direct modulation of metabolism. Moreover, biological parameters such as cell size or glycolytic rate may be used as predictors of *in vivo* T cells fitness and function, which are activated *in vitro*.

The replicative capacity and long-term survival of adoptive transfer cells may also be promoted by enhancing OXPHOS or mitochondrial biogenesis instead of limiting overly glycolysis. There is the evidence showing that by inhibiting Akt signaling pathway, *in vitro* expanded TILs increase their rates of OXPHOS and FAO, which benefit their *in vivo* survival and anti-tumor function (Crompton et al., 2015). As mentioned before, the administration of cytokines that signal via receptors containing common γ chain including IL-15 or IL-7, allows the substantial cell survival, population expansion and effector function. These *in vivo* beneficial effect induced by these cytokines has at least partially link to metabolic reprogramming. IL-15 reduces glycolysis while enhance OXPHOS and SRC in activated $CD8^+$ T cells along with increased mitochondrial mass. Observations from our lab indicate that, in addition to accumulated mitochondrial mass, the morphology of mitochondrial in memory T cells dynamically changed and appeared to be networked, which is

distinct to their counterpart effector T cells with punctate mitochondrial (Buck et al., 2016). These fission and fusion events that constantly happened in the mitochondria in T cells regulate metabolism, longevity as well as cell fitness (Chan, 2012, Westermann, 2012, Youle and van der Bliek, 2012). As a result, pharmacologically targeting mitochondria to enhance their function may present an effective way for the improvement of adoptive transferred T cell therapy. For example, Szeto-Schiller (SS) peptides, which target cardiolipin within mitochondria in order to optimize the efficiency of electron transport chain, has been tested in Phase II clinical trials potentially as the treatment for ischemic reperfusion injury (Chakrabarti et al., 2013).

Tumor microenvironment is complicated network that not only contains malignant cells but also includes stromal and epithelial cells, as well as a group of tumor associated immune cells. The presence of various cell types may result in an inhospitable nutrient environment for effector T cells to exert their optimal functions. For instance, the depletion of amino acid such as arginine and tryptophan (Uyttenhove et al., 2003, Rodriguez et al., 2004), the competition consumption of other nutrients including glucose and lipids (Wang et al., 2014, Chang et al., 2015), as well as the production of metabolites such as lactate in the tumor microenvironment could all lead to alteration of T cell metabolism thus impact their anti-tumor functions (Fischer et al., 2007). Likewise, a recent study explained that tumor-derived lactate can drive expression of arginase I in tumor-associated macrophages, result in polarization into M2 phenotype, which presents as an immunosuppressor inhibiting T cell function and promoting tumor progression. Another example of metabolic and environmental influences on T cell function in vivo is the competition model of nutrient in the tumor microenvironment, in which effector

T cells compete with tumor cells for available glucose, and this tumor cells imposed nutrient restriction dampens T cell effector cytokine production, leading to cancer progression (Chang et al., 2015). It seems like overall suppressive nature of the tumor microenvironment contributes to impaired T anti-tumor immunity.

There have been a lot of efforts put on modifying cancer metabolism with the hope of improving the efficacy of anti-tumor therapies. The problem of this strategy is that activated T cells and cancer cells often share similar metabolic traits, thus targeting tumor cell metabolism has the potential to also negatively impact infiltrating effector T cells. One way to avoid this problem is to focus on developing small molecule compounds that target metabolic pathway in a tumor-specific manner. Aforementioned BRAF V600E described before is a clear tumor-specific therapeutic target in melanoma without detrimental effect on TILs. Another experimental AGI compounds have been developed to specifically inhibit the mutation forms of isocitrate dehydrogenase (IDH) enzymes and thus have shown anti-tumor potential against glioma and leukemia *in vitro* (Rohle et al., 2013, Wang et al., 2013). The combination small molecule compounds targeting tumor metabolism with adoptive T cell transferring immunotherapy theoretically presents a way to create a tumor microenvironment that is metabolic favorable for transferred T cells to exert their optimal anti-tumor functions.

There is substantial interest in optimizing T cell function against viral infections and cancer through metabolic regulation. On the other hand, undesirable T cell activation in the setting of graph versus host disease (GvHD) as well as autoimmune disorder may also be prevented via modulation of metabolic pathway. For example,

alloreative T cells from GvHD appear to rely on OXPHOS, thus targeting TCA cycle reaction or mitochondria ATP production could specifically limit the activation of these cells. Following this idea, metformin, which is the inhibitor of complex I of the electron transport chain, could potential has the therapeutic effect on GvHD, particularly in the setting that alloreactive T cells exhibit hyperpolarized mitochondria. Mitochondria function or ATP generation can also be inhibited by limiting the activity of carnitine palmitoyl transferase (CPT)-1a, which blocks oxidation of long-chain fatty acids in the mitochondria. Pharmacological agents such as etomoxir or perhexiline, could be used to selectively target GvHD T cells with higher rates of FAO compared to their counterparts (Byersdorfer et al., 2013).

To facilitate targeting specific cell population in the microenvironment, nanotechnology is being innovated and used for the delivery of small molecule compounds for therapeutic purpose. Because this method allows the control and sustained release of a compound in a cell specific manner, it could be extremely useful tool for delivering metabolism-modifying compounds of T cells. One type of this drug encapsulation system is biodegradable poly lactide-co-glyceride (PLGA) nanoparticles, which allows controlled released of drug from days to months (Mundargi et al., 2008). With conjugated antibodies against T cell surface marker, PLGA nanoparticles can directly target T cell population and block their metabolic pathway by delivering glycolysis inhibitor 2-DG or dichloroacetate (Gerriets et al., 2015), L-type amino acid transport inhibitor JPH203 (Gerriets et al., 2015), or lactate transporter inhibitors like AR-C141990 (Pahlman et al., 2013) as a way to limit hyperactive T cell inflammatory responses in autoimmune disorder. This delivery system has proven to be effective for leukemia inhibitory factor, which is used to

oppose Th17 cell differentiation and enhance Treg cell development in a mouse model of allograph rejection (Park et al., 2011).

Last but not least, the idea of bi-specific antibodies with two independently targeted antigen binding fragmentation (Fab) regions can simultaneously bind to two type of cells together. These proteins have been primarily explored as cancer therapies, where the bi-specific antibody is used to bring tumor cells and cytotoxic T cells closed enough for antigen-specific killing. This technology could be extended to apply in modifying T cell metabolism thus modulate their functions. The use of a strong-binding antibody against T cell surface marker could be conjunction with a low-affinity antagonistic antibody against substrate transporter to inhibit T cell activation or delivery protein conjugated with carbohydrates such as glucose to enhance T cell function. The advantage of this approach would be the hierarchy of binding affinity could ensure the delivery of desired molecules is only targeted cells that express specific markers.

## § 1.7 Emerging topics and concluding remarks

Immune cells undergo dynamic changes during the course of immune response. In the past few years, generous amount of emerging findings indicate the interconnection between cell signaling and biomedical pathways. All parameters of these fields are directly intertwined, comprising an integrated network from gene expression to metabolite production. Studies on T cell provide a unique opportunity to understand how metabolism supports normal T cell biology including survival and proliferation as well as how metabolic alternation drive T cell differentiation and fate. Characterizing how metabolic pathway regulated in T cells, how perturbations in the microenvironment influence T cell responses, and how metabolic response are modulated *in vivo* in the context of systemic stress such as infection or cancer and eventually contribute to the outcome of T cell mediated immune response are still active challenging topics in this field. Applications of pharmacologic reagents as well as advanced nanotechnology that interfere with metabolic pathway with the appreciation for the role of metabolism in dictating immune cell function holds promise for revolutionary new treatments and therapies in the near future.

# CHAPTER 2

# METABOLIC COMPETITION IN THE TUMOR MICROENVIRONMENT IS A DRIVER OF CANCER PROGRESSION

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## § 2.1 Introduction

Establishing why some cancers progress while others do not is a longstanding challenge in immunology. The immune system is often capable of distinguishing nascent tumor cells, and the recognition and destruction of strongly immunogenic tumors by T cells is a critical part of the anti-tumor immune response. However, during this process, cancer cells that express weakly immunogenic antigens will evade killing and thus be selected for outgrowth. This is thought to be a primary mechanism of tumor progression (Vesely and Schreiber, 2013). Another mechanism by which tumors are known to escape anti- tumor immunity is through T cell dysfunction, or hyporesponsiveness. Hyporesponsive states such as anergy, exhaustion, and senescence, have all been described in T cells from cancer patients (Wherry, 2011, Crespo et al., 2013) – and chronic TCR stimulation, lack of proper costimulation, and active suppression by other cell types in the tumor microenvironment have all been implicated in T cell dysfunction. However, whether other mechanisms exist, or precisely how T cell hyporesponsiveness in tumors is established, remains to be fully defined.

Nutrient competition between cells can influence cell growth, survival, and function, and a fierce competition likely exists between cells in the tumor microenvironment, as the demand for resources in this niche is high. Metabolic interplay between tumor cells and immune cells has been demonstrated. For example, tumor cells can express indoleamine 2,3-dioxygenase (IDO), an enzyme that depletes tryptophan from the microenvironment and thus inhibits T cell proliferation (Munn et al., 1999, Munn and Mellor, 2013). Tumor-derived lactate can also suppress T cell function directly by blocking lactate export from T cells (Fischer et al., 2007), which disrupts

their ability to maintain aerobic glycolysis. We previously published that, via a posttranscriptional mechanism, the engagement of aerobic glycolysis is specifically required for effector function in T cells, but not necessarily for T cell activation, proliferation, or survival (Chang et al., 2013). We also showed that in culture, tumor cells can outcompete T cells for glucose, and this lack of available glucose directly impedes the production of cytokine that can be critical for tumor clearance. Since many tumors have high rates of aerobic glycolysis (Warburg, 1956, Gatenby and Gillies, 2004), we hypothesized that tumor-infiltrating $CD8^+$ T lymphocytes (TILs) could experience a loss of function during cancer due to altered metabolism resulting from tumor-imposed glucose restriction, and this subsequently could lead to defects in cytokine production and tumor clearance. Therefore we sought to formally establish whether glucose competition in the tumor microenvironment, in its own right, determines cancer progression by regulating the 'nutrient-fed' state of tumor-infiltrating T cells, and thus their functionality.

## § 2.2 Tumor glucose-restrict T cells, altering their metabolism and function

To investigate how glucose competition between tumors and T cells affects T cell function, we used an established mouse sarcoma model of regressing and progressing tumors (Matsushita et al., 2012, Gubin et al., 2014). D42m1-T2 (referred to here as 'R' tumor) is a regressor clone of the d42m1 sarcoma that expresses the major tumor- specific rejection antigen mutant spectrin-β2. After transplantation into mice, this tumor is rejected at ~day 12 in a manner that depends upon IFN-γ production from TILs (Matsushita et al., 2012). D42m1-T3 (referred to here as 'P' tumor) is a progressor clone of d42m1 that lacks this rejection antigen and grows progressively after transplantation into mice (**Figure 1A**). Consistent with our published results using other tumor cell lines (Chang et al., 2013), we co-cultured R and P tumors with C3 T cells, a cytotoxic T lymphocyte (CTL) clone that recognizes mutant spectrin-β2 (Matsushita et al., 2012), and found less glucose in the media when T cells were cultured overnight with tumor cells than when the same total number of T cells were cultured alone (**Figure 1B**), indicating that these tumors consume more glucose than T cells on a per cell basis. However, we also found that there was less glucose remaining in the media when T cells were co-cultured with P tumor cells, than with R tumor cells (**Figure 1B**). While the T cells co-cultured with R tumor cells produced less IFN-γ than T cells cultured alone, we found that production of this cytokine was dampened even further in T cells co-cultured with the P tumor cells (**Figure 1C**). We observed similar results in IFN-γ production when the tumors were co-cultured with polyclonal T cells rather than the CTL line (data not shown). Importantly, adding glucose during the restimulation enhanced T cell IFN-γ production (**Figure 1C**), indicating that glucose availability and utilization, and thus

competition for this sugar, rather than the presence of other metabolites such as lactate, had a direct role in regulating T cell effector function in these co-cultures. We further confirmed that the extracellular acidification rate (ECAR), which is an indicator of aerobic glycolysis, the process where glucose is converted to lactate even in the presence of sufficient oxygen to support glucose catabolism via the TCA cycle and oxidative phosphorylation (Nicholls et al., 2010), was higher in the P tumor than in the R tumor (**Figure 1D**, left panel), supporting the data showing that the P tumor consumes more glucose than the R tumor (**Figure 1B**). Although the ECAR of these R and P tumors differed, their rates of proliferation in vitro were similar (**Figure 1D**, right panel), demonstrating that glycolysis is not necessarily directly coupled to the proliferation of these tumor cells. These results suggested that a defect in T cell effector function in the P tumor microenvironment could be due to a metabolic deficit imposed by glucose restriction.

Although the R and P tumors differ in their antigenicity, it has been shown that tumor-specific T cells infiltrate both tumors (Matsushita et al., 2012, Gubin et al., 2014). We transplanted either R or P tumor cells into naïve 129S6 mice and analyzed activation markers on TILs ~day 12 after transplantation (**Figure 1E**). We found that TILs in the R and P tumors had a $CD44^{hi}CD62L^{lo}$ effector T cell phenotype and expressed T-bet (**Figure 1F**, top), suggesting that the TILs present in both tumors were activated and transcriptionally competent to produce IFN-γ (Parish and Kaech, 2009, Anichini et al., 2010). However, as has been previously shown (Gubin et al., 2014), TILs in the P tumors were $PD\text{-}1^{hi}$, consistent with their hyporesponsive phenotype (Ahmadzadeh et al., 2009, Baitsch et al., 2011). Grossly, the infiltrates of immune cells were similar in R and P tumor on day 12, although the relative frequency of T regulatory (Treg) cells and the balance of M1- versus M2-

polarized macrophages differed (**Figure 2**). Together these results suggested that activated TILs infiltrate both P and R tumors (Matsushita et al., 2012, Gubin et al., 2014), but that TILs in the P tumor develop a state of hyporesponsiveness. As we have shown previously that nutrient restriction can lead to hyporesponsiveness in T cells in vitro (Chang et al., 2013), we wondered whether the high glycolytic rate of P tumors effectively limits glucose availability within the tumor microenvironment and if this contributes to the hyporesponsive state of TILs in this niche.

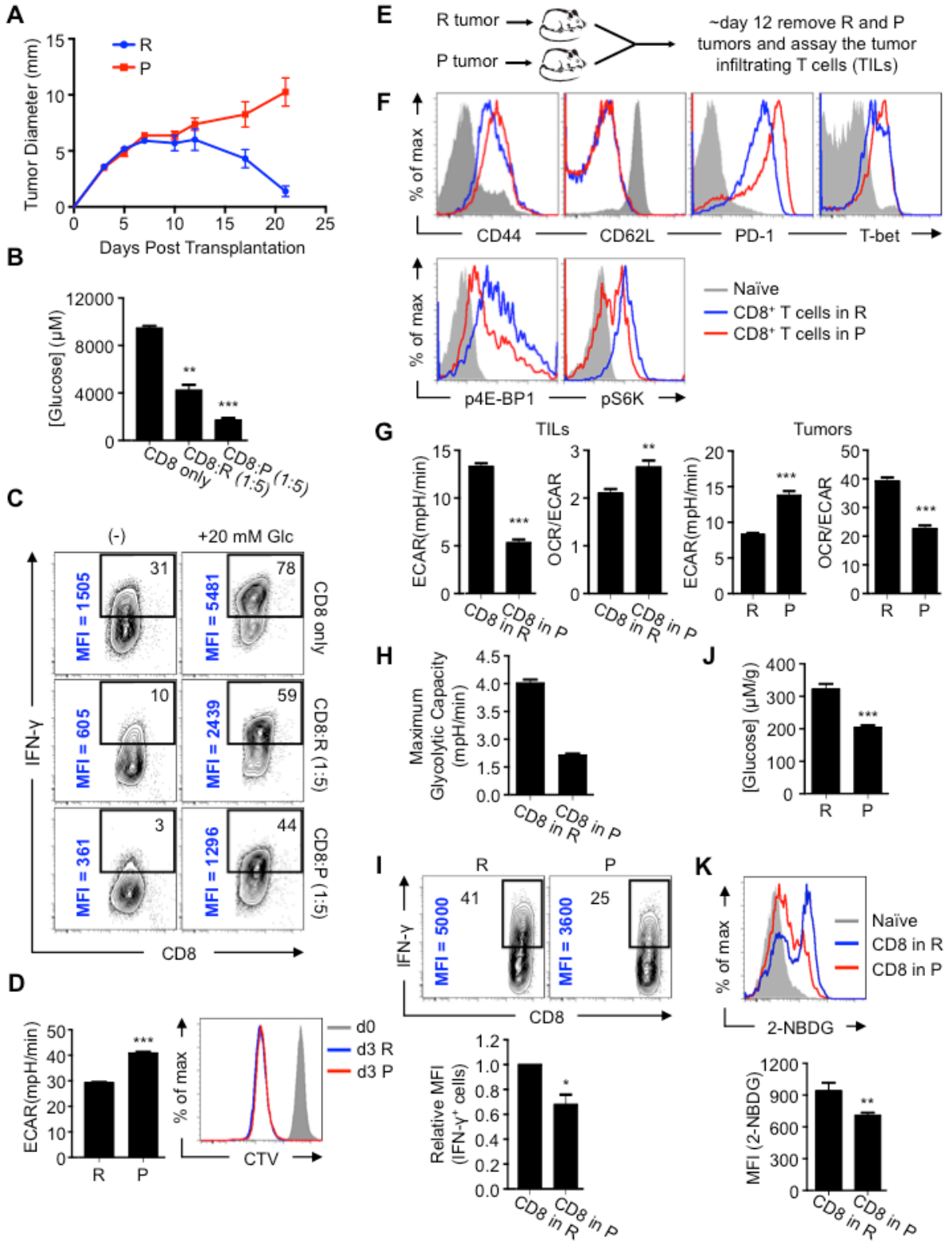
Mechanistic target of rapamycin (mTOR) is an environmental sensor, and mTOR pathway signals decrease when nutrient availability is low (Kim et al., 2002, Gatenby and Gillies, 2004). Therefore, we reasoned that mTOR pathway activity in TILs would directly reflect the nutrient status of these cells within the tumor microenvironment. We measured mTOR target activation in TILs from R and P tumors directly ex vivo and found that phosphorylation of 4E-BP1 and S6 kinase was decreased in P tumor-TILs when compared to the phosphorylation of these proteins in R tumor-TILs (**Figure 1F**, bottom). These data support the view that P tumor cells, which consumed more glucose (**Figure 1B**) and displayed a higher ECAR (**Figure 1D**, left), and thus had a higher glycolytic rate, imposed a more severe glucose restriction on TILs than did R tumor cells.

Inhibitory signals from molecules such as PD-1, or from Treg cells, also dampen T cell responses in tumors (Keir et al., 2008, Francisco et al., 2010, Simpson et al., 2013) and we speculated these signals might further inhibit the ability of TILs to compete for glucose in a tumor. However, we wanted to test the idea that metabolic competition between cells in a microenvironment is a fundamental force that drives immune cell phenotypes. Therefore, we considered that nutrient competition by P

tumor cells alters the metabolism of TILs within the same tumor niche, and in turn dampens their effector function. To begin to investigate the metabolic landscape in the tumor microenvironment, we assayed the metabolism of TILs from excised R and P tumors on day 12 post-transplantation. We found that ECAR of TILs from P tumors was lower than that of TILs from R tumors (**Figure 1G**, left), indicating less aerobic glycolysis in these cells. Notably, unlike TILs from R tumors, TILs from P tumors were impaired in their capacity to augment glycolysis when mitochondrial respiration was blocked by the ATP synthase inhibitor oligomycin (**Figure 1H**). As we found to be the case for P tumor cells in vitro, ex vivo P tumor cells also exhibited higher ECAR when compared to R tumor cells. This elevated ECAR was inversely proportional to the metabolism of the TILs that were isolated from that tumor (**Figure 1G**, right), indicating a metabolic interplay between tumors and their TILs. After restimulation, TILs from the P tumor produced significantly less IFN-γ than TILs from the R tumor (**Figure 1I**), directly illustrating a relative lack of functionality in the P tumor metabolically-restricted TILs. Importantly, the glucose concentration in the extracellular milieu of P tumors was significantly lower than in R tumors (**Figure 1J**). These data show that elevated ECAR of P tumor cells directly correlates with lower glucose availability in their tumor microenvironment, and are consistent with the idea that high glucose consumption by tumors cells can cause glucose depletion within the tumor niche. To directly address whether TILs in P tumors are glucose-restricted, we intravenously injected the fluorescent glucose analog 2-NBDG and tracked its uptake by TILs in P vs. R tumors. We found that TILs in P tumors acquired less 2-NBDG than those in R tumors (**Figure 1K**), which is consistent with their reduced ECAR (**Figure 1G**, left). Taken together, these results suggest that TILs are nutrient-restricted in the P tumor microenvironment, and that this accounts for their impaired

glycolytic capacity and effector function in this niche.

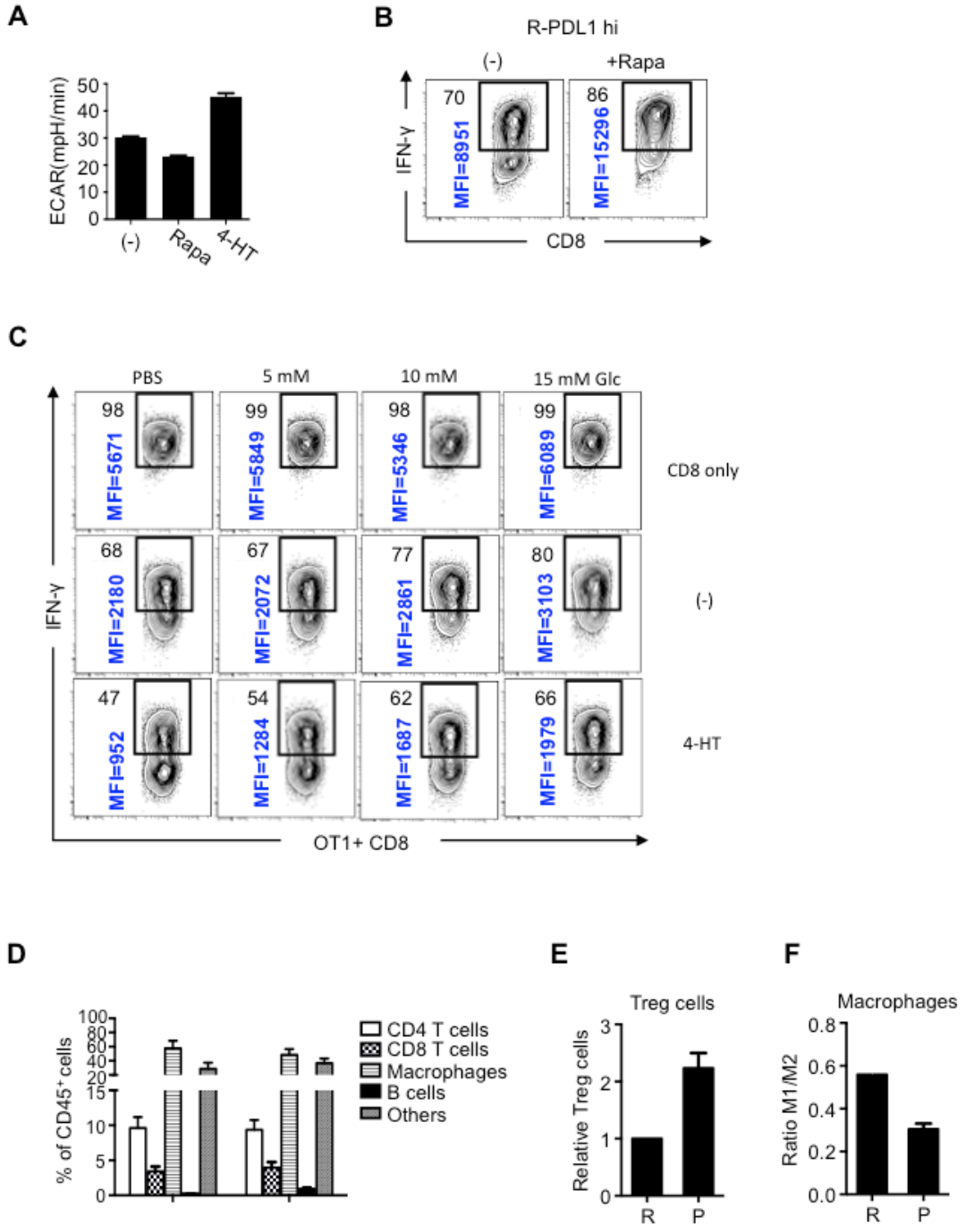
**Figure 1. Tumor mediated glucose restriction alters the metabolism of T cells and dampens their ability to produce cytokine**



(A) $1x10^6$ MCA-induced sarcoma d42m1 derived regressor (R) or progressor (P) tumor cells were injected s.c. into 129S6 mice (n=5) and tumor growth was monitored. Tumor size is shown as the average of two perpendicular diameters ± SEM from 10 mice of 2 independent experiments. (B) C3 T cells were cultured alone, or with a 1:5 ratio of P or R tumor cells for 24h. Cells were then restimulated with PMA/ionomycin for 5h and IFN-γ production was measured. At the time of restimulation, either no additional glucose or 20 mM glucose (Glc) was added to the media. The percentage of IFN-$\gamma^+CD8^+$ cells is depicted on the top right and the mean fluorescence intensity (MFI) of $CD8^+$ cells is shown vertically. The results are representative of ≥ 2 independent experiments. (C) Glucose concentrations in the media of the above co-culture were measured before stimulation. Data represent at least 2 independent experiments and are shown as mean ± SEM, **p=0.0087 and ***p=0.0011. (D) ECAR and proliferation of R or P tumor cells. R or P tumor cells were labeled with CellTrace Violet (CTV) and cultured for 3 days. CTV dilution was measured at days 0 and 3. Data represent ≥3 independent experiments, ***p=0.001. (E) R or P tumors were injected s.c. into 129S6 mice and TILs were isolated from bulk tumors ~12 days post- transplantation. (F) CD44, CD62L, PD-1, T-bet, phosphorylated 4E-BP1 (p4E-BP1), and phosphorylated S6K (pS6K) expression were measured in TILs by flow cytometry. Data are representative of ≥3 independent experiments. (G) ECAR and OCR/ECAR ratios of ex vivo tumor cells and their TILs were analyzed. Data are shown as mean ± SEM from 3 independent experiments. **p=0.003, ***p=0.001. (H) Glycolytic capacity of TILs was measured after exposure to the mitochondrial ATP synthase inhibitor oligomycin. Bar graph is shown as mean ± SEM and is representative of 2 independent experiments. (I) IFN-γ production in TILs was measured 5h after PMA/ionomycin restimulation. Contour plots (above)

and MFI of IFN-γ producing cells are shown (below). Results represent data from ≥3 independent experiments. (J) Glucose concentration in the extracellular milieu of ex vivo tumors, ***p=0.0005. Data shown are the average of 5 individual mice. (K) 100 µg 2-NBDG was injected i.v. into a tumor-bearing mice and tumors were harvested 15 min later. The representative histogram (above) depicts 2-NBDG uptake in TILs. The bar chart (below) shows mean MFI ± SEM from 3 individual mice. **p=0.0147.

**Figure 2. *In vitro* glucose competition between tumors and T cells, and composition of immune cell infiltrates in R and P tumors *in vivo***



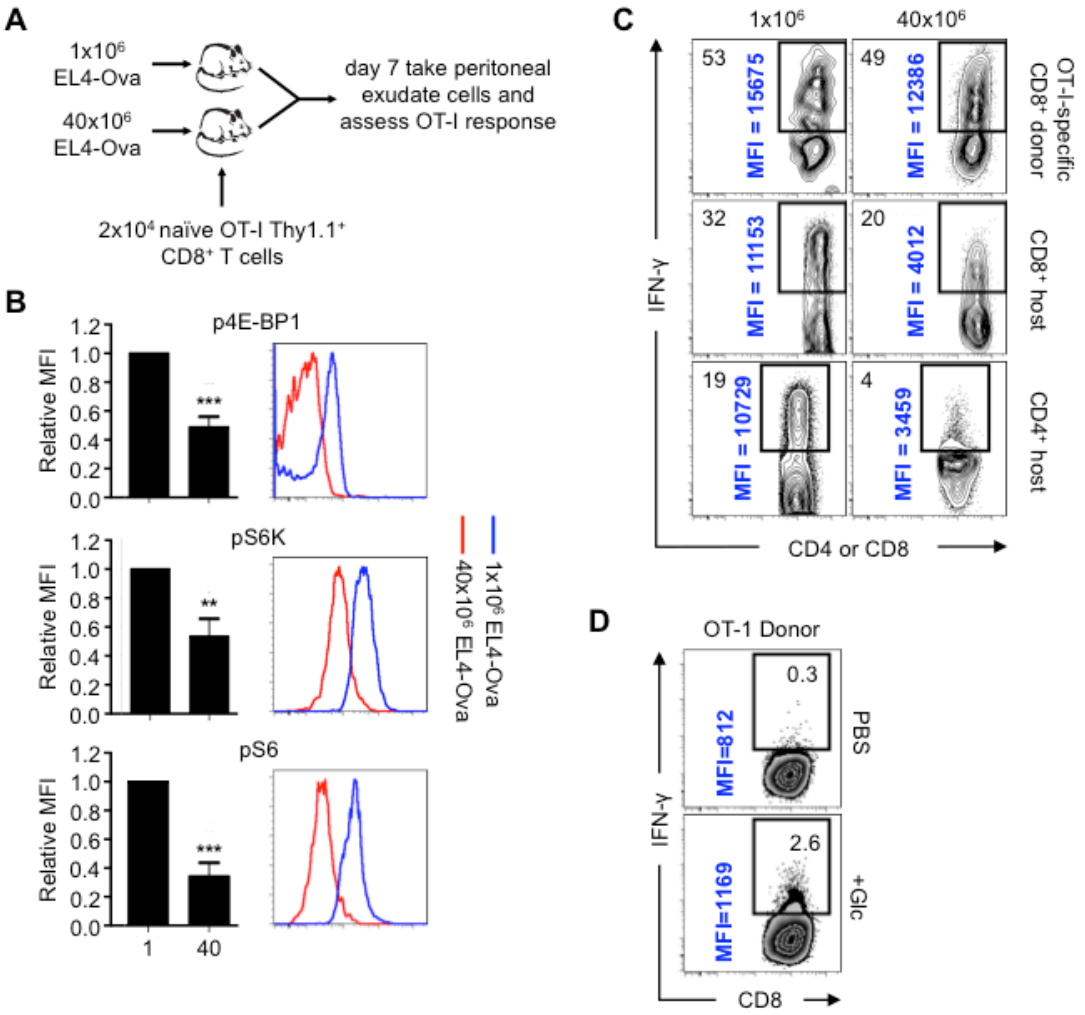
(A) R tumor cells were pre-treated with 100 nM rapamycin (Rapa) or 4-hydroxy-tamoxifen (4-HT) for 2 days and ECAR of cells were measured. Data are from one experiment. (B) Activated OT-I $CD8^+$ T cells were cultured with a 1:3 ratio of rapamycin- pretreated R tumor cells overnight. IFN-γ production was measured after PMA/ionomycin stimulation for 5h. (C) 4-HT-pretreated R tumor cells were cocultured with activated OT-I $CD8^+$ T cells at a 3:1 ratio overnight. Cells were then restimulated with PMA/ionomycin and IFN-γ production was measured. At the time of restimulation, either no additional Glc or indicated Glc concentrations were added to the media. The percentage of IFN-$\gamma^+CD8^+$ cells is depicted on the top left, and IFN-γ MFI of $CD8^+$ cells is shown vertically. Data (B and C) are representative of 3 independent experiments (D) The composition of infiltrating $CD45^+$ cells in R and P tumors was assessed at 9-13 days after transplantation. Frequencies of $CD3^+CD8^+$ and $CD4^+$ T cells, $F4/80^+CD64^+$ macrophages, $CD19^+$ B cells, and the rest of $CD45^+$ cells (Others) are shown. Bar chart is presented as percentage of mean frequency ± SEM from at least 4 independent experiments. (E) Infiltrating Treg cells in R and P tumors were determined as $FoxP3^+CD4^+$ T cells. Relative composition of Treg cells was calculated by normalizing the frequencies of the cells to those in the R tumor. Data are shown as mean ± SEM from 3 independent experiments. (F) M1 ($iNOS^+$ cells) and M2 ($RELM\alpha^+$ cells) macrophages in R and P tumors were assessed at 12 days after transplantation. Bar chart is presented as M1 verse M2 ratio ± SEM from 2 independent experiments.

## § 2.3 Tumor-imposed nutrient restrictions lead to T cell hyporesponsiveness even when tumors are highly antigenic

Antigen-specific T cell responses are critical for mediating tumor clearance (Baitsch et al., 2011, Matsushita et al., 2012) and antigenic mutant spectrin-β2 expressed by R (but not P-) tumors has been shown to be an important target for tumor rejection (Matsushita et al., 2012). Given that the antigenicity of R and P tumors differs, we designed a series of experiments that would address how nutrient competition alone could affect the activity of T cells in tumors. To begin to explore this question we used an EL4 lymphoma that expresses Ova peptide (EL4-Ova), allowing us to target the cancer with Ova- specific (OT-I) $CD8^+$ T cells, and thus determine the impact of nutrient limitation in T cells with defined tumor antigen specificity. We used a model in which we could enhance nutrient restriction simply by increasing tumor cell number, and injected either $1x10^6$ or $40x10^6$ EL4-Ova cells intraperitoneally, and then intravenously transferred $2x10^4$ naïve OT-I $CD8^+$ T cells into these mice (**Figure 3A**). Seven days later we assessed T cell responses in the peritoneal cavity and found that, while OT-I T cells infiltrated the peritoneal cavities of mice carrying high or low peritoneal tumor burdens, mTOR activity in OT-I T cells was greatly dampened in mice injected with $40x10^6$ EL4-Ova tumor cells compared to those mice which had received $1x10^6$ EL4-Ova cells (**Figure 3B**). Moreover, the OT-I T cells in mice inoculated with $40x10^6$ EL4-Ova cells exhibited reduced IFN-γ production after restimulation compared to OT-I T cells in mice which had received $1x10^6$ EL4-Ova cells, and this reduced responsiveness in the context of the larger tumor burden was also apparent in endogenous $CD8^+$ and $CD4^+$ T cells that had entered the peritoneal cavity as part of the anti-tumor response in these mice

(**Figure 3C**). Furthermore, in mice that had received OT-I cells and $40x10^6$ EL4-Ova cells seven days earlier, we twice injected a bolus of glucose, or PBS, along with Brefeldin A, 2.5 and 5 hours prior to assessing the IFN-γ production of OT-I cells in the peritoneum. We injected BFA into the mouse so that we could capture IFN-γ production, as it is in situ, without restimulation in high glucose in vitro. We found that the T cells in the mice that had received injections of a high concentration of glucose produce more IFN-γ (**Figure 3D**). Taken together, these data show that the effector function of T cells, even antigen- specific ones (i.e. Ova-specific), can be affected by an increased number of tumor cells, or glucose concentrations in vivo, suggesting that tumor-imposed nutrient-restriction of T cells can be a general mechanism for hyporesponsiveness.

**Figure 3. *In vivo* competition for glucose modulates cytokine production in antigen-specific T cells**



(A) $1x10^6$ or $40x10^6$ EL4-Ova lymphoma cells were injected i.p. into C57BL/6 mice that received $2x10^4$ naïve OT-I Thy1.1$^+$ CD8$^+$ T cells by i.v. injection. Cells from the peritoneal exudate were assessed 7 days post-inoculation. (B) Phosphorylation of 4E-BP1 (p4E-BP1), S6K (pS6K), and S6 (pS6) of OT-I cells was assessed by flow cytometry and relative MFI from mice transplanted with either $1x10^6$ EL4-Ova cells or $40x10^6$ EL4-Ova cells (40) was normalized to the MFI of T cells from mice injected with $1x10^6$ EL4-Ova cells (1). Bar graphs are shown as mean ± SEM and are from 4 independent experiments. **p=0.0085, ***p=0.001. (C) IFN-γ production by OT-I CD8$^+$ donor T cells as well as CD8$^+$ and CD4$^+$ host T cells was measured after 5h PMA/ionomycin restimulation. Dot plots showing IFN-γ production are representative of 2 independent experiments. Vertical number is the MFI of IFN-γ$^+$ cells. (D) In vivo IFN-γ production by OT-I CD8$^+$ donor T cells was measured. Mice were injected i.p. with EL4- Ova cells and i.v. with congenic naïve OT-I T cells. On day 7 post-transplatation, mice were injected i.p. with Brefeldin A along with either PBS or glucose, and then again 2.5 hours later. Cells in the peritoneal cavity were harvested 5 hours after the first injection and analyzed by flow cytometry. Dot plots showing MFI of IFN-γ$^+$ cells relative to the mice treated with PBS. Each dot represents an individual mouse and horizontal bars indicate mean ± SEM combined from 2 independent experiments. * p=0.0142.

## § 2.4 Nutrient competition between tumors and T cells can regulate cancer progression

It is important to note that in the experiments depicted in **Figure 3**, hyporesponsiveness developed in the face of increased antigen concentration. This differs from published data showing that increases in cell-free antigen concentrations promoted T cell responses until an upper limit of activation is reached, at which point IFN-γ production plateaus (Constant et al., 1995). Thus, we reasoned that when antigen is available and not limiting, tumors may assume a critical role in inhibiting immunity through the more rapid consumption of available nutrients within the tumor mass niche, leaving TILs at a metabolic disadvantage. To directly address whether nutrient competition alone, outside of other extrinsic factors, could determine cancer progression, we aimed to alter tumor cell metabolism, and then assess progression and rejection of the altered tumors. By altering tumor cell metabolism directly, we would be able to make comparisons between different groups using the same tumor (rather than comparing different tumors derived from the same parental tumor, as is the case when P and R tumors are compared). Making comparisons within one tumor line would also remove the confounding factor of differing antigenicity, as the same rejection antigens would be expressed. Furthermore, by only altering tumor cell metabolism, we would be able to administer equivalent numbers of tumor cells into mice, keeping tumor and antigen burden the same at the time of transplant, as opposed to using a larger tumor burden to facilitate nutrient competition, as was the case in the experiments depicted in **Figure 3**. To accomplish this we took advantage of a recent study that showed that tumor cells cultured for extended periods of time in low glucose adapt by increasing mitochondrial respiration (Birsoy et al., 2014),

demonstrating that modulating nutrient availability enforces changes in tumor cell metabolism. We cultured R tumor cells in high glucose (50 mM) and low serum (1% FCS) over a number of weeks to select R tumor cells with increased glycolytic capacity. During this same time period we cultured the original R tumor in control media (11 mM glucose, 10% FCS). When returned to the control media, the resulting R-1% tumor displayed enhanced ECAR and glucose uptake when compared to the original R tumor, although not to the level of that observed in P tumor cells (**Figure 4A, left**). We transplanted R-1% tumors into mice and found that 10 out of 14 recipients developed either fully progressing tumors, or exhibited delayed tumor regression (**Figure 4B and 5A**). At day 20, all 13 of the original R tumors had regressed, while only 4 out of 14 R-1% tumors had fully regressed (**Figure 4B and 5A**). Critically, all of the R-1% tumors that progressed still expressed the major rejection antigen mutant spectrin-β2 (**Figure 4C and 5B**), indicating that the gain of the 'progressor' phenotype in R-1% tumors was not due to the loss of dominant epitopes recognized by potentially protective T cells. Furthermore, we found that R and R-1% tumor cells grow at the same rate in vitro (**Figure 5C**), as well as in $RAG^{-/-}$ mice, which lack B and T cells (**Figure 4D**). These data demonstrate that the reason for tumor progression in the immune competent mice was not due to acquired differences in inherent rates of proliferation between the R and R-1% tumors, and that the adaptive immune system mediates rejection of the R tumors in this setting.

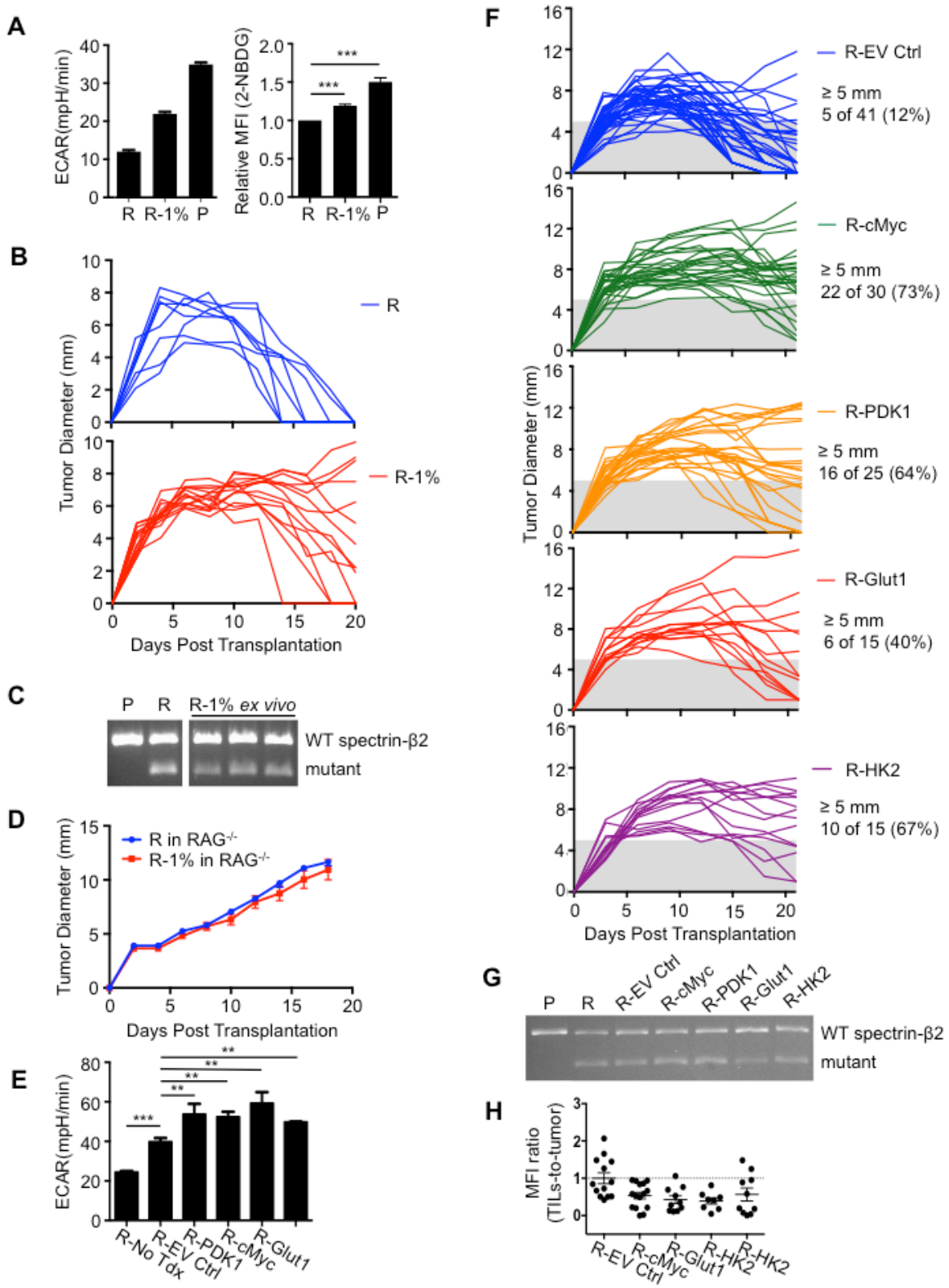
We reasoned that the R-1% tumor behaved as a progressor tumor due to its enhanced glucose uptake (**Figure 4A, right**). Therefore, we predicted that if this were the case, then genetically manipulating the glycolysis pathway directly should also turn the R tumor into a progressor tumor. To this end we performed a genetic gain-of-function experiment and transduced R tumors with a retrovirus expressing c-

Myc, a transcription factor that drives the glycolytic program (Gordan et al., 2007). We found that c-Myc expressing R tumors cells (R-cMyc) displayed enhanced ECAR in vitro (**Figure 4E**), when compared to R tumors expressing the empty control vector (R-EV Ctrl), which was consistent with their increased glycolysis. Importantly, we also observed that the R-EV Ctrl tumors displayed higher ECAR than non-transduced R tumor cells. These data suggested that while the enhanced glycolysis of c-Myc expressing tumors should confer a strong progressor phenotype when compared to non-transduced R tumors and R-EV Ctrl tumors, the R-EV Ctrl tumors might also conceivably exhibit some progression compared to non-transduced R tumors, due to their enhanced rate of glycolysis. We transplanted R-cMyc tumors into mice and found that 22 out of 30 recipients (73%) had tumors equal to or larger than 5 mm at day 21, while only 5 out of 41 mice (12%) with R- EV Ctrl tumors had tumors larger than this size (**Figure 4F and 5D**). We reasoned that the elevated ECAR in R-EV Ctrl tumors compared to non-transduced R tumors caused progression or delayed regression in a few of the mice, when compared to non-transduced R tumors, which normally fully regress by day 21 (**Figure 1A, 4B**). Therefore we compared between groups based on tumor size at day 21. Importantly, c-Myc expressing R tumors maintained expression of the major rejection antigen mutant spectrin-β2 (**Figure 4G and Figure 5B**). These data show that in spite of expressing a major rejection antigen, c-Myc expressing tumors became more glycolytic and gained a strong ‘progressor’ phenotype. Since c-Myc is an upstream regulator of the glycolysis pathway, it is possible that this transcription factor may drive other diverse programs beyond glycolysis in transduced tumor cells. To more directly test the role of tumor cell glycolysis in the progression of antigenic tumors, we transduced R tumors with a retrovirus expressing pyruvate dehydrogenase

kinase 1 (PDK1), an enzyme that sits at a key bifurcation point between glycolytic and oxidative metabolism (Gerriets et al., 2015). We also transduced T tumor cells with the glucose transporter Glut1, and hexokinase II (HK2), the first enzyme in the glycolysis pathway. We found that PDK1-, Glut1-, and HK2-expressing R tumors (R-PDK1, R-Glut1, and R-HK2) displayed higher ECAR when compared to control transduced cells, consistent with enhanced glycolysis (**Figure 4E**). We transplanted these tumors into mice and found that 16 out of 25 of R-PDK1 transduced tumors (64%), 6 out of 15 of R-Glut1 transduced tumors (40%), and 10 out of 15 of R-HK2 transduced tumors (67%) progressed, compared to only 5 out of 41 of the R-EV Ctrl transduced tumors (12%) (**Figure 4F and 5D**). Importantly, the R-PDK1, R-Glut1, and R-HK2 tumors continued to express the major rejection antigen mutant spectrin-β2 (**Figure 4G and 5B**). In addition, activated C3 T cells could still efficiently kill the R-1% and the transduced R tumors when in nutrient rich conditions in vitro, indicating that all of these tumors maintain expression of the target antigen mutant spectrin-β2 (**Figure 5E, F**). Moreover, the transduced tumors cultured in vitro (**Figure 5G**), or in $RAG^{-/-}$ mice, all grew at fairly similar rates (**Figure 5H**), indicating that the difference in progression between the tumors transduced with glycolysis genes and those transduced with EV Ctrl was not solely due to substantial inherent differences in growth rates. We also injected 2-NBDG into mice bearing transduced tumors and assessed its uptake in tumors and TILs. We found that all of the TILs in transduced tumors acquired less glucose than TILs in tumors expressing the EV-Ctrl (**Figure 4H**). Finally, we co-cultured transduced tumors with activated OT-I cells. We found that the tumors transduced with glycolysis genes dampened IFN-γ production in OT-I T cells more than the EV Ctrl transduced tumors, and that the addition of glucose to the culture substantially increased IFN-γ production in the T cells (**Figure**

**5l**). Taken together, these results demonstrate that tumor cell metabolism, which itself drives a competition for nutrients, can determine cancer progression, even in the presence of a major tumor rejection antigen recognized by the immune system.

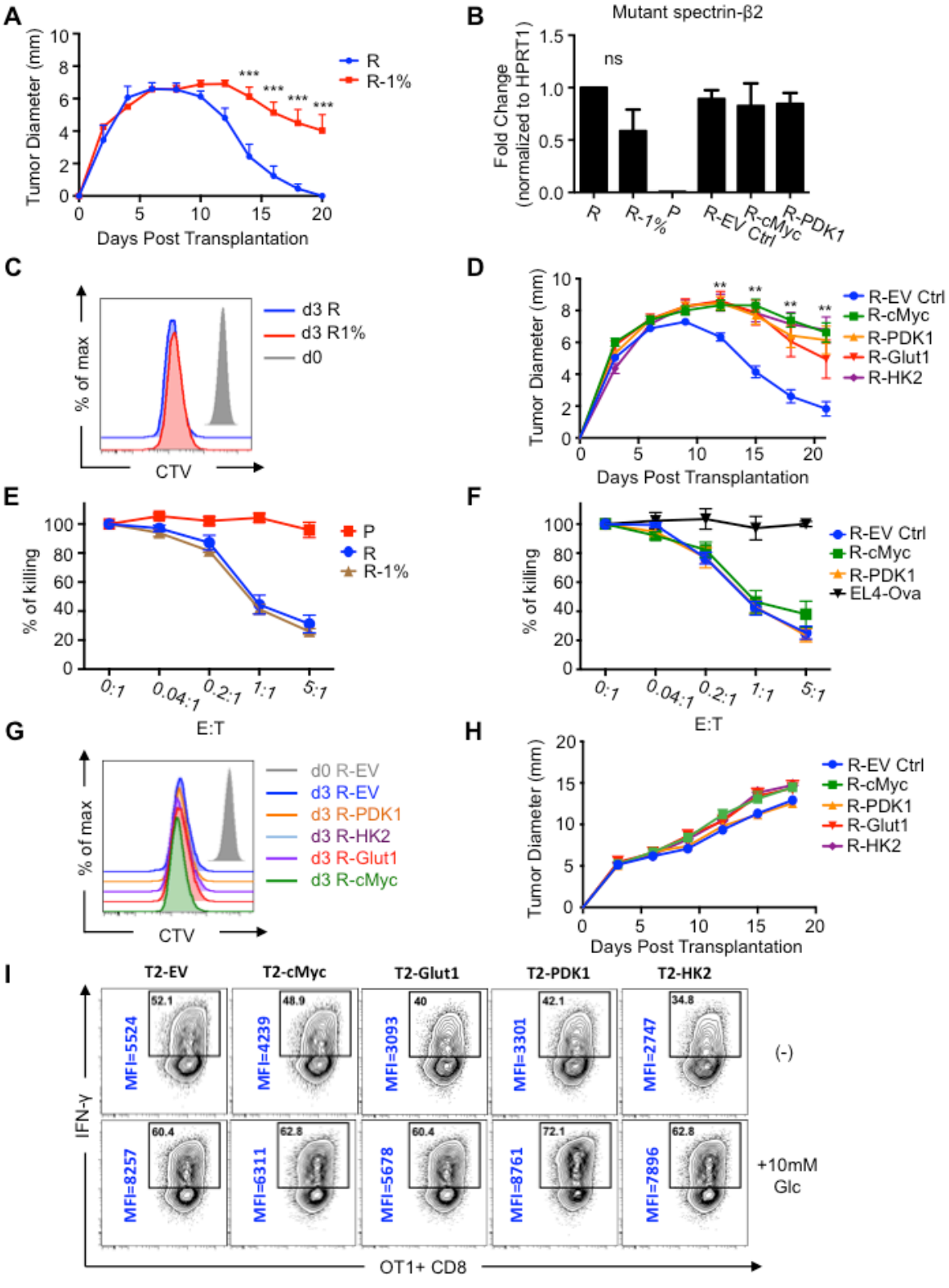
**Figure 4. Enhancing glycolytic metabolism in antigenic tumors that are normally rejected promotes tumor progression**



(A) R tumors were cultured either in complete media (11 mM glucose and 10% FCS: R) or in high glucose and low FCS media (50 mM and 1% FCS: R-1%) over 3 weeks and ECAR of the cells was measured (left). Tumor cells were cultured in 5 μg/ml 2-NBDG at room temperature for 15 min and 2-NBDG uptake was measured by flow cytometry (right). Data are the average of 3 independent experiments, with R and P group values also used in Figure 5B. 2-NBDG MFI is normalized to the MFI values of the R tumor. ECAR data are representative of 3 independent experiments, ***p=0.001. (B) 129S6 mice were injected s.c. with $1x10^6$ R or R-1% tumor cells and tumor growth was monitored. Tumor size is shown as an average of two perpendicular diameters ± SEM. (C) cDNA was made from ex vivo isolated progressing R-1% tumors and from cultured R and P tumors cells and amplified by PCR for spectrin-β2. The PCR product was then digested with the restriction enzyme Pst1 and analyzed by electrophoresis. Data presented are from 3 individual ex vivo R-1% tumors. (D) $1x10^6$ R or R-1% tumor cells were injected s.c. into $Rag^{-/-}$ 129S6 mice and tumor growth was monitored. Data (B and D) are from 2 independent experiments. (E) R tumor cells (R-No Tdx) were transduced with either an empty retroviral vector (R-EV Ctrl) or vectors expressing c-Myc (R-cMyc), PDK1 (R-PDK1), Glut1 (R-Glut1), or HK2 (R-HK2). ECAR of indicated cells were measured. Data are from at least 4 independent experiments. **p=0.0012 for R-EV Ctrl vs R-cMyc, p=0.0091 for R-EV Ctrl vs R-PDK1, p=0.0026 for R-EV Ctrl vs R-Glut1, and p=0.0196 for R-EV Ctrl vs R-HK2; ***p=0.001 for R-No Tdx vs R-EV Ctrl. (F) $2x10^6$ transduced R tumor cells were injected s.c. into 129S6 mice and tumor growth was monitored for 21 days. Data are from at least 3 independent experiments. (G) cDNA was made from ex vivo isolated R and P tumors as well as transduced R tumor cells and analyzed for spectrin-β2 as described in (C). (H) Mice bearing R-EV Ctrl, R-

cMyc, R-PDK1, R-Glut1 and R-HK2 tumors on day 12 were injected i.v. with 100 µg of 2-NBDG per mouse. Acquisition of 2-NBDG by CD8 TILs and tumor cells was measured by flow cytometry. Data are presented as 2-NBDG MFI ratios of TILs-to-tumors. Each dot represents an individual mouse and horizontal bars indicate means ± SEM of 3 independent experiments. **p=0.009 for R-EV Ctrl vs R-cMyc, p=0.0065 for R-EV Ctrl vs R-Glut1, and p=0.0066 for R-EV Ctrl vs R-HK2.

**Figure 5. Enhanced metabolis in antigenic tumors can dictate tumor progression**



(A) The average tumor growth from all mice injected with either R or R-1% tumor cells is shown. ***p<0.001. Data are from 3 independent experiments. (B) The mRNA expression of mutant spectrin-β2 expression in indicated tumors was assessed. Results are presented as mean ± SEM from 3 independent experiments. ns, not significant. (C) Cell proliferation rate of transduced R and R-1% tumors were measured by dilution of CTV at day 0 and 3. The histogram plot is shown from one experiment. (D) The average tumor growth from all mice injected with transduced R-EV Ctrl, R-cMyc, R-PDK1, R- Glut1 and R-HK2 tumors is shown. **p<0.01 for all the tumors in comparison with R-EV Ctrl tumor cells. Data are from ≥3 independent experiments. (E) CFSE labeled original R, P and R-1% targeted tumor cells were co-cultured with C3 T cells for 12h at indicated effector-to-target ratios (E:T) and the cytotoxicity efficiency analyzed by flow cytometry. (F) Cytotoxic efficiency of C3 T cells co-cultured with transduced R-EV Ctrl, R-cMyc, R- PDK1 and EL4-Ova tumors was measured as described in (E). Data (E and F) from 3-5 independent experiments are presented as the percentage of live target cells normalized to reference cells (% of survival). (G) Proliferation of indicated transduced tumors was measured by CTV dilution at day 0 and 3 post-CTV labeling. The histogram plot is shown from one experiment. (H) $1x10^6$ transduced tumor cells as indicated were injected s.c. into $Rag^{-/-}$ 129S6 mice and tumor growth was monitored. Data are an average of two perpendicular diameters ±SEM from at least 3 independent experiments. (I) Transduced tumor cells were cultured with activated OT-I $CD8^+$ T cells overnight, and then IFN-γ production was measured after 5h PMA/ionomycin restimulation. Either no additional glucose or 10 mM glucose was added back to the media during the restimulation. Representative data are shown as dot plots from 4 independent experiments. Percentage and MFI (blue vertical) values of IFN-$\gamma^+$ cells

are shown.

## § 2.5 Immune checkpoint blockade therapy corrects nutrient restriction experienced by T cells in a progressing tumor

Immune checkpoint blockade therapy is a revolutionary approach to activate anti-tumor immunity by targeting proteins that negatively regulate T cell responses (Hodi et al., 2010, Brahmer et al., 2012, Hamid et al., 2013). This treatment can affect T cell proliferation (Spranger et al., 2014), function (West et al., 2013, Spranger et al., 2014) and glucose uptake (Parry et al., 2005), but the precise mechanisms of how various checkpoint blockade inhibitors work, and why they are effective against tumors in some patients, but not in others, remain unclear (Page et al., 2014). In order to investigate whether tumor- imposed nutrient-restriction of T cells is a general mechanism leading to cancer progression, we decided to test whether checkpoint blockade therapy influences the metabolic interplay between tumors and T cells in the tumor microenvironment. We reasoned that since these treatments are effective at inducing the regression of P tumors (Gubin et al., 2014), we should observe an effect on tumor/TIL metabolism after treatment if our proposed model of metabolic competition in the tumor microenvironment was correct. Therefore we transplanted P tumors into mice and treated with isotype control antibody or CTLA-4, PD-1, or PD-L1 blockade antibodies at days 3, 6, and 9 after transplantation and assessed metabolic parameters and TIL function on day 12 (**Figure 6A**). We also transplanted R tumors into mice and treated with isotype control antibodies as a control for regression. As expected (Gubin et al., 2014), we found that treatment with antibodies against CTLA-4, PD-1, or PD-L1 all resulted in control of P tumors (**Figure 6B**). Isotype control antibodies had no effect on the outcome of P or R tumor cell growth (**Figure 6B**). We excised tumors at day 12 and measured glucose concentrations in

the extracellular milieu and found that the tumors from blockade antibody-treated mice had significantly more available extracellular glucose (similar to the concentrations found in the R tumors) when compared to isotype antibody-treated control mice (**Figure 6C**). In addition, TILs from the blockade antibody treated mice displayed enhanced ECAR in comparison to TILs isolated from P tumors of isotype control treated mice (**Figure 6D**). These data indicated that glucose availability in the tumor microenvironment correlated with the glycolytic capacity of TILs. To confirm that TILs in checkpoint blockade antibody-treated mice were in a nutrient-sufficient state, we examined their mTOR pathway activity on day 12 post-transplantation. We observed that mTOR activity, measured by the phosphorylation of 4E-BP1, S6K and S6, of TILs in P tumors from mice receiving blockade antibodies was restored to a level closer to that measured in TILs from R tumors (**Figure 6E**). Finally, we found that the increased glucose available in the tumor, and the greater ECAR observed in TILs, correlated with increased IFN-γ production by the TILs after checkpoint blockade therapy (**Figure 6F**). Together these data suggest that nutrients in the tumor microenvironment dictate the nutrient status of activated T cells and that this influences their effector function. Furthermore, our data indicate that checkpoint blockade therapy corrects the tumor- induced glucose restriction experienced by TILs and restores their glycolytic capacity and hence their ability to produce IFN-γ. These data support the notion that glucose concentration in a tumor can be a determining factor for T cell effector function. In addition to being converted to lactate, pyruvate made by glycolysis can also enter the mitochondria and be converted to acetyl-CoA, which can enter the TCA cycle and support the generation of substrates for oxidative phosphorylation (OXPHOS). We reasoned that changes in glucose availability might be reflected in changes in TIL OXPHOS as well as aerobic

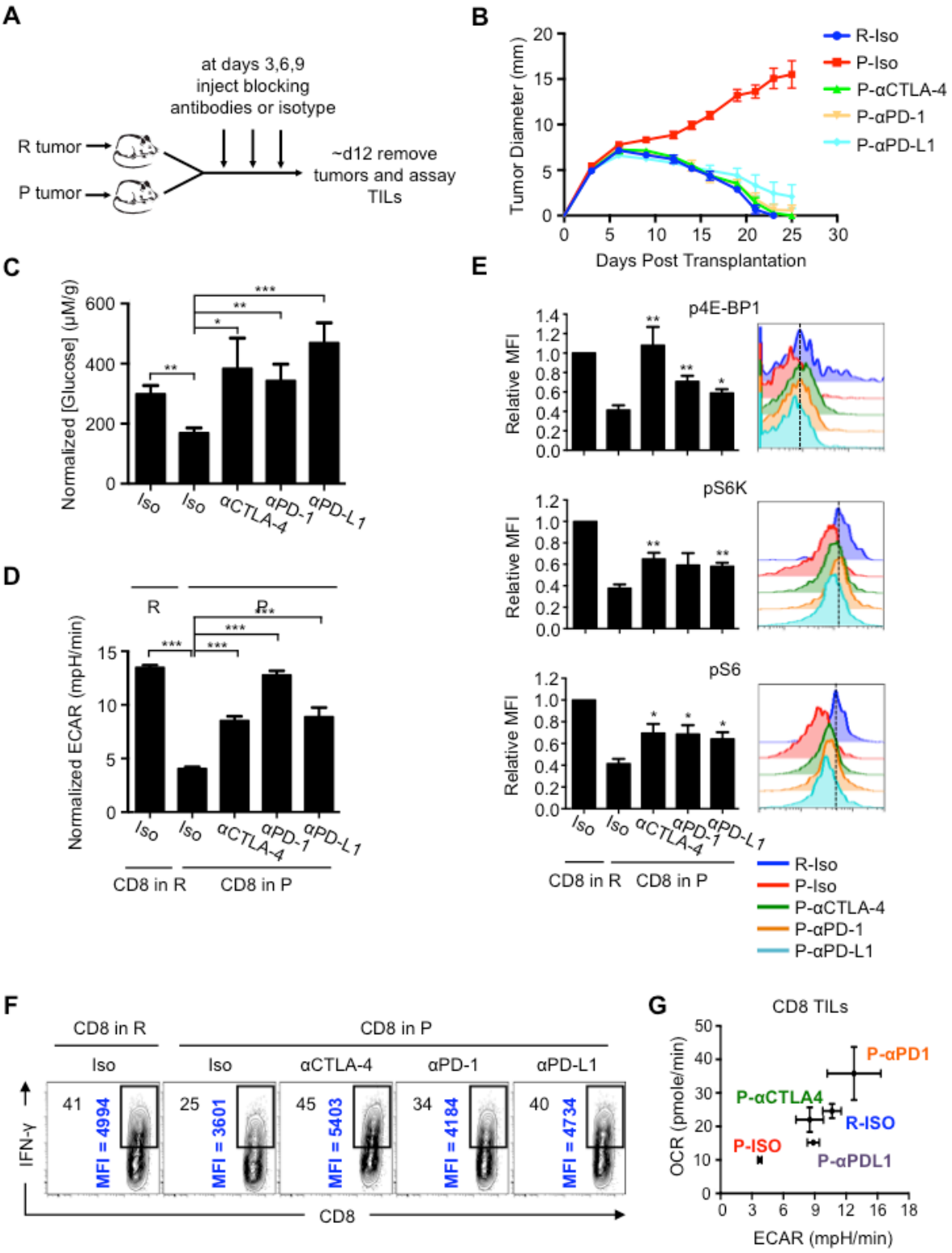
glycolysis. By plotting OCR (oxygen consumption rate, a measure of OXPHOS) vs. ECAR (the measure of aerobic glycolysis), we established a baseline value for metabolic fitness of TILs from R vs. P tumors; this measurement emphasized that TILs from R tumors have higher OXPHOS as well as aerobic glycolysis compared to TILs from P tumors (**Figure 6G**). We found that CTLA-4 and PD-1 blockade antibodies not only increased ECAR, but also increased OCR in the TILs from P tumors, to levels equal or above those observed in TILs in the R tumor, indicating that these treatments enhanced the overall metabolic fitness of the TILs (**Figure 6G**). PD-L1 blockade antibodies, however, primarily promoted aerobic glycolysis, rather than OCR, in the TILs (**Figure 6G**).

We speculated that in addition to augmenting the capacity of TILs to compete for glucose to support OXPHOS and aerobic glycolysis, checkpoint blockade treatment might increase the ability of TILs to compete for other substrates. We assessed the protein expression of glutamate dehydrogenase (Glud1), a mitochondrial enzyme that catalyzes the oxidative deamination of glutamate to α-ketoglutarate, an important process for energy homeostasis in T cells (Wang et al., 2011) .We found that Glud1 expression was increased in TILs in the P tumor that had been treated with PD-L1 blockade antibodies (**Figure 7A**). These data support the idea that, in addition to glucose, competition for amino acids, and possibly other nutrients and growth factors not examined here, occurs within a tumor microenvironment, and emphasize the fact that checkpoint blockade broadly increases the metabolic fitness of TILs and supports the view that this may be central to the beneficial effects of these treatments in cancer therapy.

Given that checkpoint blockade therapy leads to tumor regression (**Figure 6B**), we

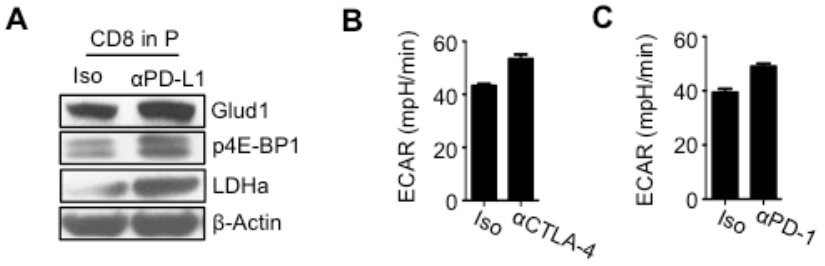
speculated that the enhanced glucose levels in the tumors of treated mice were a result of immune-mediated killing, which would lead to a reduction in the total number of tumor cells and thus a reduction in total glucose consumption within the tumor. It is known that blocking inhibitory receptors enhances T cell activation (Keir et al., 2008, Francisco et al., 2010), and we speculated that this might reflect the fact that these treatments allow T cells to better compete for nutrients like glucose in the microenvironment, allowing a greater engagement of glycolysis by TILs when competing directly against the tumor. Consistent with this idea, and with published reports (Parry et al., 2005, Staron et al., 2014, Pedicord et al., 2015), we found that even treating in vitro activated T cells, which are already highly glycolytic, with checkpoint blockade antibodies against PD-1 and CTLA-4, further increased their ECAR, although the effects were small (**Figure 7B, C**). These results could suggest that treatments that block negative signals to T cells in vivo may, in part, work by enhancing their ability to compete for nutrients in the tumor and engage aerobic glycolysis.

**Figure 6. T cells in progressing tumors regain glycolytic capacity and effector function after checkpoint blockade therapy**



(A) Naïve mice (n=6-8) were injected s.c. with R or P tumor cells followed by treatment with anti-CTLA-4 (αCTLA-4), anti-PD-1 (αPD-1), anti-PD-L1 (αPD-L1) blockade antibodies, or isotype control (Iso) antibody at days 3, 6 and 9 after tumor inoculation and tumor growth was monitored. (B) Tumor size is shown as an average of two perpendicular diameters ± SEM of at least 3 independent experiments. (C-E) Tumors were removed at day 12 after transplantation, and glucose concentrations in the extracellular milieu of ex vivo tumors were measured (C). Data are normalized to R tumors treated with isotype control antibody and depict mean ± SEM from 1–3 independent experiments. *p=0.0208, **p=0.0015 (R vs. P-Iso), **p=0.0024, ***p=0.001. (D) ECAR of TILs, isolated after checkpoint blockade therapy. Data are normalized to R tumors treated with isotype control antibody and depict mean ± SEM from 3 independent experiments. ***p<0.001. (E) Phosphorylation of 4E-BP1, S6 and S6K in TILs were measured by flow cytometry. Bar graphs (left) are shown as the mean ± SEM from 4 independent experiments, and histograms (right) are representative of 4 independent experiments. p4E-BP1: *p=0.0249, **p=0.0047 (αCTLA-4), **p=0.0050 (αPD-1). pS6K: **p=0.0024 (αCTLA-4), **p=0.0025 (αPD-L1). pS6: *p=0.0145 (αCTLA- 4), *p=0.015 (αPD-1), *p=0.0134 (αPD-L1). (F) IFN-γ production of TILs was examined after 5h restimulation with PMA/ionomycin. The percentage of IFN-$\gamma^+$ cells is depicted on the top left and the MFI of IFN-$\gamma^+$ cells is shown vertically as indicated. Data are representative of 3 independent experiments. (G) OCR versus ECAR (mean ± SEM for both parameters) of TILs, isolated after checkpoint blockade therapy. Data are from 3 independent experiments.

**Figure 7. Effects of checkpoint antibodies on T cell metabolism**




(A) Expression of Glud1, LDHa and p4E-BP1 of CD8 TILs in P tumor cells were examined by western blot. Data are representative from 2 independent experiments. Naïve $CD8^+$ T cells were activated with anti-CD3/28 for 3 days and then were treated with 10 μg/ml of either anti-CTLA-4 (αCTLA-4) (B) or anti-PD-1 (αPD-1) (C) blockade antibodies, or Isotype control antibody for 24h. ECAR of the T cells was measured. Data are shown as mean ± SEM from 3 independent experiments.

## § 2.6 PD-L1 directly regulates tumor metabolism

PD-L1 expression is induced on tumor cells in response to IFN-γ produced by T cells, and the upregulation of PD-L1 is a mechanism that allows the tumor to evade immune mediated attack by inhibiting T cell function through PD-1/PD-L1 interaction (Keir et al., 2008, Francisco et al., 2010). While there is a strong correlation between PD-L1 expression on melanomas and T cell infiltration (Quezada and Peggs, 2013), this is not the case for every cancer. For example, it has been shown that patients with glioblastoma often have high PD-L1 expression, but this expression does not correlate with levels of TILs observed in the tumors (Berghoff et al., 2015). It has also been shown that expression of PD-L1 on neurons can, through an unknown, but immune system independent mechanism, kill glioblastoma cells (Liu et al., 2013). Furthermore, another study showed that cancer cell expression of PD-L1 mediated killing of T cells in vitro, an effect that occurred independently of PD-1 (Dong et al., 2002). Collectively, these results made us consider the possibility that PD-L1 could have an additional function beyond its role in providing negative signals to T cells as a ligand to PD-1. We therefore hypothesized that blocking PD-L1 on tumor cells might directly alter tumor cell metabolism. We incubated R and P tumors with PD-L1 blockade antibody overnight and then measured the ECAR of the cells. We found that the ECAR of the P tumor cells, as well as glucose uptake as indicated by 2-NBDG acquisition, were reduced when the cells were treated in vitro with PD-L1 blockade antibody, compared to those cells treated with the isotype control antibody (**Figure 8A, B**). R tumor cells, which display lower ECAR in comparison to P tumor cells, showed a smaller reduction in ECAR when treated with anti-PD-L1 (**Figure 8A**). Antibodies against major histocompatibility complex-I (MHC-I), another surface

protein expressed by P tumors, did not affect ECAR (**Figure 9A**). We next tested whether PD-L1 blockade would inhibit ECAR in other tumor types. We found that PD-L1 blockade significantly inhibited ECAR in B16 melanoma, MC38 colon cancer, as well as L cells and other progressor-type clones derived from the d42m1 parent sarcoma (**Figure 9B**). Of note, ECAR was dampened to varying degrees across these cell lines, suggesting that there is differential sensitivity to this treatment among tumors. Our results suggested that PD-L1 had an additional and previously unrecognized role in the regulation of tumor cell glycolytic metabolism. Therefore, we examined the link between PD-L1 and glycolysis. Since mTOR is a sensor of nutrient status, and this kinase integrates input from upstream pathways (Laplante and Sabatini, 2012), we assessed whether treatment with anti-PD-L1 modulated the activity of mTOR target proteins in the P tumor cells. We treated P tumor cells with anti-PD-L1 and observed decreased phosphorylation of critical mTOR target proteins (**Figure 8C and Figure 9C**), which correlated with reductions in ECAR.

We next assessed the consequences of dampened mTOR signaling resulting from anti-PD-L1 treatment. Given that mTOR directly regulates mRNA translation and ribosome biogenesis (Laplante and Sabatini, 2012), and that we observed a reduction in ECAR following anti-PD-L1 treatment (**Figure 8A**), we analyzed expression of several glycolysis enzymes after PD-L1 blockade. Moreover, since growth factors signal to mTOR via Akt, we also assessed Akt phosphorylation. We found that the expression of glycolysis enzymes, and Akt phosphorylation, were decreased after anti-PD-L1 treatment (**Figure 8D and 9D**). Consistent with the idea that mTOR can affect the glycolysis pathway by regulating the translation of mRNAs, there were no differences in levels of transcripts from key glycolysis pathway genes following anti-PD-L1 treatment (**Figure 9E**), even though the protein levels were

decreased. These data suggest that PD-L1 regulates the Akt/mTOR pathway, which results in decreased translation of transcripts for glycolysis pathway enzymes, and thus dampened glycolysis.

Since PD-L1 has only been shown to have a clear function when signaling through PD-1, we next wanted to determine how the PD-L1 blockade antibody could dampen mTOR signals in our in vitro system, which is devoid of T cell-expressed PD-1. We reasoned that antibodies against PD-L1 could be causing PD-L1 internalization and resultant cessation of events downstream of PD-L1. After treating cells with anti-PD-L1 blockade antibody for 30 minutes at 37°C, we found that PD-L1 had moved from the surface to the interior of the cell (**Figure 8E**), indicating receptor internalization and a reduction of surface PD-L1 expression (**Figure 9F**). These results suggest that PD-L1 surface expression is important for the enhancement of Akt/mTOR signaling in tumor cells.

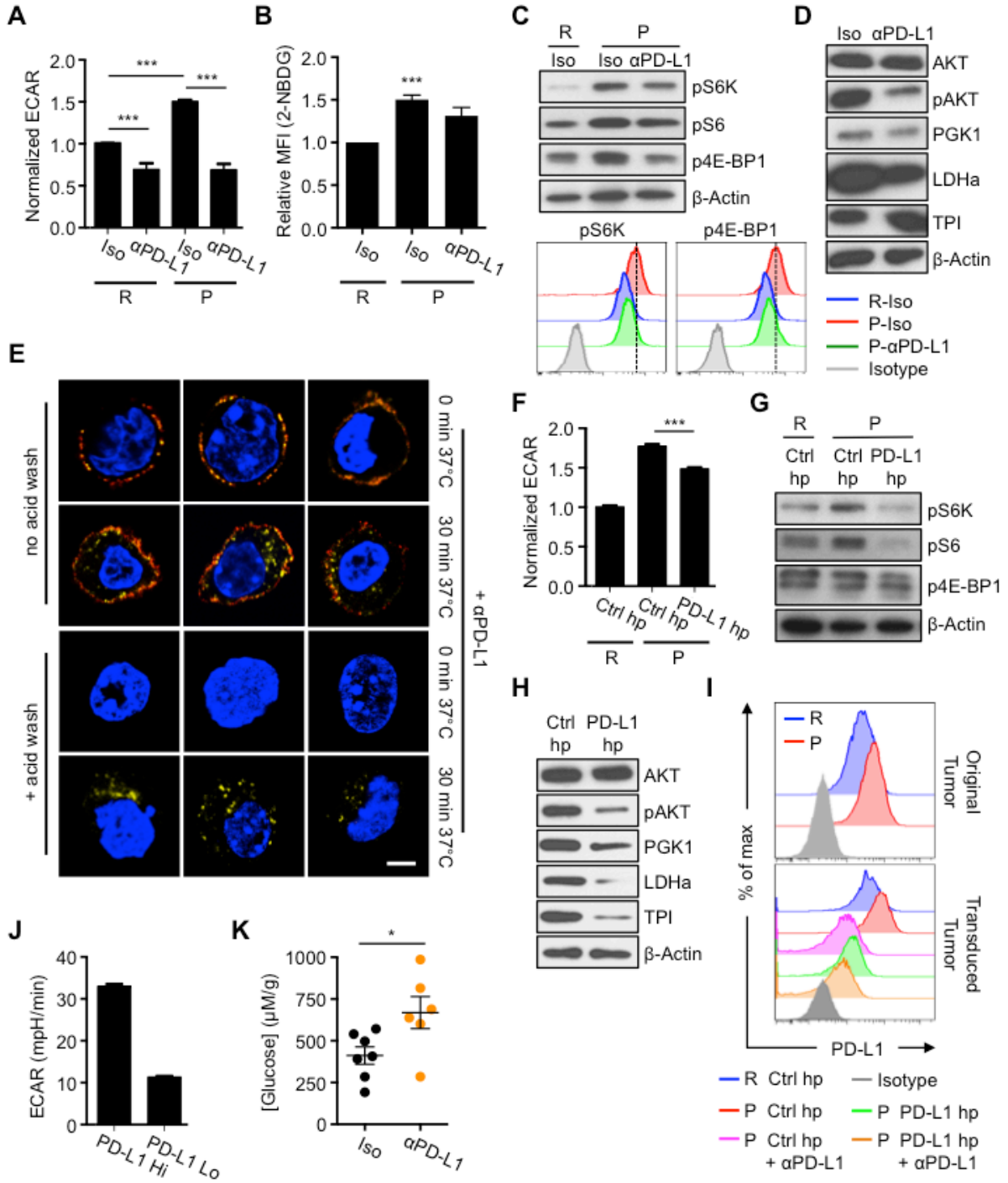
To confirm that PD-L1 can regulate glycolysis, we asked whether genetic loss of function of PD-L1 could affect glycolysis and nutrient sensing pathways in tumor cells. Using a retrovirus expressing a short-hairpin (hp) RNA against PD-L1 to decrease PD-L1 expression in P tumors, we found that ECAR, mTOR pathway activity, Akt activity, and glycolysis enzyme expression were all reduced in cells expressing PD-L1 shRNA (PD-L1 hp) compared to cells expressing a control hairpin (Ctrl hp) (**Figure 8F-H and Figure 9G**). P tumors expressed higher PD-L1 than R tumors (**Figure 8I**, upper panel), which correlated with their enhanced ECAR (**Figure 1D, G**), glucose uptake (**Figure 1B, J**), and thus glycolysis. Importantly, along with decreased ECAR, PD-L1 shRNA decreased PD-L1 expression (**Figure 8I**, lower panel), but did not affect cell proliferation in vitro (**Figure 9H**), nor did it affect tumor

growth rate when transplanted in vivo into RAG$^{-/-}$ mice (**Figure 9I**), suggesting that neither PD-L1, nor the glycolysis pathway, is necessarily coupled to tumor cell proliferation. To further verify that PD-L1 expression on tumors modulated the glycolytic pathway, we used a genetic gain of function approach, and used retroviral transduction to generate R tumor clones that expressed different levels of PD-L1 (high and low), which were sorted for subsequent analysis. We found that high PD-L1 expressing R tumors had elevated ECAR compared to low PD-L1 expressing tumors (**Figure 8J**). Together our data indicate that PD-L1 expression in tumor cells is directly associated with their glycolytic rate, and that PD-L1 engagement by mAbs can modulate glycolytic metabolism in tumor cells.

Our results suggest that PD-L1 is immunomodulatory, not only because it delivers a negative signal to T cells via PD-1 (Keir et al., 2008, Spranger et al., 2014), but also because it enhances tumor cell glycolysis and thus takes away available glucose from invading immune cells in the tumor microenvironment. To further support the idea that PD-L1 can modulate tumor cell metabolism directly, independently of the adaptive immune system, we transplanted PD-L1 expressing tumors into RAG$^{-/-}$ mice. We then treated these mice with PD-L1 blockade or isotype control antibodies. At day 12, we excised tumors and measured glucose concentrations in the extracellular milieu. We found that there was significantly more available glucose in the tumor microenvironment of tumors isolated from mice that received PD-L1 blockade antibody as compared to tumors from isotype control antibody treated mice (**Figure 8K**). Importantly, anti-PD-L1 treatment of PD-L1-expressing tumors in RAG$^{-/-}$ mice had only a minor effect on reducing tumor size (data not shown), supporting the view that in immunocompetent mice, T cell mediated clearance of tumors is critical (Matsushita et al., 2012, Gubin et al., 2014). Taken together our results show that

expression of PD-L1 on the cell surface, maintains Akt/mTOR signaling in tumors, which in turn supports the translation of glycolysis enzymes, thus promoting this metabolic pathway. Our data further show that PD-L1 blockade therapy reduces surface expression of PD-L1 on tumors and inhibits their glycolytic capacity, thereby leaving more available glucose in the extracellular tumor milieu.

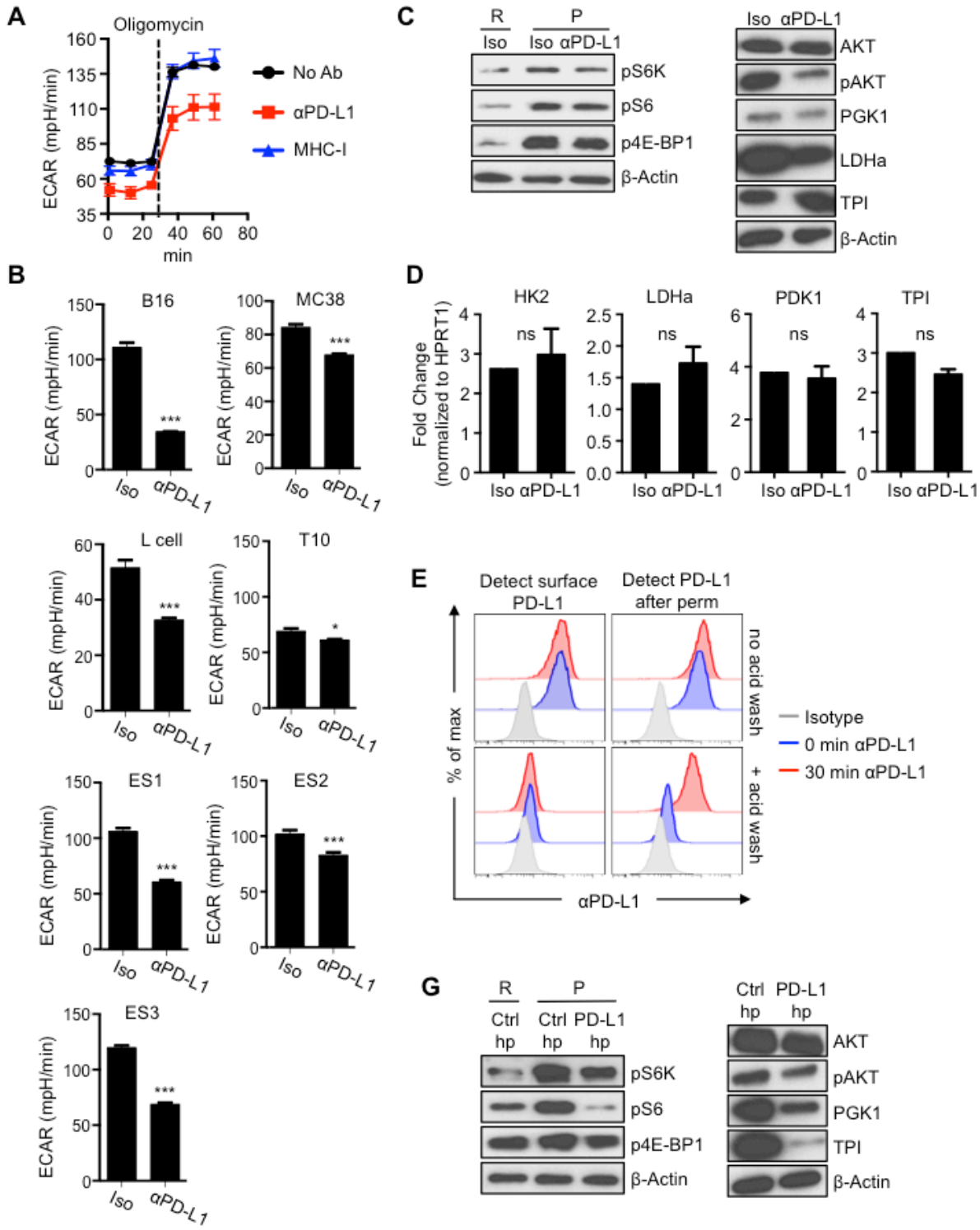
**Figure 8. PD-L1 promotes mTOR activity and glycolytic metabolism in tumor cells**

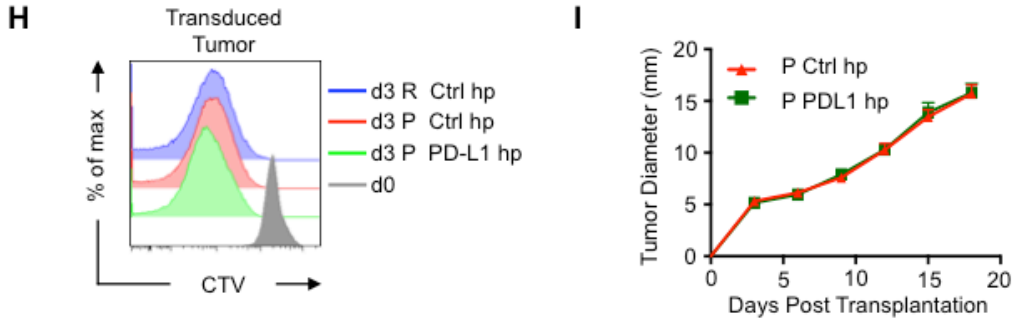


R or P tumor cells were treated with IFN-γ for 48h followed by 10 μg/ml of PD-L1 blockade (αPD-L1) or isotype control (Iso) antibodies for 24h. (A) ECAR post-treatment was assessed. Data from ≥5 independent experiments are shown as relative ECAR normalized to R tumors treated with isotype control antibody. ***p=0.0001. (B) Acquisition of 2-NBDG by tumor cells was measured by flow cytometry. Data are from 3 independent experiments and are normalized to the MFI values of the R tumor. ***p=0.001. (C) Phosphorylation of 4E-BP1, S6K and S6 was analyzed by western blot, data are representative of 3 independent experiments. Representative histogram plots of phosphorylated 4E-EP1 and S6K assessed by flow cytometry below. (D) Phosphorylation of AKT and glycolytic enzymes PGK1 and LDHa were examined by western blot. Data are representative of 2 independent experiments. (E) R tumor clones overexpressing high levels of PD-L1 were treated with PD-L1 antibody (αPD-L1) for 15 minutes on ice, then either kept on ice (0 min) or incubated at 37°C for 30 minutes (30 min). The cells were then either washed in an acid solution to dissociate antibody from the surface of the cells (+ acid wash) or left untreated (no acid wash). After fixation, cells were incubated with anti-Rat IgG A488 (red) to detect αPD-L1 present on the surface of the cells, then following permeabilization the cells were incubated with anti-Rat IgG A647 (yellow) to detect both surface expressed and internalized αPD-L1. Following staining with a nuclear stain (blue), cells were imaged by confocal microscopy. Data are representative of 4 independent experiments. (F) Tumor cells were transduced with pdl1 shRNA (PD-L1 hp) or control hp against luciferase (Ctrl hp) and ECAR of transduced tumor cells are shown (from 2 independent experiments represented as relative ECAR normalized to P Ctrl transduced tumor cells), ***p<0.0001. (G) Phosphorylation of 4E-BP1, S6, and S6K was examined by western blot. Data are representative of 3 independent

experiments. (H) Phosphorylation of AKT, total AKT, and glycolytic enzymes PGK1, LDHa, TPI were examined by western blot. Data are representative of 3 independent experiments. (I) PD-L1 expression on R or P tumors pre-treated with IFN-γ for 48h (top panel). PD-L1 expression on tumor cells transduced with PD-L1 hp or Ctrl hp and treated with anti-PD-L1 for 24 hours (bottom panel). (J) ECAR of R tumors expressing high (Hi) and low (Lo) levels of surface PD-L1 after transduction with a PD- L1 expressing retroviral construct. Data are represented as mean ± SEM (error bar) of 2 independent experiments. (K) Rag KO mice were injected s.c. with $2x10^6$ R-PD-L1 expressing tumor cells, followed by treatment with PD-L1 blockade antibodies (αPD-L1) at days 2, 5, 8 and 11 after transplantation. Tumors were removed at day 12 and extracellular glucose concentrations were measured. Data are from 2 independent experiments. *p=0.0319.

**Figure 9. PD-L1 directly regulates tumor cell metabolism**






(A) ECAR of P tumor cells treated with IFN-γ for 48h followed by 10 μg/ml of either MHC-I or anti-PD-L1 (αPD-L1) blockade antibodies for 24 hours. Data show ECAR at baseline and after injection of the mitochondrial ATP synthase inhibitor oligomycin and are representative of 3 independent experiments. (B) ECAR of B16, MC38 and L tumor cells pre-treated with IFN-γ for 48h followed by PD-L1 blockade antibody for 24 hours was measured. Multiple tumor progressor clones—T10, ES1, ES2 and ES3—derived from parental d42m1 tumor cells were treated with anti-PD-L1 or isotype control antibodies and their ECAR was measured 24h after treatment. Bar graph shows the mean ± SEM and generated from the results of 3 independent experiments. *p=0.0255, ***p=0.001. (C) Western blot analysis for the phosphorylation of mTOR targets (p4EB-P1, pS6K, and pS6) on P tumor cells treated with αPD-L1 blockade antibody. (D) Phosphorylation of Akt and glycolytic enzymes PGK1, LDHa, and TPI were examined by western blot. Blots (C and D) are representative of 3 independent experiments. (E) mRNA expression of glycolytic enzymes (HK2, LDHa, PDK1, and TPI) in P tumor cells treated with or without anti-PD-L1 antibody (αPD-L1). qPCR data are generated from 3 independent experiments and shown as mean ± SEM, n.s., not significant. (F) The R tumor clone overexpressing high level of PD-L1 was treated with αPD-L1 antibody for 30 minutes (30 min). The cells were then either either washed in an acid solution to dissociate

antibody from the surface of the cells (+ acid wash) or left untreated (no acid wash). After fixation, cells were incubated with anti-Rat IgG A488 (Detect surface PD-L1) to detect anti-PD-L1 present on the surface of the cells, then following permeabilization the cells were incubated with anti-Rat IgG A647 (Detect PD-L1 after perm) to detect internalized anti- PD-L1. PD-L1 expression was assessed by flow cytometry. Data are representative of 4 independent experiments. (G) Western blot analysis of R and P tumors transduced with pdl1 shRNA (PD-L1 hp) or Control hp against luciferase (Ctrl hp) for the phosphorylation of mTOR targets (left), and Akt and glycolysis-related enzymes Akt, PGK1, and TPI (right). Blots are representative of 4 independent experiments. (H) Cell proliferation rate of transduced R and P tumors in (F) were measured by the dilution of CTV at day 0 and 3. The histogram plot is representative of 3 independent experiments. (I) $1x10^6$ transduced tumor cells as were injected s.c. into $Rag^{-/-}$ 129S6 mice and tumor growth was monitored. Data are an average of two perpendicular diameters ± SEM from one experiment (n=4).

## § 2.7 Discussion

Antigen recognition by T cells is critical for tumor clearance, and stronger antigens lead stronger activation (Lanzavecchia and Sallusto, 2002, Rao et al., 2010) and a greater capacity to compete for nutrients. T cells must adequately compete for nutrients in order to engage the metabolism that supports their function. We (Chang et al., 2013, O'Sullivan and Pearce, 2015, Pearce et al., 2013), and others (Mellor and Munn, 2008, Srivastava et al., 2010, Mockler et al., 2014), have speculated that nutrient competition in the tumor microenvironment in vivo impacts T cell function. We establish here that tumors can dampen TIL function by competing for glucose, even when antigens that are strongly recognized by T cells are expressed by the tumor, demonstrating that metabolic competition, as a distinct mechanism, can lead to T cell hyporesponsiveness during cancer. Our data also support a model where the competition for other nutrients or growth factors beyond glucose could also present a similar metabolic competition in tumors.

It makes biological sense that nutrient competition in a tumor shapes the ability of immune cells to perform in that environment. T cells are primed in lymphoid tissues, which are likely nutrient-replete. Upon activation, T cells traffic to sites of inflammation where they have to compete with other diverse cell types for available resources. When activated T cells, which need to engage aerobic glycolysis for effector function (Cham et al., 2008, Jacobs et al., 2008, Chang et al., 2013), infiltrate a tumor, we suggest that they experience a nutrient deprivation that impairs their function, but not necessarily their survival, and this can lead to T cell hyporesponsiveness and cancer progression. Consistent with this idea, it was

recently shown that TILs that are specific for defined P tumor antigens infiltrate the P sarcoma prior to checkpoint blockade therapy, however these cells lack function and do not produce IFN-γ until after checkpoint blockade therapy is administered (Gubin et al., 2014). These results support our hypothesis, and suggest that conditions in the microenvironment, even when antigen is recognized, can impose T cell hyporesponsiveness. This view is consistent with the idea that T cell activation and costimulatory signals, first and foremost, remodel the metabolism in the T cell, and endow the cell with new features that allow it to more efficiently compete for nutrients, e.g. enhanced glucose transporter (Glut1) expression (Jacobs et al., 2008). It is not merely coincidental that signaling through CD28 – the very process that prevents T cell anergy – primarily functions to increase glucose uptake (Frauwirth et al., 2002). It is also possible that cells such as Treg cells and M2-polarized macrophages, neither of which require aerobic glycolysis, but instead use fatty acid oxidation (Vats et al., 2006, Michalek et al., 2011, Huang et al., 2014), often appear in progressing tumors because these are cells that are likely able to metabolically compete and survive well in a low glucose environment. This is also consistent with observations that M1-polarized macrophages and effector T cells, both of which require aerobic glycolysis (Pearce et al., 2013) for function, appear in regressing tumors, which we presume to be relatively glucose-replete.

Our data suggest that glucose, which is primarily thought to be stably regulated and abundantly available in metazoan organisms, can actually become a limiting factor for T cells in the tumor microenvironment. Our experiments show that there are differences between tumors in their acquisition of glucose, and that these differences do not necessarily relate to differences in their proliferation. When considering how glucose depletion alters T cell function, it is intriguing to speculate that enhanced

glucose acquisition, and even glycogen storage, is selected for within tumors (Favaro et al., 2012) as these traits could act to deprive T cells of glucose and thus reduce the effectiveness of the antitumor T cell response. Our understanding of how the competition for resources, including basic nutrients, is dynamically regulated in a particular niche in an animal, and how this can impose functional changes in cells, is only just beginning to develop. Tumor-imposed nutrient restrictions are of course not limited to glucose. Availability of amino acids, fatty acids, other metabolites like lactate, and the presence of growth factors and co-stimulatory signals that dictate whether T cells will express the appropriate transporters to allow nutrient acquisition will all influence T cell function in the tumor (Pearce et al., 2013, Mockler et al., 2014). Furthermore, other cell types, especially other immune cells in the tumor microenvironment, will not only impact, but may actively shape the metabolic balance between tumors and effector T cells, vastly affecting the immune response outcome.

We showed previously that aerobic glycolysis is required for T cells to attain full effector status and that this occurs via a posttranscriptional mechanism that involves the bi- functional enzyme GAPDH. When glucose is present, GAPDH is engaged in its enzymatic function in the glycolysis pathway; when cells are restricted from glucose, GAPDH leaves the glycolysis pathway and binds the 3'UTR of IFN-γ mRNA and prevents its efficient translation (Chang et al., 2013). We found that when T cells are glucose restricted for short periods of time, their dampened cytokine production can be rescued by re-feeding the cells glucose, as GAPDH will become re-engaged in the glycolysis pathway. However, our preliminary observations indicate that if T cells experience prolonged chronic nutrient deprivation, this dampened cytokine production becomes relatively irreversible, leading to a more permanent dysfunction

that cannot be corrected through simple re-exposure to nutrients. These data suggest that therapeutic strategies to simply elevate glucose in an established tumor microenvironment would not necessarily be effective in reversing the hyporesponsive phenotype of TILs. Not only might TILs be unable to respond to glucose readily, for example, if they have not maintained expression of the glucose transporter, but if the metabolic balance between the tumors and TILs is not perturbed in favor of the T cells prior to exposure to glucose, then the tumor will likely continue to outcompete the T cells, even in the presence of more available glucose (O'Sullivan and Pearce, 2015). Current studies in our laboratory are aimed at understanding how initial metabolic restrictions in vivo can set the scene that leads to long-term hyporesponsiveness in T cells.

We envisage that the various states of T cell hyporesponsiveness that have been described in cancer and infection may be induced by an initial metabolic restriction. This could manifest from a simple shortage of glucose, as we suspect is the case when a tumor outcompetes T cells for this nutrient, or any signal, or lack of signal, to the T cell that abrogates its ability to acquire glucose. The latter situation provides a mechanism for how these events could occur even when glucose itself is not limiting. If this model were correct, then it might suggest that there is only a short window of time when T cells already present in a tumor could be targeted to regain functional capacity. Therefore, strategies that aim to deplete immune cells in a tumor, coupled with those that aim to promote glycolysis in newly infiltrating T cells may be the most effective way to metabolically remodel the tumor microenvironment. It is conceivable that checkpoint blockade therapy against CTLA-4, which is now known to deplete Treg cells in tumors (Simpson et al., 2013) may work in part by creating space in the tumor that permits the influx of newly activated cells. Combining checkpoint blockade

therapies that target CTLA-4 with those against PD-1 may further amplify the metabolic alterations in the tumor by enhancing the activation of the infiltrating T cells as they enter the newly created, and more nutrient replete, environment. Perhaps highlighting why, at least in part, these combined therapies are proving so successful in patients (Hamid et al., 2013, Wolchok et al., 2013).

Our data showing that checkpoint blockade antibodies restore the glucose balance in the P tumor microenvironment suggest that these therapies might be most effective against tumors with higher glycolytic rates. It is likely that progressor tumors with low rates of glycolysis, such as those that rely predominantly on OXPHOS, and perhaps even use other diverse substrates for fuel, do not starve the microenvironment of glucose as drastically as those tumors that engage glycolysis highly, and thus would be less affected by these therapies. If this were the case, then it could perhaps explain why these therapies work for some patients, but not for others. We are currently investigating whether the glycolytic rate of a tumor could be used as a prognostic tool to determine the efficacy of these treatments.

Our finding that surface expressed PD-L1 directly regulates tumor cell glycolysis was serendipitous. Enhanced PD-L1 expression on tumor cells correlates with dampened immune responses and tumor progression (Keir et al., 2008), and PD-L1 is known to inhibit T cells via PD-1, but it has remained unclear whether PD-L1 expression by tumor cells serves additional biological advantages for malignant tumors to establish and grow. Consistent with our findings that PD-L1 has T cell independent function, it has been shown that neurons can inhibit the proliferation of astrocytoma cells in co-culture (Hatten and Shelanski, 1988), and that killing of murine glioblastoma cells is dependent on expression and activity of PD-L1 receptor on neurons (Issazadeh-

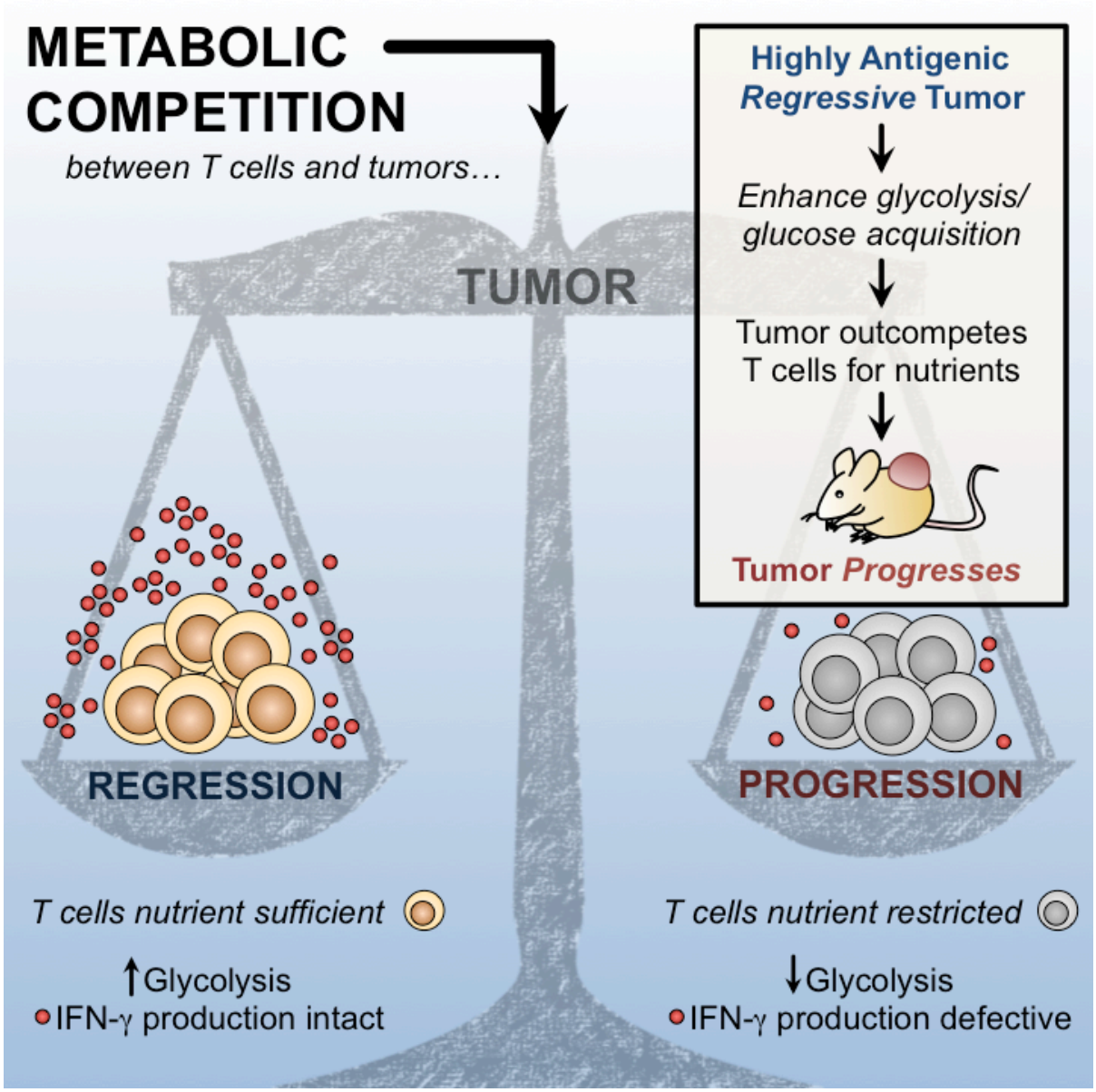
Navikas, 2013, Kingwell, 2013). The precise mechanism by which these events occur is not known, however these observations might possibly be explained if PD-L1 expression conferred a higher glycolytic metabolism to one cell type, e.g. neurons, that allowed them to deplete glucose from, and subsequently lead to the dampened survival of another cell type, e.g. cancer cells. In fact, induction of PD-L1 expression by neurons in tumor-adjacent brain tissue associates positively with glioblastoma patient survival, whereas lack of neuronal PD-L1 expression is associated with high PD-L1 in tumors and unfavorable prognosis (Liu et al., 2013). Intriguingly, the fact that PD-1 blockade increased OXPHOS and aerobic glycolysis in TILs, while PD-L1 blockade only increased aerobic glycolysis might suggest that these treatments work through distinct mechanisms to promote TIL function in vivo, but more investigation is needed to determine if this is the case.

The 30 amino acid cytoplasmic tail of PD-L1 is highly conserved across species, which suggests functional significance, however it has not yet been characterized to possess any signaling capacity (Keir et al., 2008, Francisco et al., 2010). Our data show that PD- L1 shRNA-mediated knockdown, which results in decreased overall PD-L1 expression, phenocopies our results with PD-L1 blockade antibody, which causes decreased expression of surface PD-L1 via receptor internalization. These data suggest that this protein must be expressed on the cell surface for its function. Current experiments are underway to identify precisely how surface expressed PD-L1 is signaling to Akt and mTOR, and which other proteins might be involved in this process. It is conceivable that the cytoplasmic tail of PD-L1 is phosphorylated directly and when this occurs, it interacts with other proteins that relay information to mTOR. Likewise, it is also possible that PD- L1 sits in a domain in the cell membrane that promotes its association with other signaling proteins. This association would

not necessarily be dependent on any signaling capacity inherent to the cytoplasmic domain, but rather accessory proteins could relay a signal to mTOR. We envisage that in this situation, if PD-L1 is not expressed at the surface, then the association with other proteins in the membrane is destabilized, and signaling to mTOR is blunted. Regardless of the precise sequence of events that transduces the signal from PD-L1 to the mTOR pathway, we have clearly shown that this molecule has a novel function in regulating tumor cell metabolism.

In summary, we have shown that glucose competition between tumors and T cells can directly influence cancer progression, and have discovered an unexpected role for PD- L1 in directly regulating the metabolic state of tumor cells. New efforts to target cancer should incorporate the idea that metabolic competition occurs in tumors and that this greatly influences tumor progression. In light of these findings, future therapies may consider combining treatments that dampen tumor cell metabolism with those that enhance glycolytic capacity in TILs in order to promote optimal anti-tumor immunity.

**Figure 10. Summay model of nutrient competition in tumor microenvironment**



# CHAPTER 3

# ACETATE RESCUES T CELL HYPORESPONSIVENESS INDUCED BY NUTRIENT RESTRICTION

(Manuscript submitted for review)

## § 3.1 Introduction

Metabolic fitness is intimately linked with T lymphocyte survival, differentiation, and function. T cells use glucose and glutamine as their primary fuel source for energy generation (Bental and Deutsch, 1993). Naïve T cells are quiescent and break down glucose to fuel oxidative phosphorylation (OXPHOS). Upon activation, T cells undergo rapid metabolic reprogramming and augment aerobic glycolysis, in which glucose is metabolized into pyruvate and then converted to lactate, generating 2 molecules of ATP despite the presence of adequate oxygen. These metabolic changes support their extensive cell growth, proliferation, and cytokine production allowing a functional immune response against infection and cancer. This dramatic increase of glycolysis requires increased glucose uptake by T lymphocytes, which is controlled by cell-surface expression as well as the trafficking of Glut-1, a glucose transporter primarily expressed on T lymphocytes (Frauwirth et al., 2002, Wood et al., 2003, Scheepers et al., 2004, Cham et al., 2008). An inability to access sufficient nutrients poses a significant barrier to effector T cell function. Culture of T cells in glucose-limiting conditions has been shown to inhibit mTORC1 signaling and gene expression, thus impairing cell adhesion, proliferation and survival of CD8 T cells, as well as the production of effector molecules including interferon-gamma (IFN-γ) (Cham et al., 2008, MacIver et al., 2013). CD4 T cells from fasting animals also exhibited long-lasting metabolic and functional defects in a leptin dependent manner (Saucillo et al., 2014). When effector T cells disengage from glycolysis, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) switches functionally from a metabolic enzyme to a mRNA binding protein that preferentially binds to 3' untranslated regions (UTR) of cytokine mRNAs, preventing their translation. (Nagy

and Rigby, 1995, Chang et al., 2013). In addition, inhibition of glycolysis can lead to increased expression of immune regulatory receptors, such as program death-1 (PD-1), which has been reported as an early driver of T cell exhaustion due to altered metabolism (Wherry, 2011, Patsoukis et al., 2015, Wherry and Kurachi, 2015, Bengsch et al., 2016) Naïve cells deprived of glucose enter a state of metabolic anergy even in the presence of adequate TCR engagement and stimulatory signals (Zheng et al., 2009). Finally, nutrient availability in tissue microenvironments may also impact T cell function. Studies from our group indicate that infiltrating T cells in the tumor microenvironment compete with tumor cells for available glucose and this nutrient restriction directly dampens their mTOR activity, glycolytic capacity, and IFN-γ production, leading to tumor progression (Chang et al., 2015).

Acetate has gathered increased attention as both a carbon source for cellular biomass synthesis and an epigenetic regulator of posttranslational protein modification (Wang et al., 2010, Zhao et al., 2010, Hosios and Vander Heiden, 2014, Jaworski et al., 2016). In the context of cancer where glucose and oxygen may be limiting resources, acetate is a primary source of acetyl-CoA, which is the key metabolic intermediate for lipogenesis and protein acetylation (Yoshii et al., 2009, Comerford et al., 2014, Schug et al., 2015, Bulusu et al., 2017). In addition to tumor cells, acetate also has a major impact on immune cells. Synthesis of acetate from ethanol is critical for enhancement of the inflammatory response in macrophages through the increased acetylation of proinflammatory gene histones in acute alcoholic hepatitis (Kendrick et al., 2010). A recent study also described that a systemic increase in acetate levels induced by stress is required for optimal memory $CD8^+$ T cell function (Balmer et al., 2016).

Changes in cellular metabolism due to nutrient limitation can link to alternations in gene expression through transcriptional/posttranscriptional regulation networks, which coordinate with T cell dysfunction or hyporesponsiveness (Hentze et al., 2010, Castello et al., 2012). However, exactly how T cells respond to external nutrients and how this shapes their effector function through alternation of metabolic pathways remains unclear. We set out to determine why T cell hyporesponsiveness due to short-term glucose restriction can be rescued by glucose re-exposure, while T cells restricted from glucose for longer periods of time cannot be rescued by this substrate.

## § 3.2 Glucose restriction limits T cell glycolysis and dampens effector cytokine production

Our previous studies indicate that a competition for metabolic resources exists within the tumor microenvironment. Tumor infiltrating T cells (TILs) can experience glucose deprivation imposed by tumor cells, which dampens their mTOR activity, lowers their glycolytic capacity, and impairs their cytokine production, leading to cancer progression (Chang et al., 2015). Although our study suggests a distinct mechanism that contributes to T cells hyporesponsiveness during cancer, a fundamental understanding about the extent environmental changes influence T cell metabolism, the impact of this metabolic reprogramming on T cell differentiation and function over time, and ultimately the outcome of T cell mediated immune responses under these conditions remains unclear. In order to understand how T cells metabolically adapt to nutrient restrictive environments, we started with a simple *in vitro* model by activating naive OT-I T cells with OVA peptide in complete media containing 25mM glucose for 3 days. We used this high concentration of glucose because T cells will produce higher levels of cytokines at higher glucose concentrations. Activated $CD8^+$ T cells were then either maintained in 25mM glucose media or switched to glucose-limiting media containing only 1mM glucose for either 2 more (day 5), 4 more (day 7), or 6 more (day 9) days (**Figure 12A**). To answer whether nutrient restriction could induce T cell hyporesponsiveness, we measured IFN-γ production from cultured effector $CD8^+$ T cells and found that cytokine production was dampened in cells subjected to 1mM glucose media compared to those in 25mM glucose media after PMA and ionomycin restimulation, consistent with TILs isolated from nutrient restrictive tumors (**Figure 11A**) (Chang et al., 2015). Their ability to produce IFN-γ continued to

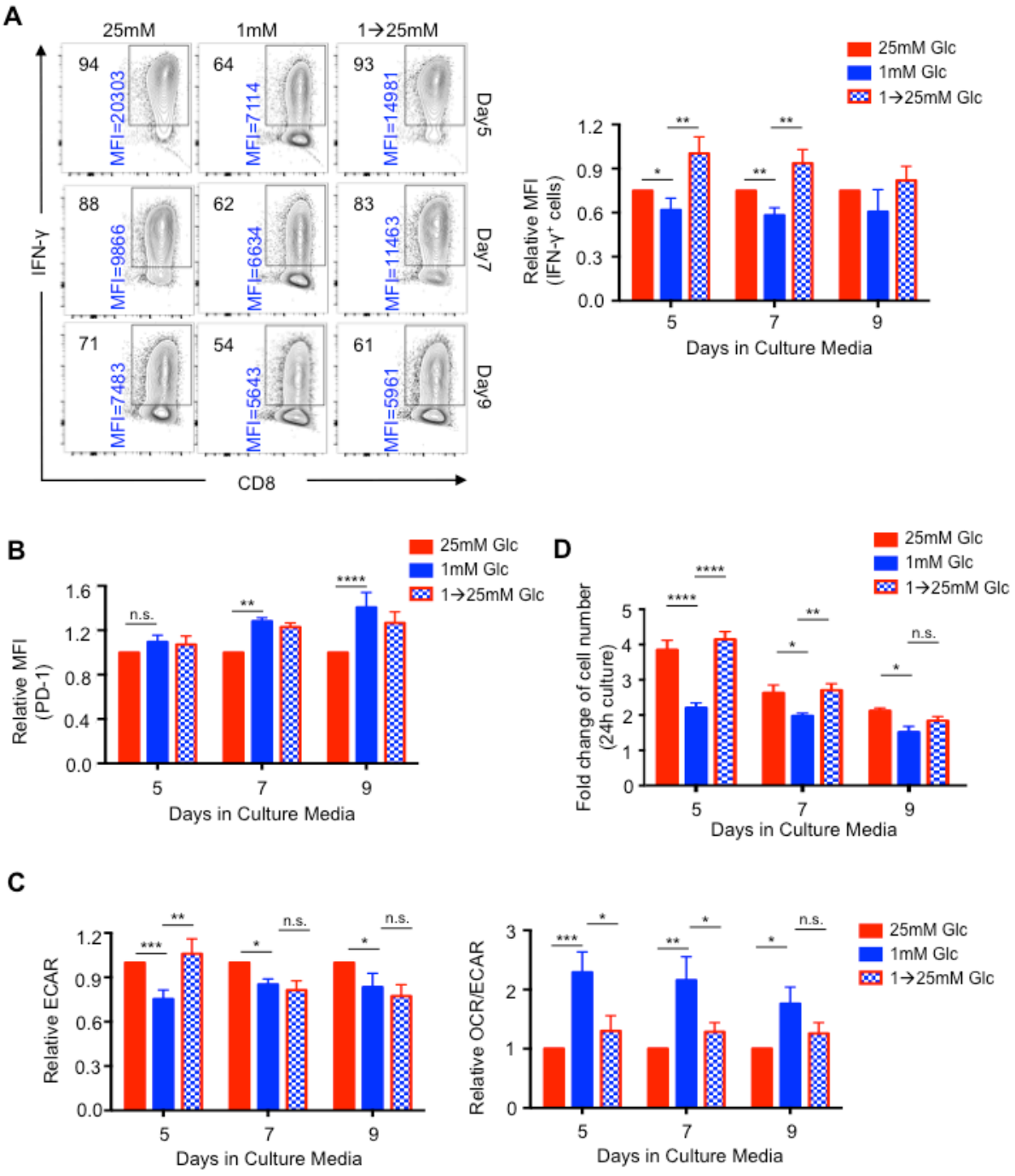
gradually wane as they were subjected to prolonged glucose restriction (**Figure 11A**). PD-1 molecule expression has been well defined as an indicator of T cell exhaustion or hyporesponsiveness during chronic infection and cancer (Crawford et al., 2014, Wherry and Kurachi, 2015). To further validate whether hyporesponsive T cells developed during extended glucose restriction *in vitro*, we examined surface expression of PD-1 and found that the level of PD-1 on acutely glucose-restricted T cells was relatively similar to their counterparts in 25mM glucose but significantly higher after longer-term glucose deprivation (**Figure 11B**), consistent with their hyporesponsive phenotype of impaired IFN-γ production.

Effector T cells need to engage glycolysis for optimal cytokine production(Chang et al., 2013). We predicted that defective IFN-γ production by glucose restricted T cells was due to their inability to engage sufficient glycolysis. We performed mitochondrial stress test assays and confirmed that the extracellular acidification rate (ECAR, correlates with glycolysis) was lower, but the ratio of oxygen consumption rate (OCR, correlates with OXPHOS) to ECAR was higher in T cells switched to culture in 1mM glucose than those in 25mM glucose, indicating that glucose-restricted effector T cells reprogramed their metabolism by switching from aerobic glycolysis to OXPHOS (**Figure 11C**). Cells maintained in 1mM glucose for an additional week continued to use OXPHOS over glycolysis. While we observed diminished cell population expansion after culturing in 1mM glucose media compared to 25mM glucose media (**Figure 11D**), cell survival was maintained (**Figures 12B)**. Altogether, these data suggest that exposing activated T cells to glucose limiting conditions can induce a functional state of hyporesponsiveness in a persisting population of T cells.

## § 3.3 The hyporesponsive phenotype of T cells restricted from glucose cannot be fully restored by re-exposure to glucose

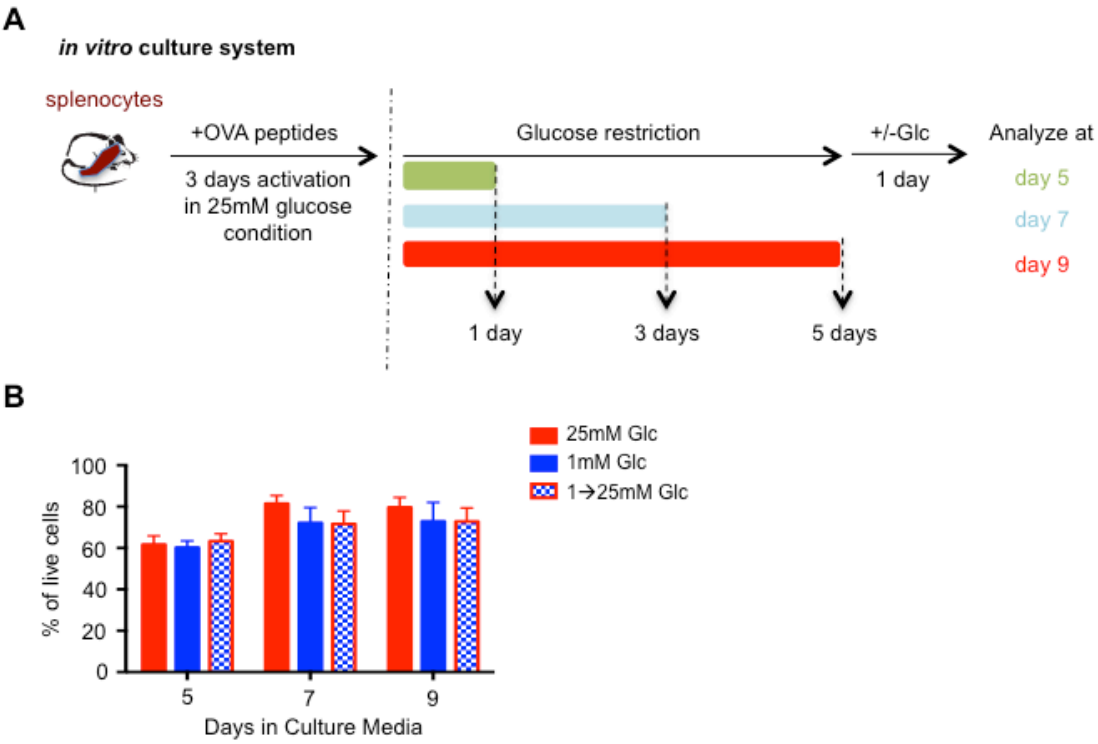
We previously observed that defects in cytokine production of TILs isolated from glucose-restrictive tumors could not be fully recovered even in the presence of sufficient glucose during re-stimulation (Chang et al., 2015). Our findings here show that glucose-restricted T cells are able to survive and proliferate, but have impaired IFN-γ production due to insufficient glycolysis *in vitro.* We questioned whether restoring glucose levels would rescue their hyporesponsive phenotype. In order to test this, we re-exposed glucose-restricted T cells to 25mM glucose for 24h and analyzed their proliferation and cellular metabolism as well as effector cytokine production. The addition of glucose rescued the dampened IFN-γ production of T cells after acute glucose starvation, which re-engaged in glycolysis (**Figures 11A and 11C**). T cell expansion was also restored when sufficient glucose was present (**Figure 11D**). However, glucose failed to correct the status of hyporesponsive T cells subjected to prolonged glucose restriction (**Figure 11A and 11C**). We observed not only continued impairment of T cell expansion, but also defects in IFN-γ production were no longer restored by additional glucose when cultured in 1mM glucose for 6 days. T cells experiencing long-term glucose restriction gradually lost the ability to re-engage in glycolysis, resulting in irreversible dysfunction of effector cytokine production (**Figures 11A and 11D**). Interestingly, glucose supplementation did not significantly change the surface expression of PD-1 on glucose-restricted T cells compared to non-treated cells (**Figure 11B**).

**Figure 11. Prolonged glucose restriction limits T cell IFN-γ production that cannot be rescued by glucose re-expose**



Naïve OT-I T cells were activated with OVA peptide for 3 days in high glucose media (25mM glucose and 10%FCS). T cells were either maintained in 25mM glucose (Glc) media or switched to low glucose condition (1mM glucose and 10%FCS) followed by re-exposure to 25mM glucose for 24h. Cells were collected at day 5, day 7 and day 9 for analysis. (A) IFN-γ production of T cells were examined after 5h re-stimulated with PMA/ionomycin. FACS plots are representative of >7 independent experiments. Numbers show relative MFI of IFN-γ producing cells normalized to 25mM glucose group as the mean ± SEM. *p=0.016, **p=0.006, **p=0.004, **p=0.008. (B) PD-1 expression of T cells was assessed by flow cytometry and data from ≥5 independent experiments are shown in bar graph as relative MFI normalized to T cells cultured in 25mM glucose media. **p=0.0029, ****p<0.0001. (C) ECAR (left) and OCR/ECAR ratio (right) of T cells in differentiate glucose media. Data are from 6 independent experiments presented as relative ECAR or OCR/ECAR ratio normalized to T cells in 25mM glucose media. ***p=0.0004, **p=0.006, *p=0.022, *p=0.025 (ECAR), ***P=0.0007, **P=0.002, *P=0.05, *P=0.01, *P=0.03. (D) The cell expansion was measured using a haemocytometer and data are presented as fold change over 24h culture. Bar graph are obtained from 4 independent experiments and shown as ±SEM, ****p<0.0001, *p=0.0125, *p=0.0185, **p=0.003.

**Figure 12. Glucose restriction does not impair T cell survival *in vitro***



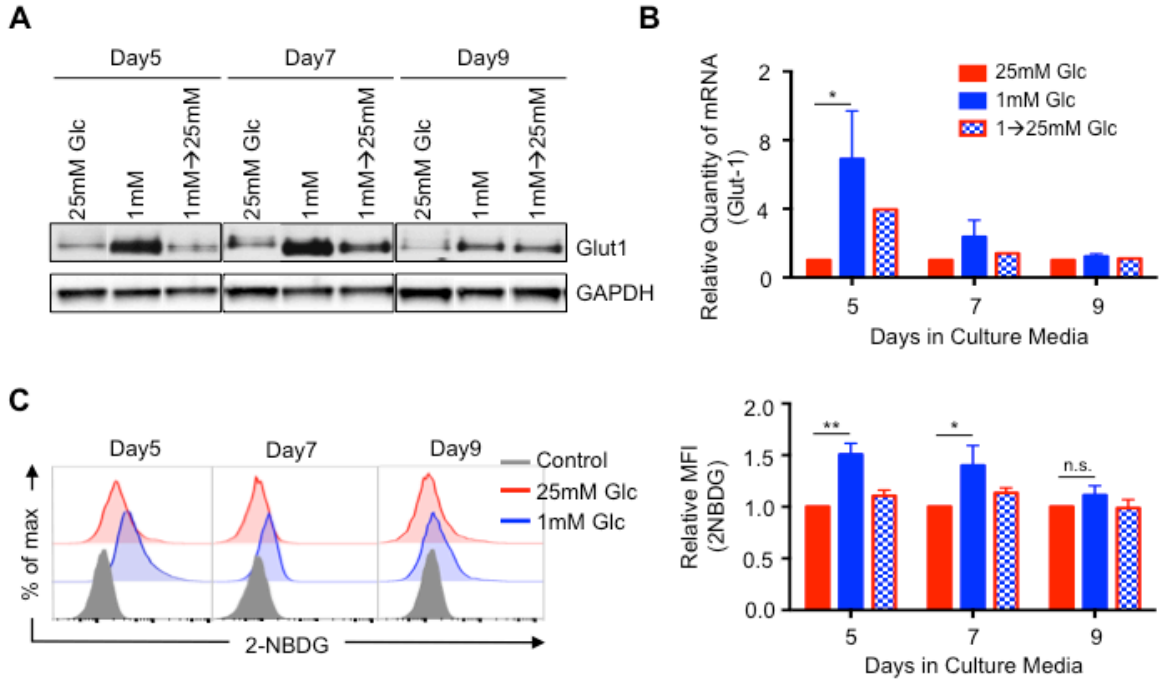

(A) The schematic diagram of *in vitro* culture system. (B) The viability of culture T cells in differentiated glucose culture media was measured by the haemocytometer with Trypan blue staining. The data is presented as the bar graph from >=4 independent experiments.

## § 3.4 Glucose availability regulates Glut-1 expression, which directly controls glucose uptake by T cells

Given the described role of the glucose transporter Glut-1 in controlling glucose uptake during T cell activation (Frauwirth et al., 2002, Frauwirth and Thompson, 2002, Scheepers et al., 2004, Jacobs et al., 2008), we assayed the total protein level of Glut-1 in differentially cultured T cells by western blot and found that cells exposed to 1mM glucose dramatically induced Glut-1 expression compared to those in 25mM glucose conditions (**Figure 13A**). Glucose-restricted cells did not maintain this high level of Glut-1 expression at later time points as observed by decreased total protein levels. Importantly, cells experiencing acute glucose restriction could respond to re-exposure of exogenous glucose and subsequently decreased their expression of Glut-1, but also became refractory to added glucose over time (**Figure 13A**). Quantification of Glut-1 mRNA exhibited a similar expression pattern (**Figure 13B**). Acute glucose deprivation led to an elevation of *Slc2a1* (Glut-1) mRNA, which was not observed under long-term glucose restriction. Accordingly, 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) incorporation by T cells supported these results showing that glucose-restricted T cells with upregulated Glut-1 expression had an increased ability for glucose acquisition. However, T cells cultured under prolonged glucose restriction were unable to mediate sufficient glucose uptake in response to lower Glut-1 expression (**Figure 13C**). Interestingly, the protein level of another glucose transporter Glut-3, which is also expressed by T cells but is not essential for T cell function (Macintyre et al., 2014), was not modulated by glucose availability (**Figure 14**). Our results suggest that glucose availability in the extracellular environment dictates expression of the glucose

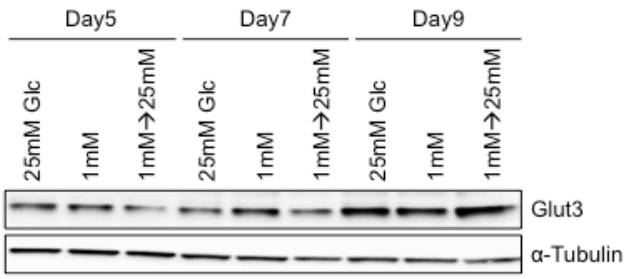
transporter Glut-1 on T cells, which may be responsible for their inability to engage glycolysis for optimal cytokine production.

**Figure 13. Prolonged glucose restriction decreases surface Glut-1 expression and prevents efficient glucose uptake**




(A) Total Glut1 expression in T cells with glucose restriction was examined by western blot, and data are representative of ≥2 independent experiments. (B) mRNA level of Glut-1 was analyzed by qPCR. Data from 2 independent experiments are relative quantity of mRNA normalized to T cells cultured in 25mM glucose media and presented as ±SEM. *p=0.013. (C) T cells in differentiate glucose media were cultured in 5ug/ml 2NBDG at RT for 15min and 2NBDG uptake was measured by flow cytometry. Representative histogram plots are shown on the left and relative mean fluorescence intensity (MFI) of 2NBDG shown on the right was normalized to the MFI of cells cultured in 25mM glucose media. Bar graphs are shown as ±SEM and from ≥3 independent experiments. ***p=0.0004, **p=0.003.

**Figure 14. Glut-3 expression maintains the same regardless of glucose perturbation**




Total protein expression of Glut-3 of T cells in glucose restriction condition was exanimated by western blot. Data is the representative image from 2 independent experiments.

## § 3.5 Acetate rescues IFN-γ production of T cells under prolonged glucose-restriction

Since supplementing glucose failed to restore IFN-γ production of T cells exposed to prolonged glucose-limiting conditions, and our data suggested that this could be due to the inability to acquire glucose due to dysregulated Glut1 expression, we asked if there might be alternative metabolites that could potentially play a role in modulating T cell function. Acetate has been recognized as an important alternative carbon source for cancer cells to support their survival and proliferation in glucose starvation (Hosios and Vander Heiden, 2014, Lyssiotis and Cantley, 2014). In addition to cancer cells, recent studies also indicate a critical role for acetate in immune cells such as macrophages and memory T cells during metabolic stress (Kendrick et al., 2010, Balmer et al., 2016). In order to investigate whether acetate can enhance effector T cell function depressed by prolonged glucose-limitation, we treated cells with 5mM sodium acetate instead of additional glucose for 24h and measured intracellular IFN-γ protein after PMA and ionomycin restimulation. Significant enhancement of IFN-γ production was observed not only in T cells exposed acutely to low glucose media, but also in those that became unresponsive to additional glucose cultured under prolonged glucose restriction (**Figure 15A**). These data suggest that unlike glucose, the metabolite acetate can rescue the hyporesponsiveness of T cells induced by long-term glucose starvation.

## § 3.6 Acetate is incorporated into histones and enhances histone acetylation in glucose-restricted T cells

In order to be utilized by cells, acetate must be enzymatically converted to acetyl coenzyme A (acetyl-CoA), which is essential for a vast majority of biological processes including protein acetylation and lipogenesis. In human livers, acetate converted acetyl-CoA enhances histone acetylation at promoter regions of proinflammatory genes in residential macrophages (Kendrick et al., 2010). Acetyl-CoA derived from acetate can also acetylate the glycolytic enzyme GAPDH, which is important for optimal function of memory CD8 T cells under stress (Balmer et al., 2016). According to these findings, we reasoned that the augmentation of IFN-γ production by acetate maybe a consequence of histone acetylation, which promotes gene expression, and not necessarily due to GADPH modification, since glycolysis is severely dampened in glucose-restricted T cells. Indeed, we were able to observe a dramatic increase in IFN-γ production when using valproic acid (VPA), which is a histone deacetylase inhibitor and results in increased levels of histone acetylation (**Figure 15A**).

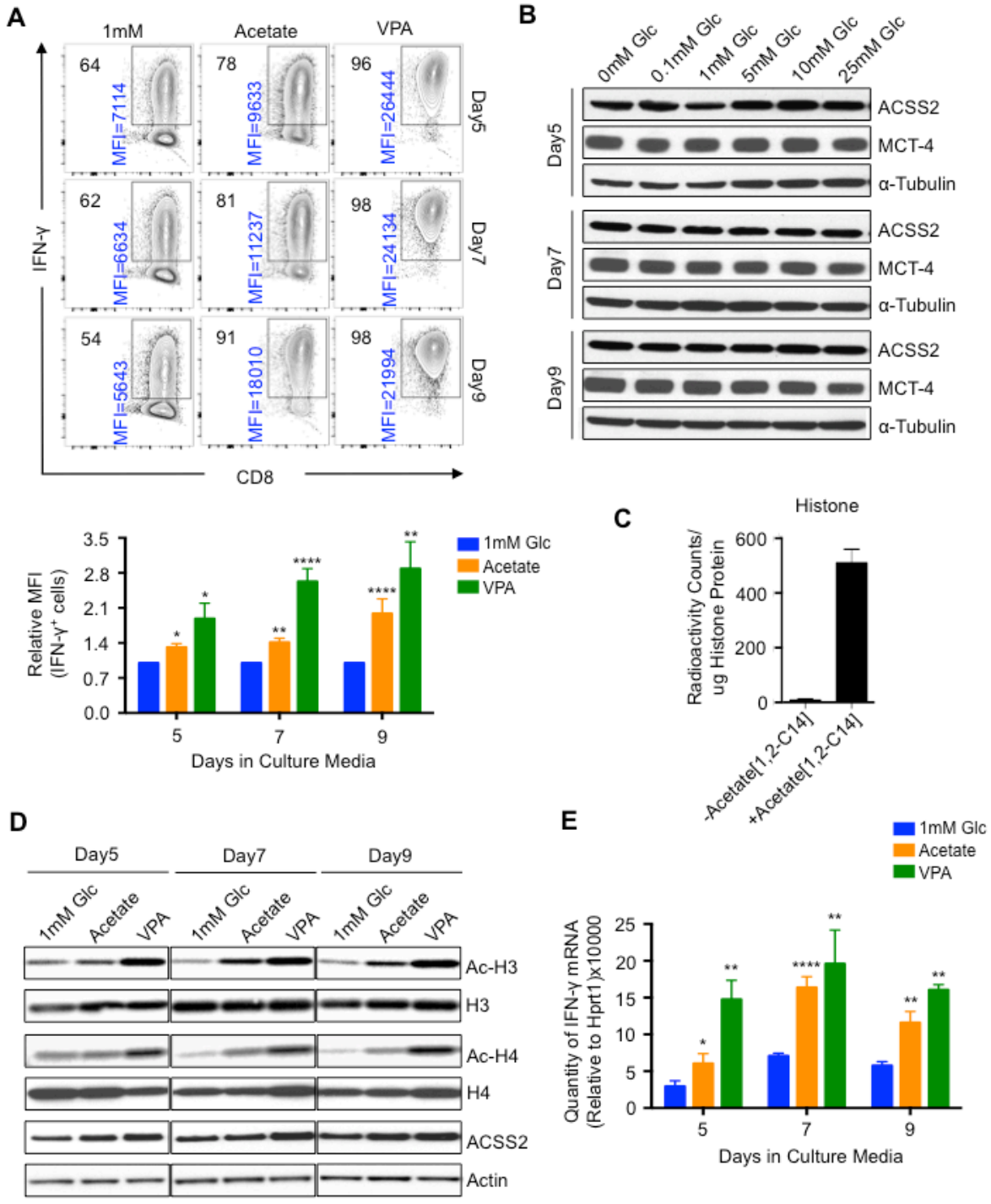
Acetate can enter cells through monocarboxylate transporters (MCTs) or facilitated diffusion via aquaporins (Kirat and Kato, 2006, Kirat et al., 2006, Halestrap and Wilson, 2012, Rae et al., 2012). After penetrating the membrane, acetate is catalyzed to acetyl-CoA in an ATP-dependent manner (Watkins et al., 2007). The mammalian genome contains genes encoding three different acetyl-CoA synthetase enzymes capable of converting acetate to acetyl-CoA, one of which, designated ACSS2, has been reported to be localized to both the cytoplasm and nucleus and is

required for acetate uptake and utilization (Fujino et al., 2001, Perez-Chacon et al., 2009, Comerford et al., 2014). We wondered whether T cells were still competent for acetyl-CoA generation from acetate in glucose-limiting conditions. Western blots assessing the protein level of MCT-4 as well as ACSS2 revealed that the membrane transporter and catalyzing enzyme responsible for cellular acetate uptake and utilization were consistently expressed, regardless of glucose concentration in the media (**Figure 15B**), unlike Glut-1, the expression of which is modulated by glucose availability (**Figure 16A**).

To test whether T cells use acetate for histone modifications, we assessed the incorporation of acetyl groups into histones by exposing cells to $^{14}C$ labeled acetate. The radioactivity count of $^{14}C$ was enriched in extracted histones compared to those without $^{14}C$ labeled acetate treatment (**Figure 15C**), suggesting that T cells were able to take up exogenous acetate and catalyze it into intermediate metabolites. Another major role of acetate metabolism is to generate acetyl-CoA in cytoplasm for lipid synthesis. The incorporation of $^{14}C$ labeled acetate into lipid compartments further confirmed the ability of glucose-restricted T cells to uptake and utiliize exogenous acetate (**Figure 16B**). Next we extracted histones from glucose-restricted T cells and measured global acetylation of histones by western blot. As expected from our radiolabeled studies and consistent with previous reports (Kendrick et al., 2010, Lee et al., 2014, Shen et al., 2015, Shi and Tu, 2015), we observed significantly elevated acetylation on histone proteins H3 and H4 in acetate treated T cells during glucose restriction (**Figure 15D**). We also found that ACSS2 expression was increased by the addition of acetate, which is consistent with previous reports that acetate consumption is dictated by functional expression of

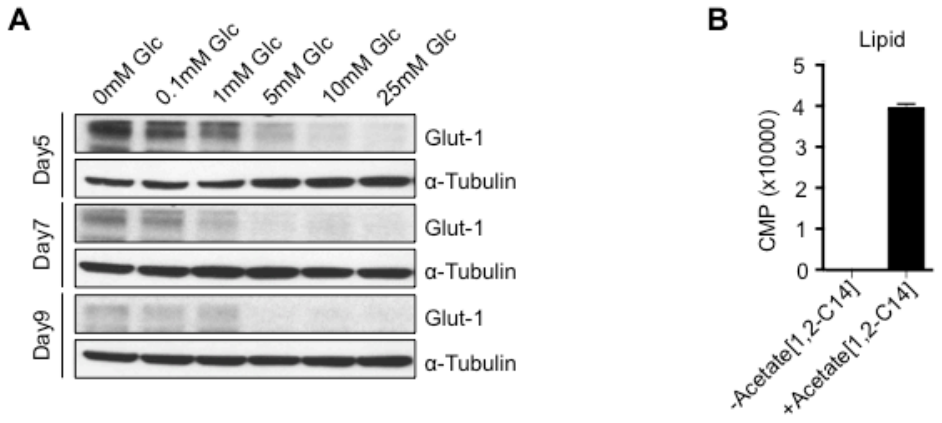
ACSS2 (**Figure 15D**) (Comerford et al., 2014). Accordingly, the quantification of mRNA expression of the IFN-γ gene significantly increased in T cells exposed to acetate compare to non-treated group, which supports our hypothesis that augmentation of histone acetylation enhances cytokine gene expression and thus reverses the hyporesponsive phenotype of T cells subjected to nutrient stress (**Figure 15E**).

**Figure 15. Exogenous acetate enhances IFN-γ production from T cells during prolonged glucose restriction in an ACSS2 dependent manner**



(A) Naïve OT1 T cells were activated as descried before and treated with 5mM sodium acetate or 1mM valproic acid for 24h. IFN-γ production of T cells was measured 5h after PMA/ionomycin re-stimulation. Dot plots showing MFI of IFN-γ as the representative of >=5 independent experiments and bar graph is presented as relative MFI of IFN-$\gamma^+$ cells normalized to untreated cells. *p=0.034, **p=0.006, ****p<0.0001 (Acetate), *p=0.013, ****p<0.0001, **p=0.006. (B) T cells are differentially cultured in the media containing 0, 0.1,1,5,10 and 25mM glucose. The expression of ACSS2 and MCT-4 enzymes were exanimated by western blot at day 5,7 and 9 post-activation. (C) Cultured T cells were assayed for their ability to utilized [$^{14}$C]acetate for histone acetylation. Bar charts are shown as mean ± SEM (error bar) from ≥2 independent experiments. (D) Global histone acetylation was measured western blot and graphs are shown as representative of >=2 independent experiments. (E) IFN-γ mRNA was analyzed by real-time quantitative PCR and relative expression of mRNA of each time point and culture condition over the levels expressed in untreated cells and data presented as ±SEM.

**Figure 16. Glucose restricted T cells maintain the ability for acetate uptake**




(A) The expression of membrane bond Glut-1 in T cells cultured in differentiated glucose condition was also exanimated by western blot. Data is the representative image from 2 independent experiments. (B) The incorporation of [$^{14}$C]acetate into lipids was measured and Bar charts are shown as mean ± SEM (error bar) from ≥2 independent experiments.

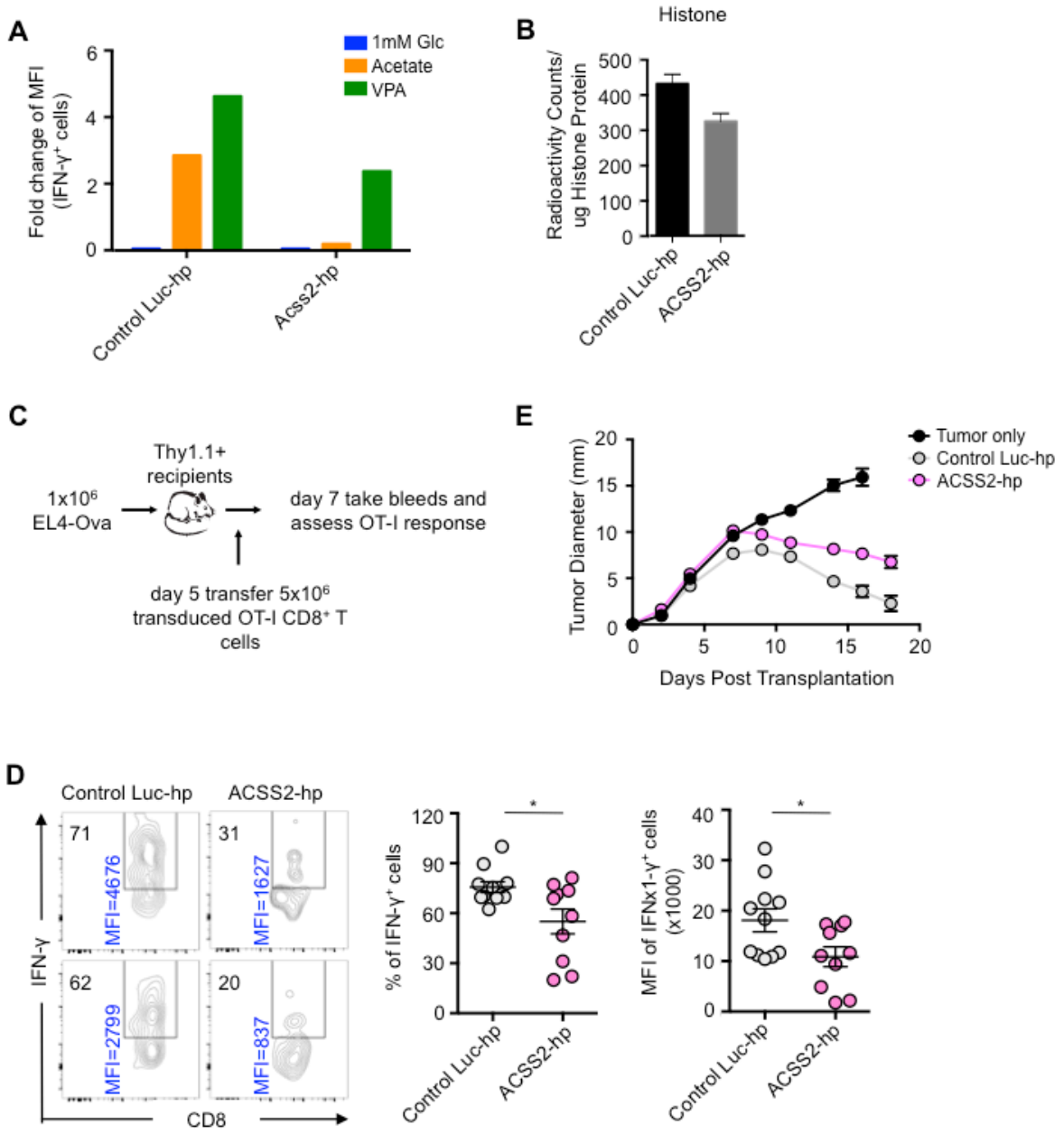
## § 3.7 The effect of acetate on enhancing IFN-γ production is dependent on ACCS2

Based on evidence that ACSS2 is the primary enzyme that converts cytosolic acetate to acetyl-CoA, which can be used by histone acetyl transferases for histone acetylation, we reasoned that the rescue of cytokine production driven by acetate would be dependent on ACSS2. In order to test this hypothesis, we used short-hairpin RNAs to selectively silence ACSS2. Suppression of ACSS2 by shRNA rendered glucose-restricted T cells unresponsive to acetate-induced increases in IFN-γ production compared to control cells, indicating a requirement of ACSS2 for acetate in rescuing cytokine production in glucose-restricted T cells (**Figure 17A**). The knockdown efficiency of ACSS2 protein was confirmed by western blot (**Figure 18B**). Furthermore, we measured incorporation of acetyl units into histonse or lipids by exposing transduced T cells with $^{14}C$ acetate and found that T cells with reduced ACSS2 enzyme exhibited an impaired ability to utilize radiolabeled acetate and assimilate it in both histone and lipid components (**Figure 17C and 18**). Together, our data suggest that ACSS2 is important for allowing T cells to respond to exogenous acetate by converting it into acetyl-CoA for subsequent metabolic utilization and chromatin modification.

## § 3.8 ACSS2 is required for optimal effector T cell function *in vivo*

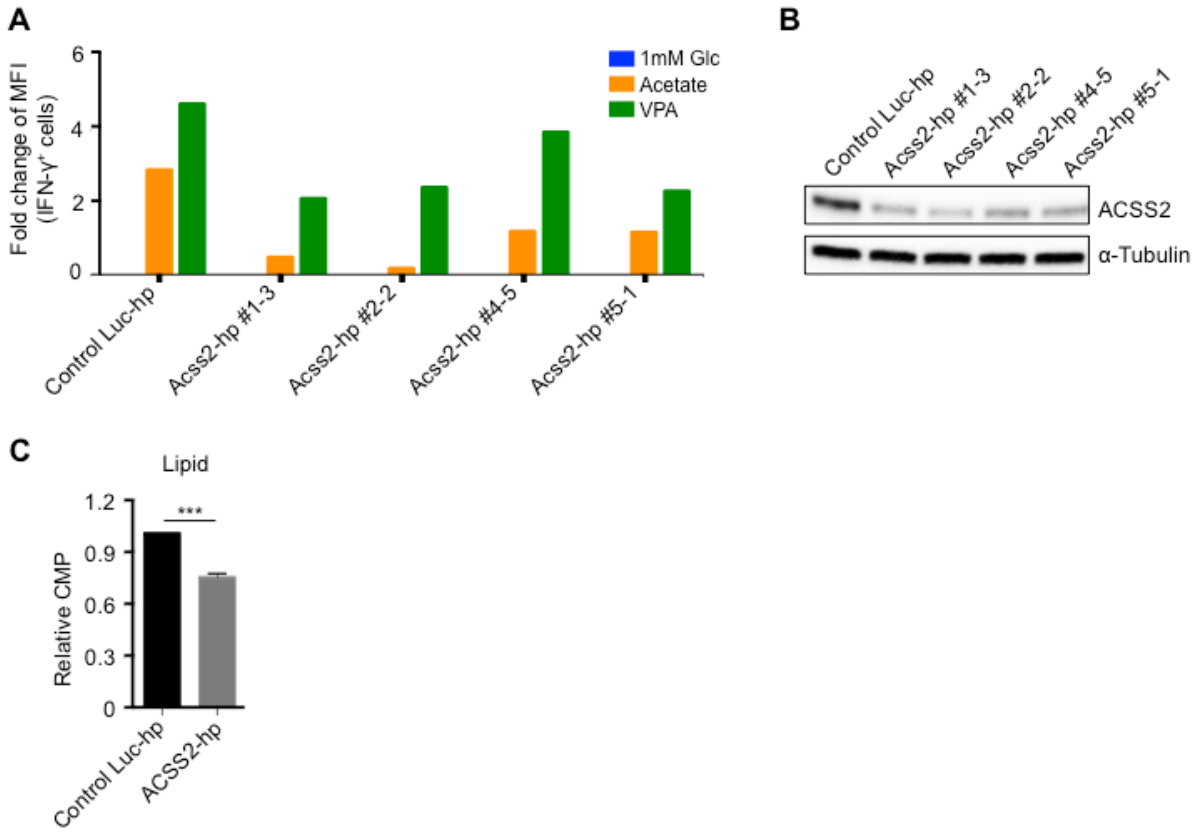
As reported previously, systemic acetate concentrations present in serum are transiently increased under metabolic stress induced by bacterial infections in mice (Balmer et al., 2016). The observation that hyporesponsive T cells with prolonged glucose restriction maintained ACSS2 expression to be able to process exogenous acetate instead of glucose led us to consider the importance of ACSS2 for antigen specific T cell responses during infection or cancer. In order to address this question, we took advantage of EL4 lymphoma tumors expressing OVA antigen (EL4-OVA) and OT-I T cells that recognize OVA. We injected $1x10^6$ EL4-OVA cells subcutaneously and then intravenously transferred $5x10^6$ activated OT-I cells expressing either control or ACSS2-shRNA into Thy1.1$^+$ recipient mice 5 days post-tumor transplantation (**Figure 17C**). XX days later we measured Donor OT-I T cells in the peripheral blood and found that cells expressing ACSS2-shRNA exhibited dampened IFN-γ production after restimulation compared to those expressing the control hairpin (**Figure 17D**). Although mice that received activated OT-I T cells were able to control tumor growth, tumor clearance was impaired in mice that received OT-I T cells expressing the ACSS2-shRNA (**Figure 17E**). These data indicate that antigen specific T cell effector function can be affected by the loss of ACSS2 *in vivo* due to its inability to utilize endogenous acetate under metabolic stress (**Figure 18C**).

**Figure 17. ACSS2 is important for effector T cell function *in vivo*.**



(A) OT-1 T cells transduced with either control Luc shRNA (Luc-hp) or constitutive shRNA knockdown of *ACSS2* (ACSS2-hp). IFN-γ production of transduced T cells treated with acetate or VPA was measured 5h after PMA/ionomycin restimulation. Bar graph showing fold change of IFN-γ MFI was normalized to 1mM Glc control group from 1 experiment. (B) Their ability to incorporate [$^{14}$C]acetate into histone was also assayed and data presents the mean± SEM and n=3. (C) $1x10^6$ EL4-Ova lymphoma cells were s.c. injected into C57BL/6 Thy1.1$^+$ congenic mice that received $5x10^6$ transformed OT-I Thy1.1$^+$ CD8$^+$ T cells containing control Luc-hp or Acss2-hp 5 days post tumor transplantation. (D) Mice were bled 7 days post-transplantation and IFN-γ production of donor transduced T cells was assessed after overnight OVA peptide re-stimulation. FACS plot showing MFI of IFN-γ as the representative of 2 mice and dot graph of of from 2 independent experiments and dot graph is presented as percentage and MFI of IFN-γ$^+$ cells (n>=10). *p=0.0343. (E) Tumor growth was monitored and data are an average of two perpendicular diameters ± SEM. (n≥4).

**Figure 18. The incorporation of acetate into cells and thus drive IFN-γ production is mediated by ACSS2.**



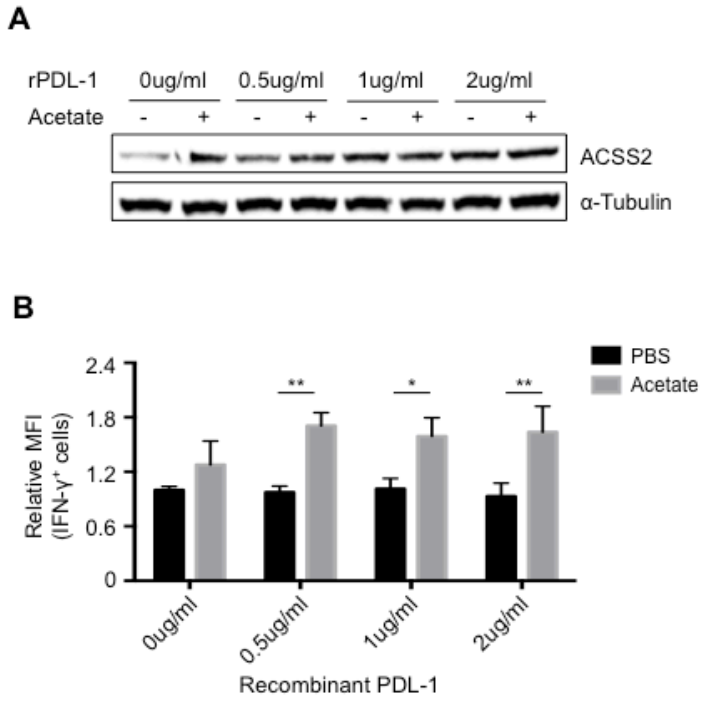

(A) OT-1 T cells transduced with 4 different short hairpin against ACSS2 and IFN-γ production of acetate or VPA treated T cells were measured 5h after PMA/Ionomycin re-stimulation. (B) The knock-down efficiency of short hairpin against ACSS2 were confirmed by western blot. (C) The incorporation of [$^{14}$C]acetate into lipid in transduced T cells were assessed and bar graph in shown as the relative CMP normalized to control Luc-hp from 3 independent experiments. ***p=0.0004.

## § 3.9 PDL-1/PD-1 signals promote ACSS2 expression and enhance T cell responsiveness to acetate

The PDL-1/PD-1 signal pathway has been recognized as one of many inhibitory mechanisms that contribute to T cell hyporesponsiveness during infections and cancer (Barber et al., 2006, Day et al., 2006). Higher and sustained expression of PD-1 is a hallmark of hyporesponsive T cells, which mediate downstream signaling cascade that dampen T cell function (Odorizzi and Wherry, 2012, Okazaki et al., 2013). We showed here that glucose-restricted T cells with defective cytokine production augment PD-1 expression on the cell surface in our *in vitro* model. From a metabolic aspect, PD-1 can alter T cell metabolism by promoting fatty acid oxidation and inhibiting glycolysis, thus preventing optimal effector function (Patsoukis et al., 2015). As a result, PD-1 blockade may induce metabolic reprogramming by enhancing cellular glycolysis in order to reinvigorate their function. New studies on exhausted or hyporesponsive T cells with high PD-1 expression suggest a distinct but stable epigenetic profile, which indicates a potential limitation of current PD-1 pathway blockade immunotherapies to reverse their dysfunctional state (Pauken et al., 2016). In line with our finding that acetate can rescue cytokine production in T cells with prolonged glucose restriction through histone modification in an ACSS2 dependent manner, we considered the possibility that elevated PD1 signaling may have an effect on mediating cellular responses to alternative nutrients such as acetate when available glucose is insufficient. In order to test this hypothesis, we applied recombinant PDL-1 antibody (rPDL1) to induce PD-1 signals on T cells. Surprisingly, we observed increased protein expression of ACSS2 in a dose dependent manner. Upregulation of ACSS2 was also observed on cells after

administration of acetate (**Figure 19A**). Accordingly, significantly more cells produced IFN-γ in response to acetate supplementation when exposed to rPDL1 (**Figure 19B**). These data suggest that PDL-1/PD1 signals modulate ACSS2 expression on T cells, which promotes their ability to utilize acetate to potentially enhance cytokine production.

**Figure 19. PDL-1/PD-1 signals promote ACSS2 expression in T cells, which contributes to the cellular response to acetate**




(A) *in vitro* activated T cells were treated with recombinant PDL-1 protein along with either PBS or acetate for 24h and protein level of ACSS2 enzyme was measured by western blot. The graph is representative of 2 independent experiments. (B) IFN-γ production of T cells was examined after 4h re-stimulated with α-CD3 and α-CD28 antibodies. Data is shown as mean ± SEM and n=4. **p=0.007, *p=0.03, **p=0.009.

## § 3.10 Discussion

Access to sufficient nutrient depots is critical for effector T cell function. T cells must acquire nutrients to engage appropriate metabolism to support their functions to fight against infection and cancer (Chang et al., 2013, Pearce et al., 2013, Buck et al., 2015, O'Sullivan and Pearce, 2015). Our group and others have highlighted a model of nutrient competition, whereby glucose depletion by tumors in the tumor microenvironment *in vivo* can lead to T cell hyporesponsiveness despite the presence of robust antigens recognized by T cells (Mellor and Munn, 2008, Mockler et al., 2014, Chang et al., 2015). However, simply re-exposing hyporesponsive T cells isolated from a progressing tumor to glucose ex vivo cannot rescue dampened cytokine production. In order to understand how T cells sense and adapt to a glucose-depleted environment, we established an *in vitro* model and demonstrate that glucose restriction limits cellular glycolysis of T cells and dampens their ability to produce IFN-γ. While this defect in cytokine production can be reversed after initial short-term glucose restriction, it cannot be reversed by glucose re-exposure when T cells undergo prolonged glucose restriction. Our data suggest that this inability to respond to glucose associates with the eventual down-regulation of Glut-1 expression, which directly prevents efficient glucose uptake even in glucose replete conditions. Glut-1 has been reported to be selectively essential for T cell activation and effector function despite the expression of other glucose transporters, such as Glut-3 (Macintyre et al., 2014). In line with this finding, we also observed that Glut-3 is constantly expressed, independent of exogenous glucose level. Availability of nutrients and other metabolites, growth factors, and costimulatory signals that dictate whether T cells will express appropriate transporters to allow nutrient acquisition, will

impact T cell function *in vivo.* However, a fundamental understanding about how different glucose transporters are differentially regulated and facilitate T cell responsiveness to glucose in vivo remains a subject of ongoing investigation.

Research in cancer metabolism for the last decade has greatly extended our understanding of the metabolic requirements of proliferating cells, as well as metabolic alternations that promote cell growth. As a primary energy resource, glucose plays an essential role in supporting cellular bioenergetics and maintaining normal cell function. When transiting to environments with limiting nutrients and oxygen, cancer cells reprogram their metabolism to cope with environmental changes in a manner dependent on alterative substrates, as do immune cells. We found that acetate, a unique metabolite positioned at the intersection of metabolism and epigenetic regulation, enhances IFN-γ production from T cells during prolonged glucose restriction. The maintenance of MCT expression, as well as the enzyme ACSS2, independent of glucose availability suggests that T cells maintain the ability to acquire and utilize acetate in situations when glucose becomes limiting. It is possible that the observed enhancement in cytokine production is the result of differential expression of ACSS2 in the nuclear compartment, which is specifically responsible for histone acetylation as suggested by studies in cancer cells under serum and oxygen limiting conditions (Bulusu et al., 2017). Together with our results that PDL-1/PD-1 signals regulate ACSS2 levels suggest that, acetate metabolism presents a distinct target for reinvigorating responses from dysfunctional T cells.

In summary, we have shown that prolonged glucose restriction contributes to T cell hyporesponsiveness characterized by defects in IFN-γ production, which cannot be

corrected by simple re-exposure to glucose. However, administration of acetate can rescue cytokine production of T cells in an ACSS2 dependent manner. We also demonstrate a role of PDL-1/PD-1 signal in regulating ACSS2 expression, which may present as an alternative mechanism for preserving effector T cell function *in vivo*. Understanding nutrient competition in the microenvironment and ways of enhancing T cell nutrient acquisition should be considered in the generation of future therapies in order to promote optimal T cell immunity.

# CHAPTER 4

# SUMMARY AND FUTRUE REMARKS

## § 4.1 Summary

It has long been appreciated that, unlike bacteria or yeast, which respond directly to nutrients in their environment, mammalian cells must be instructed by growth factors in order to efficiently utilize nutrients. This presents a fundamental mechanism for the regulation of cell growth in metazoans. One of the classical examples of this it how IL-2 promotes glucose transports expression for mediating glucose uptake. It is also equally important to consider that available nutrients, substrates, or other resource may also become limited or immune cell niches, affecting immune cell metabolism and thereby, cell function and fate. A well-characterized example of this is tryptophan catabolism by tumors or antigen presenting cells negatively affects infiltrating T cell function and survival. Moreover, competition for arginine that rapidly depleted by myeloid-derived suppressor cell populations may also fall into this general category. Given these findings, it would seem likely that substrate availability, whether concentrations of nutrient itself or of the growth factors that allows its acquisition, fluctuates dramatically depending on the location of the immune cells. By using the MCA-induced sarcoma tumor cell line, we have been able to demonstrate a general nutrient competition model in which glucose consumption by antigenic tumors can metabolically restrict T cells, directly dampening their effector function and allowing tumor progression. Checkpoint blockade therapy may present a way to correct this nutrient imbalance through a direct effect in the tumor cells.

However, our further study suggests that the plasticity of T cell response to additional glucose dynamically changes overtime. Simply re-expose T cells with

prolonged glucose restriction cannot fully restored effector T cell function, which may due to its inability of regulating the expression of glucose transporters for the sufficient nutrient uptake. Taking lessons from cancer metabolism that cancer an cells derive energy from alternative carbon sources and metabolic pathway to maintain cell survival and proliferation under nutrient limiting conditions, we considered the opportunities by the utilization of fatty acids, amino acids, ketone bodies and acetate in addition to glucose for therapeutic manipulation. Given the central role of acetyl-CoA in cellular metabolism, acetate has garnered increased attention in the context of cancer as both an epigenetic regulator of posttranslational protein modification, and as a carbon source for cellular biomass accumulation. Our data suggest that unlike glucose, metabolism acetate can rescue T cell hyporesponsiveness under prolonged glucose restriction. The augmentation of cytokine production by acetate is dependent on acetyl-CoA synthesis enzyme ACSS2 through histone acetylation enhancement.

## § 4.2 Future remarks

Our initial observation that limiting glucose condition dampens cellular glycolysis in T cells thus impairs their effector function. However, the primary data suggests that glucose-restricted T cells do not increase the utilization of glutamine or lipids to compensate the shortage of glucose. Further studies on glucose tracing and quantification of metabolites should be conducted to understand the metabolic plasticity of T cells under metabolic stress. Moreover, glucose restricted T cells upregulates glut-1 expression, which coordinates with increased glucose uptake. However, this increased ability of glucose acquisition did not help compensate their dampened cellular glycolysis. Exactly how T cells regulate the surface nutrient transporter and how the expression of these transporters associates with the whole cell metabolism in response to environmental cues thus affect their function need to be addressed.

One of our novel findings is that the presentation of acetate can rescue impaired cytokine production in T cells with prolonged glucose restriction dependent on ACSS2 enzyme. However, there are two other isoforms encoded by the genes in the same family ACSS2, which locate in the mitochondria and differentially expressed in terms of tissue distribution. Although it has been reported that ACSS1 and 3 are not as important as ACSS2 to convert acetate to acetyl-CoA for energy derivation in cancer cells, the contribution of these enzymes to acetate metabolism T cell in the glucose limiting condition has not well characterized. Given that acetate incorporate into TCA cycle and contributes to the cellular acetyl-CoA pool, mitochondrial enzyme ACSS1 and 3 could potentially play a role in T cell metabolism as well as their

function under metabolic stress. Further studies are required to investigate this hypothesis.

T cell hyporesponsiveness described in chronic infections or cancer is usually marked by elevated surface PD-1 expression. The ligation of PD-L1 with PD-1 can transmit a negative signal to T cells thus inhibit its function. PD-1/PD-L1 pathway presents one of the inhibitory mechanisms that contribute to impaired effector T cell function. We Surprisingly found that PDL-1 induced PD-1 signals in glucose restricted T cells could modulate ACSS2 expression, which promote their ability to response to acetate. Beyond the well-appreciated PD-1/PDL1 signaling, our study might suggest a distinct role of PD1 plays during T cell hyporesonsiveness. However, the underlying mechanism of how PD-L1/PD-1 pathway involved in contributing T cell response to alternative nutrients and how that affected T cell functions in nutrient restricted environment requires further investigated.

Because cellular metabolism is linked to immune cell function, understanding more about metabolic pathway in T cells will likely illuminate new ways to exploit these pathways to harness immunity *in vivo*. Our studies have provided new insights regarding the contributions of primary as well as alternative carbon sources to T cell metabolism and function in the tumor microenvironment. With the appreciation for the role of metabolism in dictating immune cell function, our research could result in revolutionary effective immunotherapies by targeting metabolism to prevent T cell dysfunction in cancer.

# CHAPTER 5

# MATERIALS AND METHODS

## § 5.1 Mice and tumor cells

129S6 mice from Taconic Farms and C57BL/6, C57BL/6 CD90.1 and major histocompatibility complex (MHC) class I-restricted OVA specific TCR OT-I transgenic mice from The Jackson Laboratory were used for all experiments. Animals were housed in our specific-pathogen free animal facility and studies were performed in accordance with procedures approved by the AAALAC accredited Animal Studies Committee of Washington University School of Medicine in St. Louis. An established methylcholanthrene (MCA)-induced mouse sarcoma model of regressing (d42m1-T2 or R tumor) and progressing tumors (d42m1- T3 or P tumor) were used in this study, both of which originated from d42m1 parental sarcoma cells (Matsushita et al., 2012). Other progressing tumor clones (T10, ES1, ES2, and ES3) derived from the d42m1 parental sarcoma cells were kindly provided by Dr. Robert Schreiber. B16 cells, L cells and MC38 cells were kindly given by Dr. Marco Colonna and were used for anti-PD-L1 blockade antibody treatment experiments.

## § 5.2 Tumor Transplantation

For the sarcoma model, 1-2x$10^6$ R or P tumor cells unless otherwise indicated were injected subcutaneously (s.c.) in 150 µl of sterilized PBS into the right flank of mice. Recipient mice were 6-8 week old males on the 129S6 background. Tumor size was measured and quantified as the average of two perpendicular diameters. For the EL4-Ova model, a total of 1x$10^6$ or 40x$10^6$ EL4-Ova cells were injected into the peritoneal cavity of recipient C57BL/6 wild-type (Thy1.$2^+$) mice. Splenocytes from naïve OT-I Thy1.$1^+$ mice were stained with $K^b$/OVA tetramer to determine the

proportion of OVA-specific $CD8^+$ T cells. Splenocytes containing $2x10^4$ naïve $Thy1.1^+$ OVA-specific $CD8^+$ T cells were transferred intravenously (i.v.) to the recipient mice on the same day of EL4-Ova tumor transplantation.

For ACSS Knock-down experiment in EL4-Ova model, a total of $1x10^6$ EL4-Ova cells were subcutaneously (s.c.) in 150ul sterilized PBS into right flank of recipient C57BL/6 $Thy1.1^+$ mice. Splenocytes from naïve OT-I $Thy1.2^+$ mice were activated by OVA peptides and transduced with either control Luc-hp or Acss2-hp. Transformed OVA-specific $CD8^+$ T cells were transferred intravenously (i.v.) to the recipient mice 5 days post EL4-Ova tumor transplantation. For the sarcoma model, $1x10^6$ progressing tumor cells unless otherwise indicated were injected subcutaneously (s.c.) in 150 µl of sterilized PBS into the right flank of the mouse. Recipient mice were 6-8 week old males on the C57BL/6 background. Tumor size was measured and quantified as the average of two perpendicular diameters.

### § 5.3 Tumor harvest

Established sarcoma tumors were excised from mice at ~12 days post-transplantation. Isolated tumors were chopped and treated with 1 mg/ml type IA collagenase (Sigma) and DNase I (Sigma) in HBSS (Hyclone) for 1h incubation at 37°C. In order to keep phosphorylated proteins stable, tumor harvest was performed at 0–4°C. Cells were filtered through a 70-micron strainer to obtain single-cell suspensions.

## § 5.4 In vivo checkpoint blockade treatment

Tumor bearing mice were treated intraperitoneally (i.p.) with 200 µg of anti-CTLA4 (9H10) or anti-PD-1 (clone RMP1-14) or anti-PD-L1 (clone 10F.9G2) on days 3, 6, and 9 post-tumor transplantation. Tumor bearing mice in the control group were injected with 200 µg each of IgG2a and IgG1 isotype antibodies.

## § 5.5 Flow cytomoetry and intracellular staining

All fluorochrome-conjugated monoclonal antibodies were purchased from BioLegend or eBioscience, except Alexa Fluor® 647-conjugated phospho-4E-BP1 (Thr37/46), Alexa Fluor® 488-conjugated phospho-S6 ribosomal protein (Ser235/236), and phospho-p70 S6 kinase (Thr389) antibodies (Cell Signaling). Intracellular cytokine staining was performed as previously described (Chang et al., 2013). Briefly, cells were stimulated at 37°C for 5h in complete medium supplemented with 100 U/ml IL-2 and 1.0 µl/ml GolgiStop (BD Pharmingen) with or without PMA and ionomycin (Sigma). After stimulation, cells were fixed in Cytofix/Cytoperm fixation (BD Biosciences) at 4°C for 20 min before intracellular staining. Staining for phosphorylated signaling proteins was carried out with Phosflow kits (BD Biosciences). Ex vivo bulk tumors were directly fixed with Phosflow Lyse/Fix buffer (BD Biosciences) at RT for 10 min and then permeabilized on ice for 30 min before intracellular staining. Tumor infiltrating $CD8^+$ T cells (TILs) represent cells gated on $CD45^+TCR\beta^+Thy1.2^+CD8^+$ and tumor cells were gated on $CD45^-FSC^{hi}$. $CD45^+TCR\beta^+Thy1.2^+CD4^+$ cells were gated as $CD4^+$ T cells, $CD45^+CD19^+$ cells as B cells, and $CD45^+CD64^+F4/80^+$ cells as macrophages. Macrophages expressing iNOS were

referred to M1 type and those expressing RELMα referred to M2 type. For 2-NBDG uptake in vivo, mice were injected i.v. with 100 µg 2-NBDG/mouse diluted in PBS. For proliferation assay, cells were fluorescein-labeled with 5 µM CellTrace Violet (Life Technologies) at 37°C for 30 min. Anti-PD-L1 antibody (clone MIH5) was used to detect surface PD-L1 expression on tumor cells. Data were collected on FACSCalibur (BD Biosciences), FACSCanto II (BD Biosciences), LSRFortessa (BD Biosciences) and analyzed by FlowJo software (TreeStar).

**§ 5.6 Cell culture**

R-tumor-specific CTL clone (C3) cells were kindly provided by Dr. Robert Schreiber and maintained by co-culturing with irradiated d42m1 parental sarcoma cells. C3 cells were co-cultured for 24h with or without sarcoma tumor cells at different ratios as indicated. Cultured cells were supplemented with or without 25 mM glucose during PMA/ionomycin stimulation and cytokine production by T cells was analyzed by intracellular staining. For *in vitro* antibody treatment assay, tumor cells were treated with 100 U/ml of recombinant murine IFN-γ (PeproTech) for 24h following by 10 µg/ml anti-PD-L1 treatment for an additional 24h before further experiments. All tumors were cultured in RPMI 1640 media containing 10% FCS. Naïve $CD8^+$ T cells were isolated from spleen and lymph nodes of 129S6 mice, and purified by MACS microbeads (Miltenyi Biotec). T cells were stimulated with 0.5 µg/ml anti-CD28 and 100 U/ml IL-2 in 5 µg/ml anti-CD3 coated plates for 3–4 days. Activated $CD8^+$ T cells were used for the experiments as indicated in the main text.

Naïve T cells were isolated from spleens and lymph nodes of OT-1 transgenic mice and activated with ovalbumin peptide SIINFEKL. T cells were cultured in no glucose

PRMI 1640 media containing 10% FCS and additional 25mM glucose was added during 3d activation and then switch to the media containing 1mM glucose for 1, 3 and 5 days. Cells were plated at same concentration everyday when fresh media was supplied. Cultured cells were supplemented with glucose, acetate and valproic acid for 24h as indicated concentration in the main text. Cytokine production by T cells was analyzed by intracellular staining after 5h PMA/Ionomycin re-stimulation.

### § 5.7 Metabolism assay

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were analyzed by a XF96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were plated in nonbuffered RPMI 1640 media with 10 mM glucose. Measurements were obtained under basal conditions and after the addition of 1 μM oligomycin (an inhibitor of ATP synthase).

### § 5.8 Glucose assay

Supernatant from tumor and T cell co-culture experiments was collected, and glucose concentrations were measured by Glucose Assay Kit (Eton Bioscience). For determining glucose levels in established tumors, harvested tumors were weighed and minced in fixed amounts of PBS. Ex vivo glucose concentration was quantified in accordance with the weight of tumors and the volume of collected supernatant, and normalized with glucose concentrations in the R tumor.

## § 5.9 Transduction

For knocking-down PD-L1 expression, sarcoma tumor cells were transduced with GFP-reporting virus expressing shRNA against luciferase (Ctrl hp), or virus expressing shRNA against CD274 (PD-L1 hp) in media containing 8 μg/ml Polybrene (Sigma) and 20 mM HEPES (Hyclone) for 5h, followed by additional transduction with the same virus overnight. Transduced tumor cells were sorted by GFP expression on a FACSAria II (BD Biosciences). For overexpression, R tumor cells were transduced with retrovirus expressing c-Myc (R-cMyc), or PDK1 (R-PDK1), or with empty vector only (R-EV Ctrl), and puromycin was used to select for stably transduced tumor cells. R tumor cells with differential PD-L1 expression were kindly provided from Dr. Robert Schreiber. They were generated by retroviral transduction and subcloned according to their surface PD-L1 expression.

For knocking-down ACSS2 expression, OT-1 T cells were transduced with GFP-reporting virus expressing shRNA against luciferase (control), or virus expressing shRNA against Acss2 (Acss2 hp) in media containing 8 μg/ml Polybrene (Sigma) and 20 mM HEBES (Hyclone). Cells were spinned down at 2500rpm for 90min at 30°C followed by additional transduction with the same virus overnight. Transduced T cells were sorted by GFP expression on a FACSAria II (BD Biosciences).

## § 5.10 RT-PCR and western blotting

Total RNA was isolated with the mRNeasy mini kit (QIAGEN) and cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All quantitative RT-PCR was performed by the Taqman method, except

for mutant spectrin-β2 mRNA, which was assayed by the SYBR green method, with an Applied Biosystems 7500 sequence detection system. For detecting the expression of mutant spectrin-β2, RNA was isolated as described above. After RT-PCR amplification, cDNA fragments were cut by restriction enzyme Pst1 and analyzed by electrophoresis as described (Matsushita et al., 2012). For western blotting, cell lysate preparation, SDS–PAGE, electrophoretic transfer, immunoblotting, and development using enhanced chemiluminescence were accomplished as previously described (Pearce et al., 2009). All antibodies for western analysis were purchased from Cell Signaling, except Triosephosphate isomerase (TPI) from Abcam and Phosphoglycerate kinase 1 (PGK1) from Thermo Scientific. For detecting mTOR pathway signaling, antibodies against phosphorylation of 4E-BP1 (p4EBP1) at Thr37/46, p70 S6 kinase (pS6K) at Thr389, and S6 ribosomal protein (pS6) at Ser235/236 were used. For detecting AKT signaling, antibody against phosphorylation of AKT (pAKT) at Ser473 was used. For detection of histone acetylation, cells were lysed with PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsufonyl fluoride (PMSF) and 0.02% $NaN_3$ followed by acid extraction by 0.2N HCl overnight. Antibodies against total H3 and H4 were from Cell Signaling but acetyl-histone H3 and acetyl-histone H4 were purchased from EMD Millipore.

### § 5.11 Detection of PD-L1 internalization

R tumor clones overexpressing high levels of PD-L1 was treated with αPD-L1 antibody (αPD-L1) for 15 min on ice, then either kept on ice or incubated at 37°C for 30 min. The cells were then either washed in an acid-strip solution (0.2M acetic

acid, 0.5M NaCl) twice to dissociate antibody from the surface of the cells or left untreated. After fixation in 4% paraformaldehyde for 10 min, cells were incubated with Alexa Fluor® 488-conjugated (A488) anti-Rat IgG antibody to detect αPD-L1 present on the surface of the cells. Then, following permeabilization (Perm/Wash; BD Biosciences), the cells were incubated with Alexa Fluor® 647-conjugated (A647) anti-Rat IgG antibody to detect both surface expressed and internalized αPD-L1. Surface and internalized PD-L1 were then assessed by flow cytometry. Following staining with a nuclear stain (DAPI), cells were mounted in anti-fade (Prolong Diamond; Life Technologies) and imaged by confocal microscopy.

## § 5.12 [$^{14}$C]Acetate incorporation into histones and lipids

For histone incorporation, cells were treated with 1µCi/ml sodium [1,2-$^{14}$C] acetate (PerkinElmer) for overnight. After two washes with ice-cold PBS, cell pellets were re-suspended in 500µl NP-40 buffer (0.1% NP-40, 10mM HEPES, 5mM $MgCl_2$, 0.25M Sucrose) and incubated on ice for 10min. Lysates were washed with buffer without NP-40 and spun down at 6000g for 10min. Histone were acid extracted in 500µl of 0.8M HCl overnight with shaking and then centrifuged at 4°C 20,000xg for 30min. Supernatants were neutralized with 40ul of 10N NaOH and counted for radioactivity using Ultima Gold scintillation fluid.

For lipid incorporation, cells were treated with 1µCi/ml sodium [1,2-$^{14}$C] acetate (PerkinElmer) for overnight. After two washes with ice-cold PBS, cells were lysed with 0.6ml MeOH solution. 0.4ml $CHCl_3$ was added to lysate and vortexed for 30s. Lysates were then centrifuged for 5min at 1000rpm for phase separation. Soluble lipid fraction was collected at lower layer and counted for radioactivity.

## § 5.13 Statistical analysis

Comparisons for two groups were calculated by using an unpaired, two-tailed Student's t-test. Comparisons for more than two groups were calculated using 1-way ANOVA followed by Bonferroni's multiple comparison tests.

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## EDUCATION

| | |
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| 08/2012-Present | Ph.D. Candidate in Immunology<br>Washington University in St. Louis, St. Louis, Missouri USA<br>Max Plank Institute for Immunobiology and Epigenetics, Freiburg, Germany |
| 01/2009-05/2010 | Master of Science in Biology<br>New York University, New York, NY |
| 09/2001-07/2005 | Bachelor of Science in Electrical Engineering<br>Nanjing University of Posts & Telecommunication, Nanjing, Jiangsu, P.R.China |

## RESEARCH EXPERIENCE

**08/2012-present** **Ph.D. Student**
Department of Pathology and Immunology at Washington University
Department of Immunometabolism at Max Plank Institute for Immunobiology and Epigenetics
*(PI: Dr. Erika L. Pearce)*

*Project: To understand the metabolic regulation of $CD8^+$ T cell function in tumor microenvironment*

* We first established that tumor-imposed metabolic restriction can mediate T cells hyporesponsiveness during cancer. The glucose competition in tumor microenvironment can determine cancer progression by regulating the nutrient statue of tumor infiltrating T cells and thus their functionality. The checkpoint blockade therapy could restore glucose in the tumor microenvironment, permitting T cells engage glycolysis for cytokine production. Our results were published on *Cell* in 2015.

* The ongoing project is focusing on how to rescue the function of T cell with long-term hyporesponsiveness. We are investing the metabolic plasticity of effector T cells in glucose limiting conditions. We are also discovering the interplay between metabolic adaptation and posttranscriptional modification as a critical regulator on effective T cell functions.

**08/2012-12/2012** **Rotation Student**
Department of Pathology and Immunology at Washington University
*(PI: Dr. Robert D. Schreiber)*

*Project: To identify specific antigens potentially driving tumor rejection in methylcholanthrene(MCA) induced sarcoma*

**05/2010-08/2012** **Research Assistant**
Cold Spring Harbor Laboratory
*(PI: Dr. Mikala Egeblad)*

*Project: To understand how chemotherapy influences tumor-associated myeloid cells and how changing innate immune cells functions affect drug response in the tumor microenvironment*

**10/2008-05/2010** **Research Assistant**
Department of Basic Science at New York University College of Dentistry
(*PI: Dr. Daniel Malamud)*

*Project: To develop a novel oral-based point-of-care diagnosis system to detect bacteria and viral pathogens based on Up-converting Phosphor Technology (UPT)*

**06/2009-07/2009** **Visiting Student**
Department of Mechanical Engineering at University of Pennsylvania
*(PI: Dr. Haim H. Bau)*

*Project: To develop a user-friendly interface for a microfluidic diagnosis system*

**10/2009-05/2010** **Research Assistant**
Department of Biology at New York University
*(PI: Dr. Kristin C. Gunsalus)*

*Project: To develop a web-start application within the Gaggle network for the purpose of analyzing gene lists based on the Gene Ontology (GO) Consortium*

## PUBLICATIONS

Buck, M. D., O'sullivan, D., Klein Geltink, R. I., Curtis, J. D., Chang, C. H., Sanin, D. E., **Qiu, J**., Kretz, O., Braas, D., Van Der Windt, G. J., Chen, Q., Huang, S. C., O'neill, C. M., Edelson, B. T., Pearce, E. J., Sesaki, H., Huber, T. B., Rambold, A. S. & Pearce, E. L. 2016. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. *Cell,* 166**,** 63-76.

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Chang, C. H*., **Qiu, J***., O'sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., Chen, Q., Gindin, M., Gubin, M. M., Van Der Windt, G. J., Tonc, E., Schreiber, R. D., Pearce, E. J. & Pearce, E. L. 2015. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell,* 162**,** 1229-41. (*co-first author)

Park, J. H., Rasch, M. G., **Qiu, J**., Lund, I. K. & Egeblad, M. 2015. Presence of insulin-like growth factor binding proteins correlates with tumor-promoting effects of matrix metalloproteinase 9 in breast cancer. *Neoplasia,* 17**,** 421-33.

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Chang, C. H., Curtis, J. D., Maggi, L. B., Jr., Faubert, B., Villarino, A. V., O'sullivan, D., Huang, S. C., Van Der Windt, G. J., Blagih, J., **Qiu, J**., Weber, J. D., Pearce, E. J., Jones, R. G. & Pearce, E. L. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell,* 153**,** 1239-51.

Nakasone, E. S., Askautrud, H. A., Kees, T., Park, J. H., Plaks, V., Ewald, A. J., Fein, M., Rasch, M. G., Tan, Y. X., **Qiu, J**., Park, J., Sinha, P., Bissell, M. J., Frengen, E., Werb, Z. & Egeblad, M. 2012.

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Qiu, X., Liu, C., Mauk, M. G., Hart, R. W., Chen, D., **Qiu, J**., Kientz, T., Fiene, J. & Bau, H. H. 2011. A Portable Analyzer for Pouch-Actuated, Immunoassay Cassettes. *Sens Actuators B Chem,* 160**,** 1529-1535.

## FELLOWSHIP

2013~2015 Lucille P. Markey Special Emphasis Pathway in Human Pathobiology Fellowship

## PRESENTATIONS

06/2014 Golden Research Conferences—Immunochemistry & Immunobiology
Poster presentation
02/2016 Keystone Symposia—Immunometabolism in immune function and inflammatory disease
Poster presentation

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